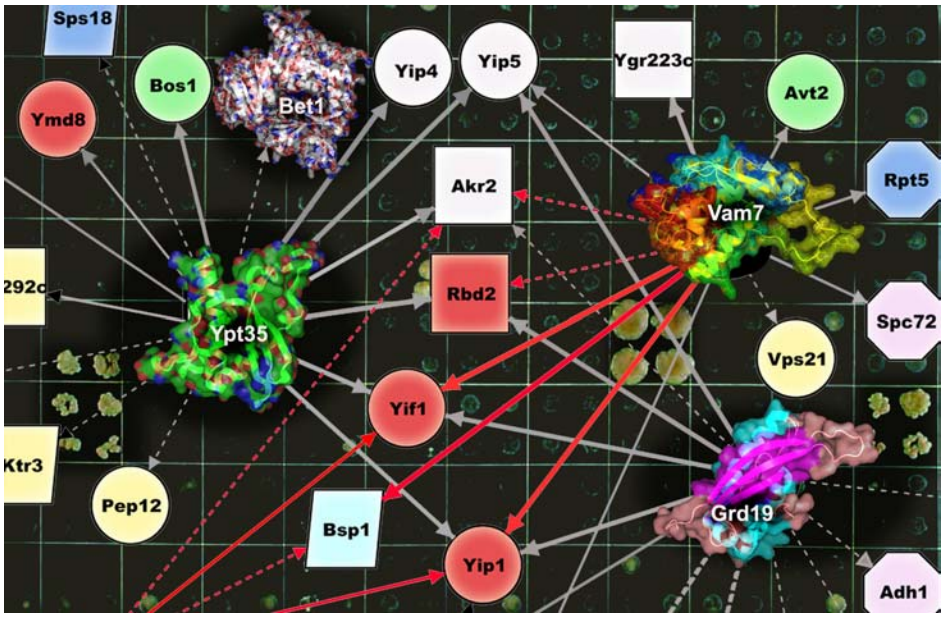
The PX domain protein interaction network in yeast



Zur Erlangung des akademischen Grades eines

DOKTORS DER NATURWISSENSCHAFTEN

(Dr. rer. nat.)

der Fakultät für Chemie und Biowissenschaften der

Universität Karlsruhe (TH)

vorgelegte

DISSERTATION

von

Dipl. Biol. Carolina S. Müller

aus Buenos Aires

Dekan: Prof. Dr. Manfred Kappes
Referent: Dr. Nils Johnsson
Korreferent: HD. Dr. Adam Bertl
Tag der mündlichen Prüfung: 17.02.2005

I dedicate this work to my Parents and Alex

Table of contents

Introduction

Yeast as a model organism in proteome analysis

Budding yeast was the first eukaryotic organism to be completely sequenced (Goffeau et al. 1996). It has a relative small genome size (12 Mb compared to 3,2 Gb in humans) and contains 16 chromosomes encoding approximately 6000 genes. The fact that introns in yeast genes are rare (5% of genes) makes *in silico* gene prediction relatively straight forward and systematic gene nomenclature simplifies data comparison. Moreover, despite that this unicellular eukaryotic organism is only one tenth the size of a white blood cell, many of the cellular functions of higher species are present in yeast. *S.cerevisiae* has nearly 1800 genes homologous to human genes of which approximately 200 are related to human disease genes including 23 related to cancer (Rubin et al. 2000). For these reasons and the fact that yeast cells allow efficient homologous DNA recombination, *S.cerevisiae* has served as the prime model organism for post-genome functional analysis.

Many systematic approaches have been used to study various aspects of the function and dynamics of the genome ranging from the analysis of deletion phenotypes to biochemical activities (Table 1). The yeast community has the most complete repertoire of physical resources: chips, reagents, plasmids, and mutant strains, covering the whole genome of this organism. This reflects the power of forward and reverse genetic approaches in yeast and hence yeast is certainly the best understood eukaryote.

Project	References
Systematic gene deletion EUROFAN I (European Functional Analysis Network) 1995-1999 800 open reading frames were deleted and functionally analysed	(Wach et al. 1994; Oliver 1996)
Systematic gene deletion The Saccharomyces genome deletion project (EUROFANII): Creation of a complete set of bar-coded yeast deletion strains (1996-2002) & phenotypic analysis of sporulation and mating efficiency	(Winzeler et al. 1999; Giaever et al. 2002)
Random transposon mutagenesis TRIPLES (Transposon-Insertion Phenotypes, Localization and Expression in Saccharomyces). >20 000 yeast mutants were generated by random transposon mutagenesis and their phenotypes and localization were subsequently assigned to genes by sequencing.	(Ross-Macdonald et al. 1999)
Large-scale yeast two-hybrid studies of protein-protein interactions (yeast protein interactome)	(Uetz et al. 2000; Ito et al. 2001)
Systematic identification of protein complexes in yeast by mass spectrometry	(Gavin et al. 2002; Ho et al. 2002)

Project	References
Microarray gene expression studies at different conditions	(Hughes et al. 2000)
Global analysis of protein expression in yeast (quantitative immunobloting)	(Ghaemmaghami et al. 2003)
Yeast genetic interaction network (double deletions)	(Tong et al. 2004)
Yeast proteome subcellular localization (GFP and immunolocalization)	(Kumar et al. 2002; Huh et al. 2003)
100 essential genes of unknown function have been fused to a 'heat-inducible-degron' cassette that targets the protein for proteolysis at 37 degrees (degron strains). Integrated analysis (affinity purification and mass spectrometry, two-hybrid analysis, fluorescence microscopy and structure prediction methodology) of 100 unknown essential genes.	(Sanchez-Diaz et al. 2004), (Kanemaki et al. 2003), (Hazbun et al. 2003)

Table 1: Systematic approaches to functional analysis in *S.cerevisiae*

Mark Johnston, president of the Genetics Society of America, predicted at the Yeast Genetics and Molecular Biology meeting in July 2003 in Gothenburg, that the organism's 6,000 genes will be “known” (i.e. annotated) by 2007. This was based on the annotations in the Yeast Proteome Database which have followed a remarkably linear upward trajectory, reaching 4,679 functionally annotated genes in July 2003.

According to the Saccharomyces Genome Database (Dwight et al. 2004) still 1677 (Sept 2004) genes have no known biological function (GO annotations, Ashburner et al. 2000). In addition, even a 'known' gene (i.e. annotated) can still be pretty mysterious. This means that despite the overwhelming information available, there are plenty of unsolved questions in yeast biology.

Hoping to contribute to our understanding of how each yeast gene and each network of genes works together to create a living cell, this thesis deals with a yeast protein domain of “little known” function, the PX domain.

Protein-protein interactions

Protein-protein interactions affect all processes in a cell. Proteins rarely function in isolation. It has been proposed that all proteins in a given cell are connected through an extensive network where non-covalent interactions are continuously forming and dissociating. The forces that are responsible for such interactions include electrostatic forces, hydrogen bonds, van der Waals forces and hydrophobic effects. It is believed that hydrophobic effects drive protein-protein interactions, while hydrogen bonds and electrostatic interactions govern the specificity of the interface. Water is usually excluded from the contact region.

Note that many proteins are known to interact although at present it remains unclear whether all of these interactions have any physiological relevance.

In a single-cell organism such as yeast each of the roughly 6000 proteins interact with 3.3 or more other proteins on average, which adds up to a total of ~20,000

interactions or more (Grigoriev 2004). By extrapolation, there may be approximately ~100,000 interactions in the human body.

Implications about function can be made via protein-protein interaction studies. The function of unknown proteins may be discovered through their interaction with known protein partners of known function.

Protein Domains in Yeast

Proteins are constructed in a modular fashion from a combination of interaction and catalytic domains. A protein domain is defined as a part of a polypeptide chain that can fold into a three-dimensional structure independently.

Protein-protein interaction domains are independently folding modules of 35-150 amino acids that can be expressed in isolation from their host proteins while retaining their intrinsic ability to bind their physiological partners. Protein interactions are usually mediated by defined domains.

The cell uses a limited set of interaction domains (Fig. 1), which are joined together in diverse combinations, achieving in this way the different properties of proteins.

Classification of protein interaction domains

Interaction domains can be identified through their amino acid sequences, allowing the binding properties and biological functions of a protein to be predicted on the basis of its domain composition.

There are several domain databases which base their classification on different computational approaches. Some examples are listed below (Table 2).

Database	Computational approach	URL
PROSITE	patterns (regular expressions)	http://www.expasy.org/prosite/
BLOCKS PRINTS	blocks (ungapped HMMs)	http://blocks.fhcrc.org/blocks/ http://www.bioinf.man.ac.uk/dbbrowser/PRINTS/
SBASE	annotated subsequences	http://hydra.icgeb.trieste.it/sbase/
PRODOM	consensus sequences	http://protein.toulouse.inra.fr/prodom/current/html/home.php
PFAM, ISREC PROFILES SMART	full alignments	http://www.sanger.ac.uk/Software/Pfam/ http://www.isrec.isb-sib.ch/profile/profile.html http://smart.embl-heidelberg.de/
PROCLASS	classification by means other than alignments	http://pir.georgetown.edu/gfserver/proclass.html

Table 2: Domain databases and their computational approaches

Protein domains can be divided into separate families that are related either by sequence, structure or ligand-binding properties (Fig.1).

In addition to interaction domains that engage specific peptide motifs, a growing number of modules have been identified that recognize selected phospholipids, nucleic acids or other molecules. Further, a number of modular domains undergo homo- or heterotypic domain-domain interactions rather than binding short peptide motifs. A subset of interaction domains are illustrated in Figure 1 and their general binding properties are indicated.

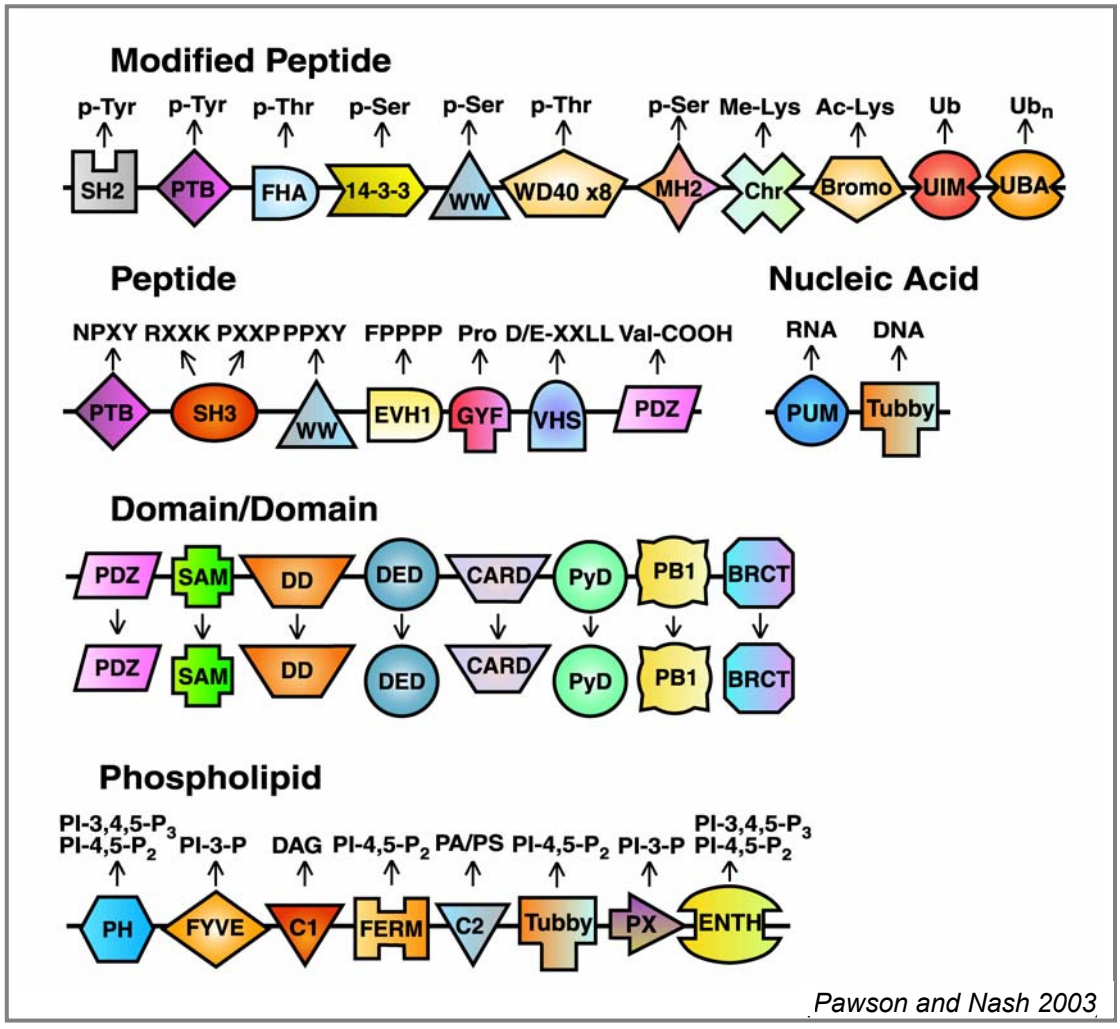


Figure 1: A subset of interaction domains and their general binding properties

According to the SMART Database (Letunic et al. 2004) approximately 300 protein domains have been described in yeast, of which ~20 are still of unknown- or little known- function. Studying the function of these domains will reveal important information about the proteins that contain them.

Phosphoinositides

Function

Cellular membranes composed primarily of lipids and proteins, not only serve as structural boundaries that separate the cell from its extracellular milieu and compartmentalize its interior into discrete functional organelles but also participate in the regulation of many cellular processes. While the functions of a myriad of membrane proteins, such as signaling receptors and ion channels, have been well studied, it is increasingly recognized that the lipid constituents of the membrane are also involved in many regulatory events including cell proliferation, cell survival, differentiation, signal transduction, cytoskeleton organization and membrane trafficking (Odorizzi et al. 2000; Simonsen et al. 2001; Sprong et al. 2001; Vanhaesebroeck et al. 2001; Toker 2002; Gruenberg 2003). Phosphoinositides can serve as a platform for molecular assemblies recruiting and/or activating spatial and temporally effector proteins, but also as a precursor of intra-cellular signaling molecules such as diacylglycerol (DAG) and inoslitol (1,4,5)P_3 (Berridge 1981).
PI is a collective term for Phosphatidyl inositol (PtdIns) and its phosphorylated derivatives (Figures 2 and 3) (Odorizzi et al. 2000; Vanhaesebroeck et al. 2001) .

Structure

PtdIns contains a 1d-myo-inositol phosphate group linked to diacylglycerol (Fig. 2).

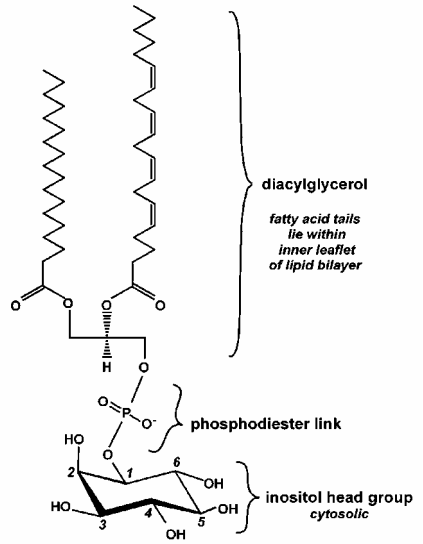
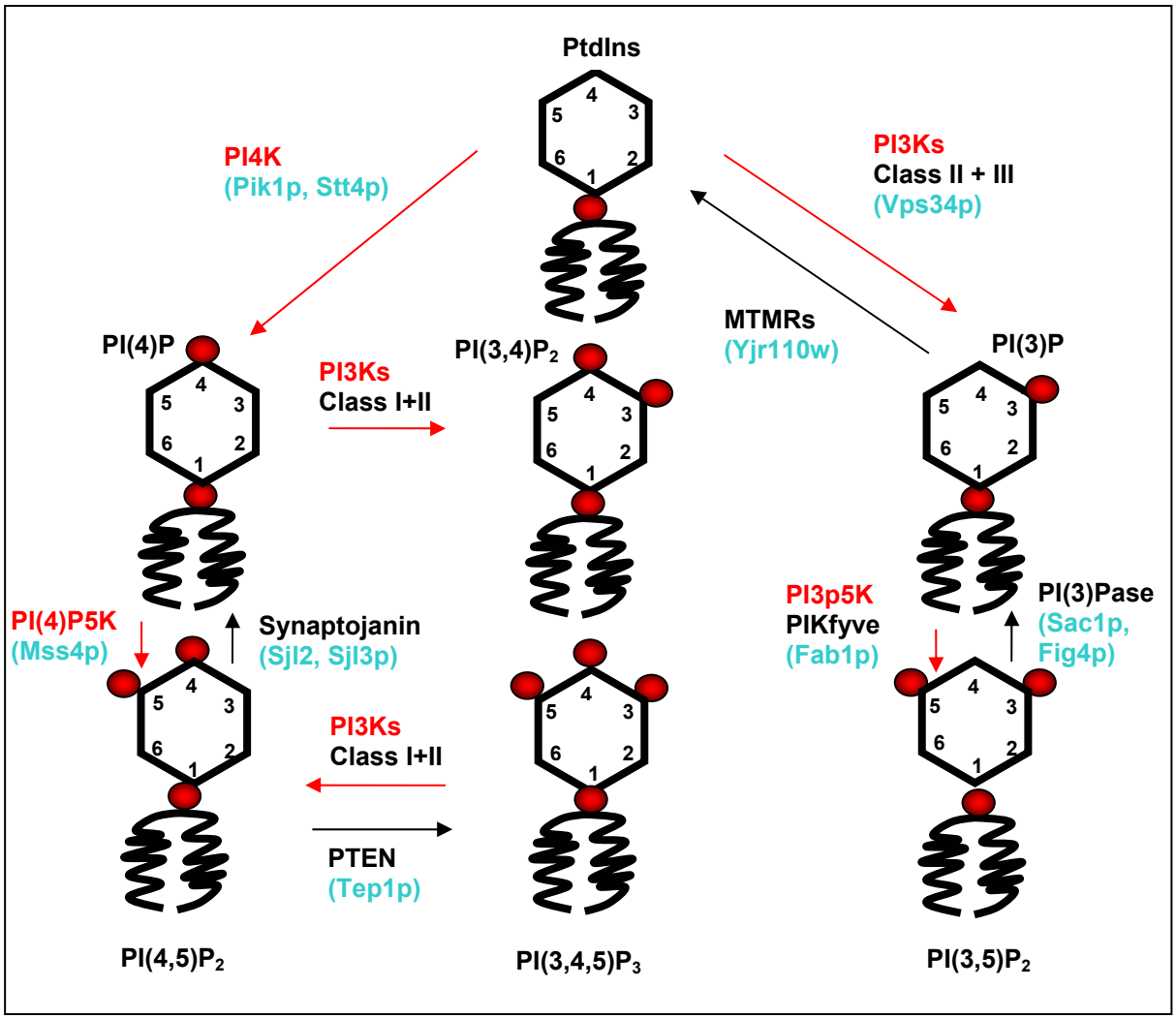


Figure 2: Chemical structure of Phosphatidyl Inositol (PtdIns)

The d-myo-inositol head group of PtdIns contains five hydroxy groups (at positions 2, 3, 4, 5 and 6), three of which (at positions 3, 4 and 5) are known to be targets of phosphorylation. These positions may be reversibly phosphorylated singly, doubly (in various combinations) or triply, yielding a myriad of PtdIns derivatives: PtdIns3P, PtdIns4P, PtdIns5P, PtdIns(3,4)P_2, PtdIns(3,5)P_2, PtdIns(4,5)P_2 and PtdIns(3,4,5)P_3 (Fig. 3).



Adapted from Simonsen et al 2001

Figure 3: Pathways for the synthesis and turnover of phosphoinositides.

The most important phophorylation and dephosphorylation pathways are indicated, with kinases in red and phosphatases in black. Yeast homologues are blue.

Biochemistry

Among the eight PI species known in mammalian cells (including non-phosphorylated PI), PtdIns is most abundant and its concentrations can be 10-20-fold higher than those of PtdIns4P and PtdIns (4,5)P_2, which are present at roughly equal amounts. Among the singly phosphorylated PIs, PtdIns(4)P is the most abundant (90-96%), while PtdIns(3)P and PtdIns(5)P each make up approx. 2-5%. PtdIns(4,5)P_2 is the most abundant of the doubly phosphorylated PIs (99%), while PtdIns(3,4)P_2 and PtdIns(3,5)P_2 each make up about 0.2%. The cellular levels of triply phosphorylated PtdIns(3,4,5)P_3 vary dramatically in response to both external and internal regulation and can be up-regulated to levels comparable to those of PtdIns(3,4)P_2 and PtdIns(3,5)P_2 (Cockcroft S., ed. 2000. Biology of Phosphoinositides. (xford Univ. press, pp. 32–108, (Rameh et al. 1997). The

abundance and presence of the different derivatives vary in different species (see Table 3).

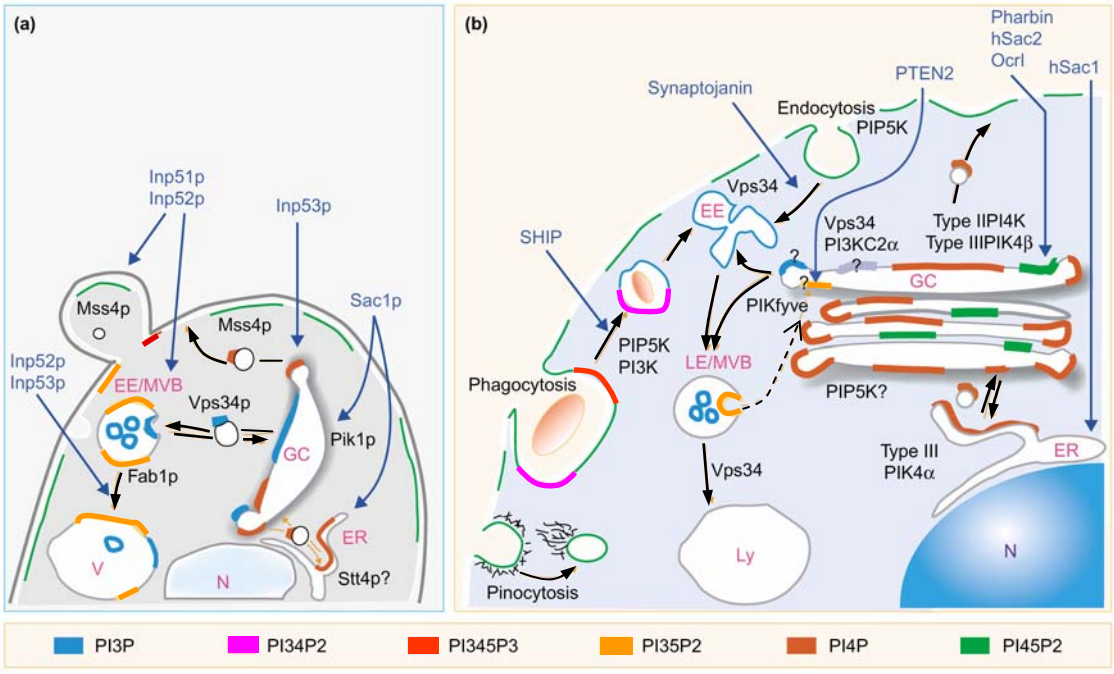
Organism	PI(3)P	PI(3,5)P_2	PI(3,4)P_2	PI(4,5)P_2	PI(3,4,5)P_3	PI(4)P	PI(5)P
Mammals	+	+	+	+	+	+	+
D. melanogaster	NI	NI	NI	+	NI	+	+
C. elegans	NI	NI	NI	+	NI	+	+
D. discoideum	+	NI	+	+	+	+	NI
Plants	+	+	+	+	-	+	NI
Yeast	+	+	-	-	+	+	+

Table 3: Occurrence of PIs in eukaryotic species
NI: not investigated.

Localization

All of these lipid products are membrane-bound, and thus compartmentalized. Direct evidence for this comes from the use of PI binding domains that are recruited to specific subcellular compartments. Although we have only a limited understanding of the distribution of these lipids, it is clearly a critical factor in their selective action. The regulation of their activities and the mechanisms of their actions have therefore been the subject of much investigation. The activities of specific PIs are regulated by intricate networks of proteins that control their synthesis, transport and degradation (PI kinases, PI phosphatases (Fig. 3) and PI transfer proteins) which in turn are also localized to discrete membrane sites restricting signaling to a specific compartment.

Generally, PtdIns(3,4)P_2, PtdIns(4,5)P_2 and PtdIns(3,4,5)P_3 are enriched in the plasma membrane, PtdIns(4)P in the Golgi and PtdIns(3)P is enriched in the endosomal compartments (Fig.4), (De Matteis et al. 2002).



De Matteis et al. 2002

Figure 4: Subcellular distribution of the phosphoinositides in (a) yeast and (b) mammalian cells

PI are colour-coded for identification. EE, early endosomes; LE, late endosomes; Ly, lysosomes; MVB, multivesicular body; N, nucleus; V, vacuole.

Lipid Binding Domains

PI signaling is mediated by protein-lipid interactions with proteins that usually contain one or several lipid binding domains. In the past decades several of this domains have been described (reviewed by Hurley and Meyer 2001; Xu et al. 2001; Itoh and Takenawa 2002; DiNitto et al. 2003; Lemmon 2003). Table 4 summarises lipid-binding domains and their lipid-binding specificities. Figure 5 shows representative lipid-binding domains structures.

Phospholipid-binding domains serve both to concentrate signaling proteins at specific sub-regions of the plasma membrane, and to regulate the enzymatic activities of their host proteins, either directly or by co-recruitment of another regulatory protein.

Domain	Name	Examples	Lipid binding
PH	**P**leckstrin **H**omology	PLC-δ	$PI(4,5)P_2$
		Btk	$PI(3,4,5)P_3$
		Dapp1, PKB	$PI(3,4)P_2$, $PI(3,4,5)P_3$
		3G Grp1, 3G ARNO, 3G Cyt	$PI(4,5)P_2$, $PI(3,4,5)P_3$
		2G **Grp1**, 2G ARNO, 2G Cyt	$PI(3,4,5)P_3$
		Tapp1, Tapp2	$PI(3,4)P_2$
		Centaurin b2	$PI(3,5)P_2$
		PEPP1	PI(3)P
		FAPP1	PI(4)P
AP2-α	AP2-α adaptor complex	**AP2-α_{1-80}**	$PI(4,5)P_2$
FYVE	**F**ab1p **Y**OTB **V**ac1p **E**EA1	Vps27, **EEA1**, Hrs	PI(3)P
PHD	**P**lant **H**omeo **D**omain	ACF, MLZF	PI(5)P
		ING1, ING2,WSTF	PI(5)P, PI(3)P
PX	**Phox** Homology	**p40phox**, yeast PX domains	PI(3)P
		p47	$PI(3,4)P_2$, PS, PA
		CISK	$PI(3,5,)P_2$, $PI(4,5,)P_2$, $PI(3,4,5,)P_3$
		CPK ,PI3K,TCGAP	$PI(4,5)P_2$
ANTH	**A**P180 **N**-**T**erminal **H**omology	**AP180**	$PI(4,5)P_2$
C2		**Syt1**, PKC-α, PTEN	PS, anionic membranes
		cPLA2	neutral membranes
FERM	**4**.1-Ezrin **R**adixin **M**oesin	Ezrin, **Radixin,** PTPL1	$PI(4,5)P_2$
ENTH	**E**psin **N**-**T**erminal **H**omology	**Epsin1**	$PI(4,5)P_2$
		Ent3p, Ent5p	$PI(3,5)P_2$
Others		MARCKS	$PI(4,5)P_2$, $PI(3,4)P_2$, PS
		Tubby	$PI(3,4)P_2$, $PI(4,5)P_2$

Table 4: Lipid-binding domains

Lipid-binding domains and lipid-binding specificities of some examples**.**
PS: Phosphatidyl Serine, **PA:** Phosphatidic Acid. (Proteins in **bold**: structure shown in Fig. 5).

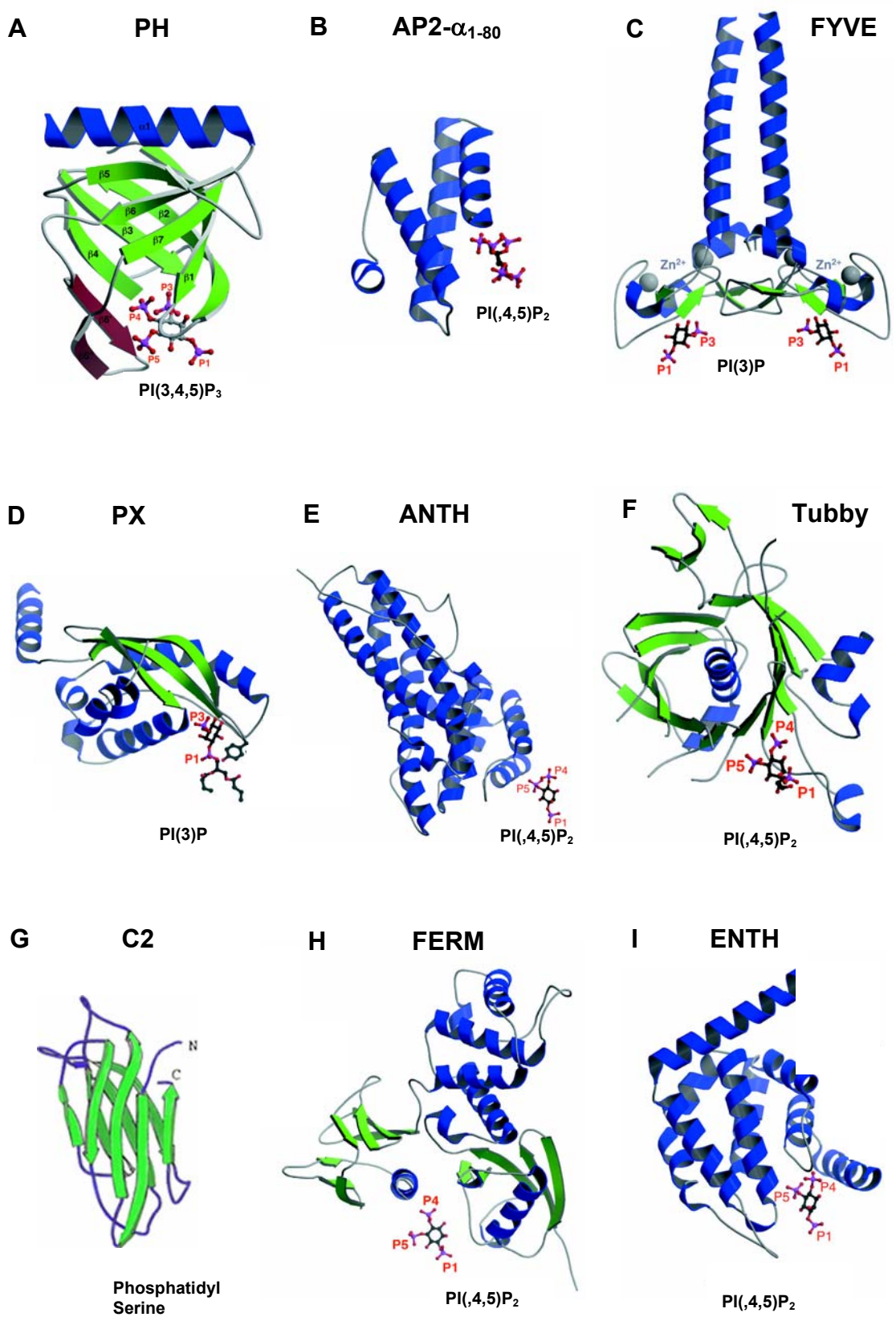


Figure 5: Representative structures of lipid-binding domains and their bound lipid

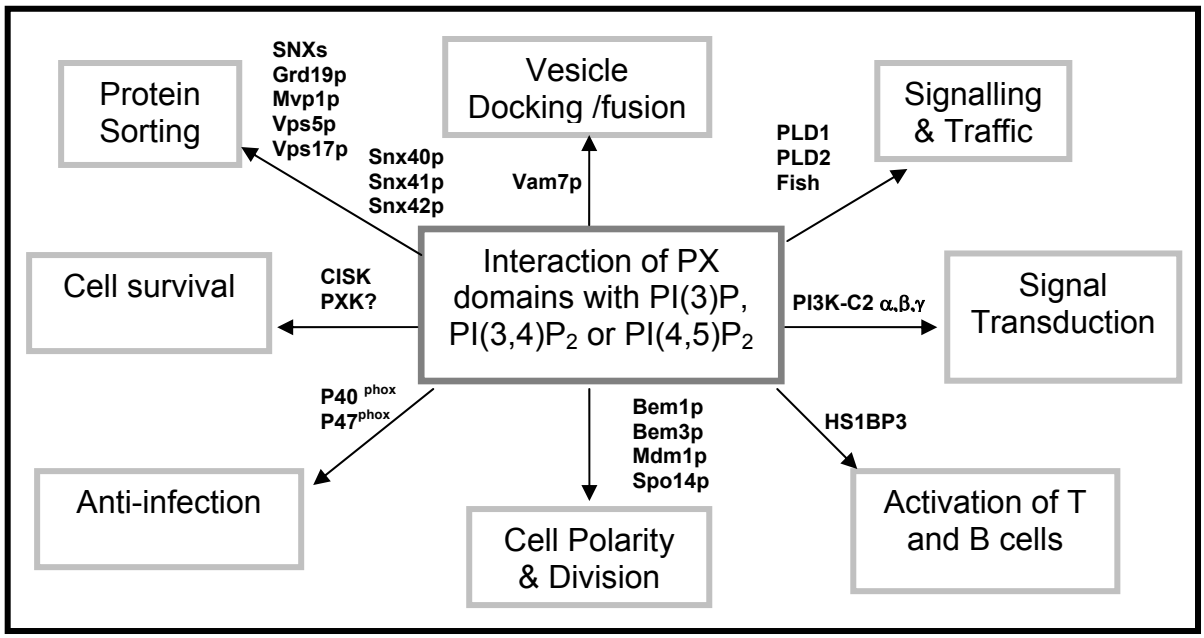
A: PH domain of Grp1, **B:** AP2-$\alpha_{1\text{-}80}$ of AP2-α, **C:** FYVE domain of EEA1, **D:** PX domain of p40phox, **E:** ANTH domain of AP180, **F**: Tubby domain of Tubby, **G:** C2 domain of Syt1, **H:** FERM domain of Radixin, **I:** ENTH domain of Epsin1

The PX domain

The PX domain has only recently been shown to be a novel PI (phosphoinositide)-binding domain. PX domains have been originally identified as a common motif of about 120 amino acids in the subunits $p40^{phox}$ and $p47^{phox}$ of the neutrophil NADPH oxidase (Phox) complex (Ponting 1996), (Fig. 8). This domain is conserved from yeast to man and more than 400 domain family members have been annotated in sequence databases (e.g. SMART, Letunic et al. 2004).

Functions of PX domain containing proteins

PX proteins have been implicated in highly diverse functions such as cell signalling, vesicular trafficking, protein sorting and lipid modification (reviewed in Odorizzi et al. 2000; Xu et al. 2001; Worby and Dixon 2002), (Fig. 6).



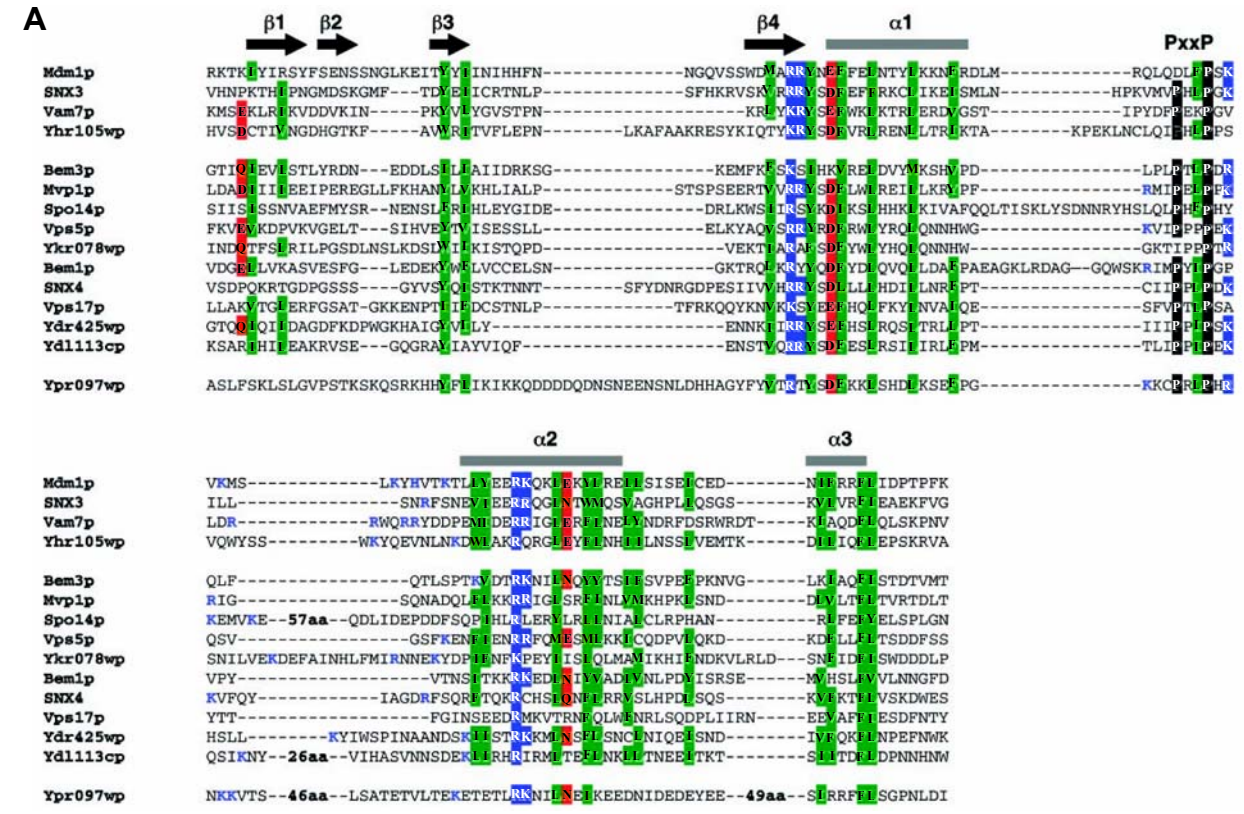
Xu et al. 2001

Figure 6: Diverse cellular functions regulated by interactions of PtdIns with PX domain-containing proteins

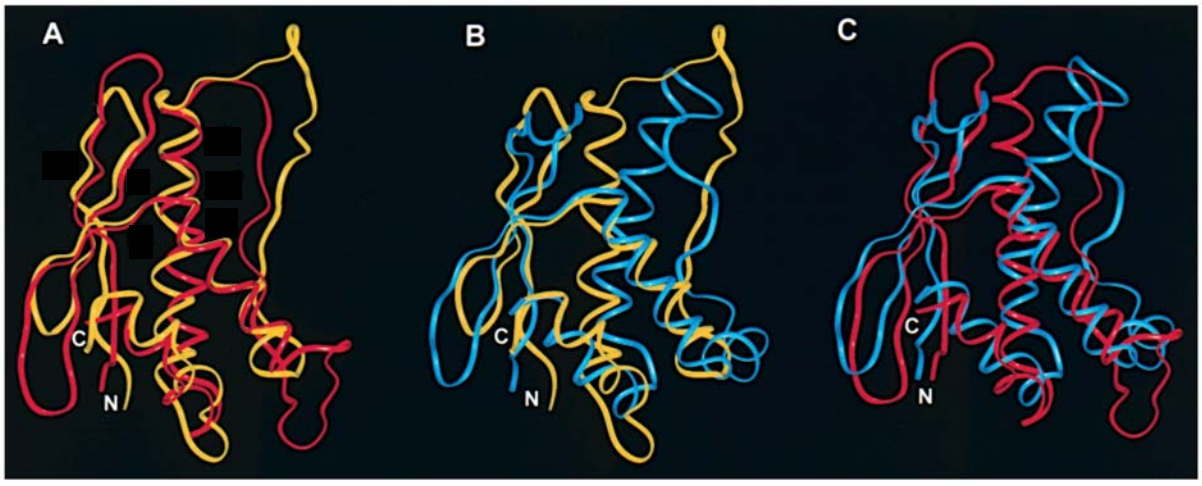
PX domain structure and PI binding affinities

PX domains show surprisingly little sequence conservation (Fig.7 A) yet their structure appears to be highly conserved based on several available crystal structures (Bravo et al. 2001; Hiroaki et al. 2001; Lu et al. 2002; Zhou et al. 2003; Xing et al. 2004) (Fig.7 B). All published crystal structures exhibit a clearly defined PI-binding pocket and in at least one case a second binding pocket has been found to bind phosphatidic acid (Kanai et al. 2001; Karathanassis et al. 2002).

PX domains represent distinct PI binding specificities. For example, the PX domains of $p40^{phox}$ and Snx3 bind to PtdIns(3)P (Ago et al. 2001; Kanai et al. 2001; Song et al. 2001; Xu et al. 2001; Karathanassis et al. 2002), while the PX domain of $p47^{phox}$ binds to PtdIns(3,4)P_2 and phosphatidic acid (Kanai et al. 2001; Karathanassis et al. 2002),(Table 4).



B



Lu et.al 2002

Figure 7: (A): Sequence alignment of all yeast PX domains

Elements of secondary structure as depicted *above* the sequences. Conserved hydrophobic and aromatic residues are highlighted in *green*, conserved basic residues are highlighted in *blue*, conserved amide and acidic residues are highlighted in *red*, and prolines in the P*XX*P motif characteristic of PX domains are highlighted in *black* (Yu and Lemmon 2001).

(B): Overall comparison between PX domain structures

Superposition of Vam7p, p40^{phox}, and p47^{phox} PX domain structures. (A) Vam7p (yellow) vs p40 ^{phox} (red), (B) Vam7p (yellow) vs p47 ^{phox} (blue), and (C) p40^{phox} (red) vs p47phox (blue) (Lu et al. 2002). Note that the crystal structure of p40 ^{phox} PX domain is PtdIns(3) bound and that the PX domain of p47^{phox} contains an additional anionic phospholipid binding pocket (Karathanassis et al. 2002).

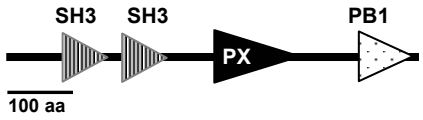
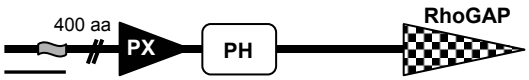
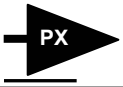
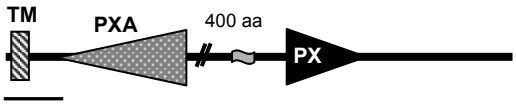
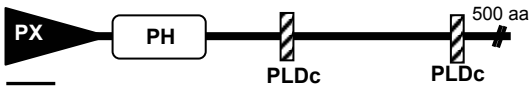
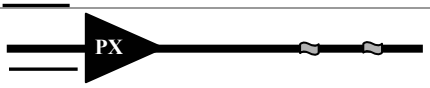
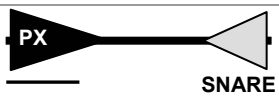
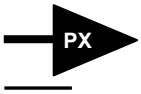
Name ORF	Domain Structure	Function	Loc.
Bem1p YBR200W		Cell polarity, bud formation	BN, B,P
Bem3p YPL115C		Cell polarity, bud formation	BN, C
Grd19p YOR357C		PVC - Golgi retrieval	VM,C, PVC
Mdm1p YML101C		Cytoskeleton organization, mitochondrial morphology and inheritance	C
Mvp1p YMR004W		Vacuole targeting	C, E, PVC
Spo14p YKR031C		Pospholipase D. Meiosis, spore formation	C , PM
Snx4p YJL036W		PGE – Golgi retrieval	E, C, PVS
Snx41p YDR425W		PGE – Golgi retrieval	E, C
Snx42 YDL113C		PGE – Golgi retrieval	E, C
Vam7p YGL212W		Vacuolar morphogenesis	C, V, VM
Vps17p YOR132W		PVC - Golgi retrieval.	E, C , PVC
Vps5p YOR069W		PVC - Golgi retrieval	E , C , PVC
Ypt35p YHR105W		Unknown	E
Ykr078p YKR078W		Unknown	C
Ypr097p YPR097W		Unknown	U

Table 5: PX-domain containing yeast proteins

: Predicted coiled-coil regions, **PVC:** prevacuolar compartment, **PVS:** Perivacuolar structure, **PGE:** Post-Golgi endosome, **BN:** budneck**, B:** bud, **C:** cytoplasm, **VM:** vacuolar membrane, **E:** endosome, **U**: unknown, **PM:** prospore membrane, **V:** vacuole. Localization data (Loc.) is retrieved from the references (McConnell and Yaffe 1992; Ekena and Stevens 1995; Sato et al. 1998; Voos and Stevens 1998; Sciorra et al. 1999; Burda et al. 2002; Nice et al. 2002; Seaman and Williams 2002; Hettema et al. 2003; Huh et al. 2003; Vollert and Uetz 2004).

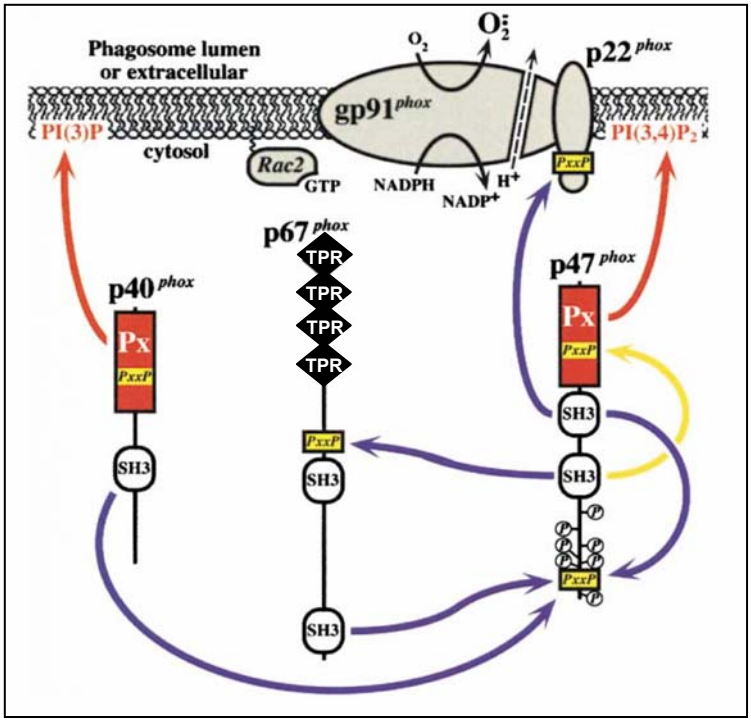
Yeast PX domain containing proteins

The yeast genome encodes 15 PX domain containing proteins involved in vesicular trafficking (Grd19p, Mvp1p, Snx4p/Cvt13p, Snx41p, Snx42p/Cvt20p/Atg20p, Vps5p, Vps17p, Vam7p), cell signalling (Spo14p), control of bud emergence and cell polarity (Bem1p, Bem3p, Mdm1p) and several proteins of largely unknown function (Ypt35p, Ykr078p, Ypr097p) (Table 5). It has been shown that all yeast PX proteins (except for the sequence outlier Ypr097p) bind specifically to PtdIns(3)P with different affinities (Yu and Lemmon 2001).

PX domain and protein-protein interactions

Although most PX domains contain additional domains or at least additional protein sequences, two yeast proteins, Grd19p and Ypt35p, consist almost exclusively of the PX domain (Table 5). The assumption that a signaling protein does not only bind to a membrane for its own sake suggested that the PX-only proteins are involved in protein-protein interactions in order to transduce a signal from the membrane to some effector. This is supported by several genetic observations in mutant PX proteins. For example, yeast strains with a deletion of their GRD19 gene missort the TGN membrane proteins A-ALP, Pep12p and Kex2p to the vacuole (Voos and Stevens 1998; Hettema et al. 2003). A direct involvement of the PX domain in protein-protein interactions has been observed in the subunit $p47^{phox}$ of the neutrophil NADPH oxidase complex. $p47^{phox}$ is held in an inactive state by an *intramolecular* interaction between its C-terminal SH3 domain and its PX domain. Upon activation by invading microorganisms $p47^{phox}$ becomes phosphorylated and the PX-SH3 interaction is disrupted (Leto et al. 1994; Sumimoto et al. 1994). The C-terminal SH3 domain interacts with a proline-rich region of the $p47^{phox}$-PX domain (Hiroaki et al. 2001), (Fig. 8).

Another PX-domain mediated protein interaction may involve the PX-only protein Grd19p. It was shown to bind to the cytosolic domain of DPAP A. However, this study used yeast extracts containing a His-tagged DPAP A fusion protein (Voos and Stevens 1998). Hence it is possible that Grd19p did not interact directly with DPAP A but rather bound via some other protein such as Vps35p (Nothwehr et al. 2000). Finally, a recent large-scale two-hybrid screen has shown that both PX-only proteins from yeast, Grd19p and Ypt35p (YHR105W) interact with a number of other proteins (Uetz et al. 2000). Nevertheless, as these proteins also contain small N-terminal extensions it could not be ruled out rigorously that these sequences were actually mediating the observed interactions.



Wishart et al. 2001

Figure 8: Model of Phosphoinositide and SH3:PxxP Interactions for PX Domain-Containing Proteins Involved in Targeted Membrane Assembly of the Phagocyte NADPH Oxidase

Selected regions of NADPH oxidase subunits gp91phox, $p22^{phox}$, $p40^{phox}$, $p67^{phox}$, $p47^{phox}$, and p21Rac1/2 are schematically represented as follows: phox domain, PX (red); src-homology 3 domain, SH3; proline-rich motif, PxxP (yellow); tetratricopeptide repeat, TPR; phosphorylation site, P. Specific phosphoinositides (orange) are depicted within a lipid bilayer. Dark arrows denote SH3:PxxP interactions previously identified for $p47^{phox}$. Red and yellow arrows indicate lipid and protein interactions involving $p40^{phox}$- and $p47^{phox}$-PX domains.

Lipid binding domains and protein-protein interactions

Protein interaction additionally to lipid binding seems to be a common phenomenon for PI binding domains. Analysis of PH domains, identified further unknown components (possibly connected by protein-protein interactions) besides their phospholipid-interaction which may play a role in their localisation to certain organelles (Levine and Munro 1998; Levine and Munro 2001; Levine and Munro 2002). For several PH domains interacting partners have been described although up to date, no physiological relevance has been reported (Cozier et al. 2004). Recently De Matteis and co-workers have demonstrated a direct interaction of the PH domains of FAPP1 and FAPP2 (four-phosphate-adaptor protein) with the small GTPase ADP-ribosylation factor (ARF) in mammalian cells. Both proteins localize to the trans-Golgi network (TGN) on nascent carriers. Additionally they were able to prove that a displacement or knockdown of FAPPs inhibits cargo transfer to the plasma membrane and overexpression of FAPP-PH impairs carrier fission (Godi et al. 2004). Protein-protein interactions have also been found in FYVE domains. Corvera and co-workers were able to demonstrate that the integrity of the FYVE domain of EEA1 (early endosome antigen 1) in conjunction with a N-terminal stretch of 30 amino acids are essential for Rab5 association (Lawe et al. 2000).

The PX-only proteins Grd19p and Ypt35p and their phenotypes

Grd19p and Ypt35p consist almost exclusively of the PX domain. Therefore, their mutant phenotypes are of particular interest with respect to the function of their PX domains.

Grd19p was identified by Voos and Stevens (Voos and Stevens 1998) in the course of a Golgi retention defective (Grd) screen (Nothwehr et al. 1996). This screen used A-ALP (a model late-Golgi membrane protein consisting of the cytosolic domain of DPAP-A = dipeptidyl amino peptidase A) fused to the transmembrane and luminal domains of ALP [Alkaline phosphatase]) as an assay for loss of retention of late-Golgi membrane proteins. The yeast mutants with mislocalization of A-ALP (from the late Golgi to the vacuole) were classified into different Grd complementation groups.

Further proteins found in the yeast TGN are the integral membrane proteins Kex1p and Kex2p, which are involved in the proteolytic processing of the secreted mating α-factor. The carboxy peptidase Y (CPY) sorting receptor (Vps10p), also predominantly localizes to the yeast TGN. Kex2p was also found missorted from the TGN to the vacuole in *GRD19* deletion strains. This was not the case for CPY and its receptor Vps10p which were sorted normally (Voos and Stevens 1998). Therefore, mutations in *GRD19* do not affect the general vesicle traffic between the yeast Golgi and vacuole, but rather specific sorting processes involving the resident late-Golgi membrane proteins DPAP A and Kex2p.

Voos and Stevens were also able to demonstrate the mechanism of correct localization of A-ALP, which is due to retrieval of the protein from the late endosomes rather than its static retention at the TGN (Voos and Stevens 1998).

Hettema and co-workers (Hettema et al. 2003) described missorting of Pep12p, a TGN SNARE protein (soluble N-ethyl-maleimide-sensitive fusion protein attachment protein receptor, mediator of fusion of vesicles from the TGN to the late endosomes) to the vacuole in *GRD19* deletion strains. Contrary to the SNAREs Snc1p, Tgl1p and Tgl2p, which are normally distributed at the early endomosmes when *GRD19* is deleted (Hettema et al. 2003). The knock-out of *GRD19* did not result in any growth defects nor any perturbation of vacuolar morphology or vacuolar biogenesis (Voos and Stevens 1998).

Based on the sequence homology within the PX domain, Voos and Stevens analysed possible genetic interactions between the *GRD19*, *VPS5*, *YPT35* and *MVP1* genes. The Golgi localization defects of *grd19*Δ cells could not be suppressed by overexpression of *VPS5* or *MVP1*. Additionally, analysis of the double deletion mutants *grd19*Δ *vps5*Δ and *grd19*Δ *mvp1*Δ did not reveal any synthetic phenotypes concerning growth, CPY secretion, or A-ALP localization. A double deletion of *grd19*Δ and *ypt35*Δ (gene with the greatest sequence homology, 26% identity, 48% similarity) did not amplify or suppress any of the phenotypes due to the *grd19*Δ mutation.

Analysing the sorting of Snc1p (a SNARE localized at early endosomes) Hettema and co-workers were not able to find an interaction between Grd19p and Snx4p, Snx41p and Snx42p.

Summarizing this data, the precise mechanistic role of Grd19p remains uncertain. Grd19p is required for the retrieval of Kex2p, A-ALP and Pep12p, all of which also require the retromer complex (Reddy and Seaman 2001). This suggests that Grd19p

may associate with the retromer complex, improving the recruitment of some but not all cargo molecules. Such an association has not been demonstrated, and whether Grd19p directly recognizes cargo is also unclear. Hettema and co-workers have not observed binding of Grd19p to Pep12p, either by cross-linking or by affinity chromatography. Although Grd19p has been reported to bind the cytoplasmic tail of A-ALP (Voos and Stevens 1998), there is also evidence that recognition of A-ALP is mediated by the retromer subunit Vps35p (Nothwehr et al. 1999; Nothwehr et al. 2000). Grd19p may therefore act indirectly, by modulating retromer binding.

Ypt35p deletion shows no obvious growth defect as the double mutant *grd19*Δ/*ypt35*Δ did not amplify or suppress any of the phenotypes of the *grd19*Δ mutation (Voos and Stevens 1998). Further, no misslocalization of Pep12p, Tgl1p, Tgl2p and Snc1p was observed (Hettema et al. 2003). Additionally CPY, PrA and ALP processing was normal in *grd19*Δ strains (Bonangelino et al. 2002). Up to now no phenotype could be observed by the deletion of *YPT35*. Analysis of large scale two-hybrid data predicted this protein to be involved in protein modification and being a oxidoreductase (Hishigaki et al. 2001). This is in contrast to a possible function in Golgi to vacuolar transport, as suggested by network-based statistical analysis (Samanta and Liang 2003). An analysis of interacting partners could shed light on the function of this protein.

Aim of my PhD work

The aim of this PhD work was to investigate the role of the PX domain as a bona fide protein interaction module. This would open a new perspective on lipid binding domains and their regulation.

Project outline

Searching for interacting partners

In order to identify interacting partners of the PX-domain in yeast, my starting point was to perform genome-wide two-hybrid screens. The 15 PX domain containing proteins in yeast and their isolated domains (except for Ypt35p and Grd19p which are composed almost exclusively of their PX domain) were constructed and used as baits (see below for definition of two-hybrid terms).

Confirmation of obtained interactions via a second independent method

In a second step the two-hybrid interactions should be confirmed by an alternative technique. This was done *in vitro* by means of GST pulldowns.

Mapping the interacting region

In a third step the exact binding region for the PX domains should be mapped. This was done by means of prey fragmentation, point mutation on the lipid binding pocket and peptide arrays.

This work shows that the PX domain is a protein-protein interaction module binding predominantly to membrane proteins that are involved in vesicular transport (Vollert and Uetz 2004)

The Two-Hybrid System

Definition

The two-hybrid technique is an experimental procedure to detect protein-protein interactions that uses two fusion proteins ("hybrids") which reconstitute an active transcription factor when the two fusion proteins interact (Fig. 9). The technique was developed by Fields and Song (1989). It has been estimated that more than 50% of all protein interactions described in the literature have been detected using the yeast-two hybrid system.

Basic Principle of the classical Yeast-Two Hybrid System

In order to test if two proteins X and Y interact, they are expressed as fusion proteins with a transcriptional activating domain (AD, **prey**), and a DNA-binding domain (DBD, **bait**), respectively. The term "two-hybrid" derives from these two chimeric proteins. The bait and prey fusions are co-expressed in yeast where a physical interaction between X and Y leads to the reconstitution of a functional transcription factor that binds to the promoter of a reporter gene (Fig. 9). Consequently, transcription of the reporter gene is switched on. A protein-protein interaction is therefore translated into reporter gene activity which can be detected or measured. The interaction must take place in the nucleus for the subsequent activation of the reporter gene. For this purpose non-nuclear proteins are targeted to the nucleus. The two-hybrid system can be used for the detection of essentially any protein-protein interaction, independent of the function of the corresponding proteins.

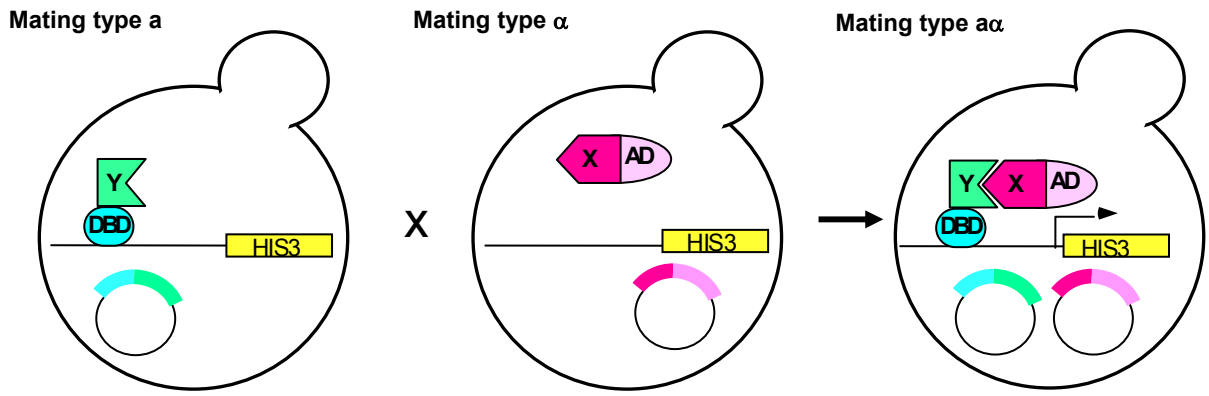


Figure 9: Classical Yeast Two-Hybrid System

A protein of interest Y is expressed in yeast as a fusion to a DNA-binding domain (DBD, "bait"; circles denote expression plasmids). Another protein of interest X is fused to a transcriptional activation domain (AD, "prey"). The two yeast strains are mated to combine the two fusion proteins in one cell. If proteins Y and X interact in the resulting diploids cells, they reconstitute a transcription factor which activates a reporter gene (here: HIS3) and therefore allows the cell to grow on selective media (here: media lacking histidine).

Peptide Synthesis

SPOT synthesis which was originally introduced by Frank (1992) is an easy and flexible technique for simultaneous and parallel chemical synthesis of peptides at distinct positions on a membrane support. The SPOT method has opened up unlimited opportunities to synthesize and subsequently screen large arrays of synthetic peptides (eg., Otte et al. 2003; Landgraf et al. 2004). Peptide arrays prepared by SPOT synthesis can be used to study molecular recognition events, such as epitope mapping, the analysis of protein-protein and protein-nucleic acid interactions, and identification of biologically active peptides. Peptide arrays can be applied to precisely depict molecular recognition events on the single amino acid level.

SPOT synthesis technique

Figure 10 illustrates of SPOT synthesis method on cellulose. A commercially available filter paper (e.g. Whatman) with hydroxyl functional groups can be uniformly converted to amino functions through coupling with activated Fmoc-ß-Ala-OH and the subsequent removal of the Fmoc-group (**I**). The spots are then predefined by dispensing small droplet of Fmoc-ß-Ala-pentafluorophenyl ester solution onto the distinct sites on the membrane (**II**). This process can be done either by a pipetting robot (Intavis Bioanalytical Instruments AG, Bergisch Gladbach, Germany) or manually. After the removal of the Fmoc group, Fmoc-ß-Ala active ester or other activated building blocks can be coupled to the defined spots as spacer/linker (**III**). The following peptide chain elongation steps are conventional solid phase peptide synthesis (Merrifield 1963). It is a repetitive procedure, each cycle starting with removal of N-terminal protecting group of the immobilized peptide chain on cellulose (**IV**), followed by coupling of the pre-activated amino acids with N-terminal and side chain suitably protected (**V**). The peptide chain is growing in the direction from carboxyl to amino-terminal (C->N). In between each step, the washing step is carried out by using respective reagents and solvents (**VI**). Finally, the peptide is generated by the removal of all side chain protecting groups (**VII**), which is ready for subsequent chemical or biological screening directly on the cellulose membrane.

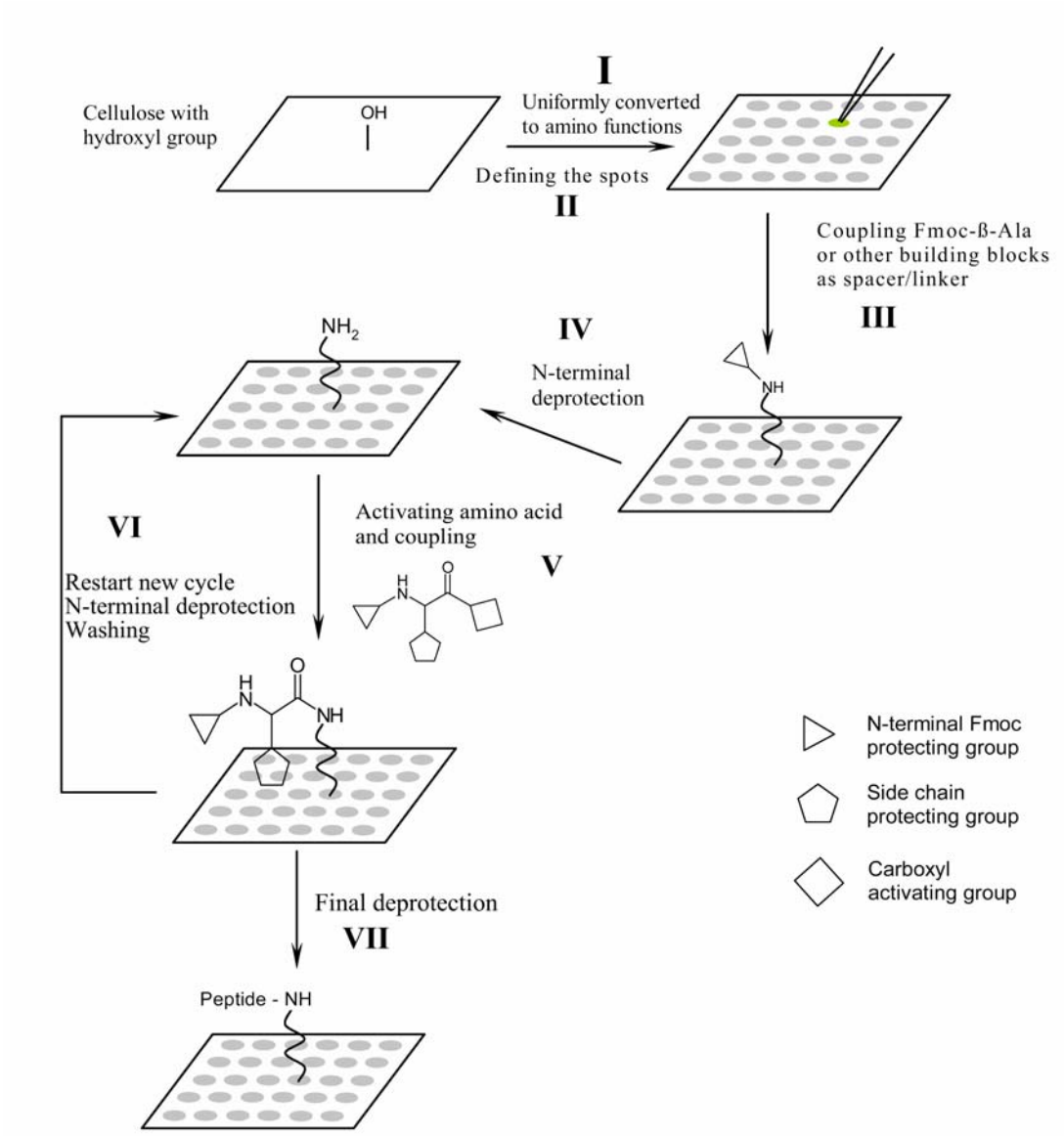


Figure 10: Scheme of SPOT synthesis method in the application of synthesizing peptides on cellulose. Steps **I-VII,** see text.

Analysis of protein- peptide contact sites based on SPOT synthesis

Protein interactions can be studied at amino acid level since the use of protein-derived scans of overlapping peptides (Geysen et al. 1984). SPOT technology allows the analysis and mapping of interaction sites of protein complexes in a rapid and straightforward way. Proteins interact via surface-accessible interaction sites which involve amino acid residues and backbone contacts along a continuous segment of the protein chain (linear epitopes) or involve amino acid residues from at least two segments close in space by the folded conformation (conformational or discontinuous epitopes) but separated in the primary sequence (Fig. 11). The term “epitopes” mentioned here is defined as the contact sites of a protein that interacts with its binding partner. In linear epitopes, the key amino acids mediating the contacts with the binding partner are located within one part of the primary structure and comprise no more than 15 amino acids in length, usually 8-12 amino acids. Peptides covering such epitopes are usually able to compete with the protein-protein interaction and have similar affinities as the intact protein from which they are derived (Reineke et al.

1999). The mapping of linear epitopes by standard SPOT method using overlapping peptides derived from the entire primary sequence of a protein is an easy and efficient approach. The whole protein sequence is fragmented and synthesized on cellulose with short overlapping peptides (normally 13-15 amino acids in length and shifted by 3 amino acids), the so called peptide scan, which is subsequently probed for binding to the respective partner protein. The binding assay is either performed directly on the peptide membrane through immunodetection of bound probe protein or autoradiography of radiolabeled probe protein, or after transfer to a nitrocellulose membrane. The mapping of a linear epitope is shown in Figure 11(A). The sequences colored red are the linear contact site with detectable signals in binding assay.

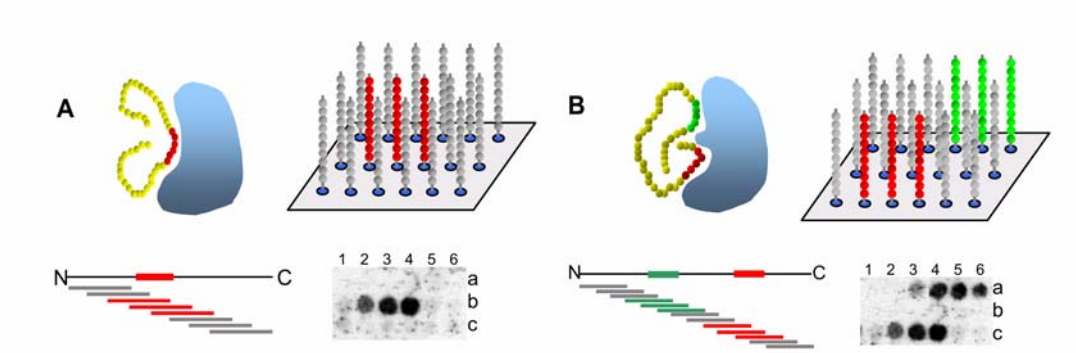


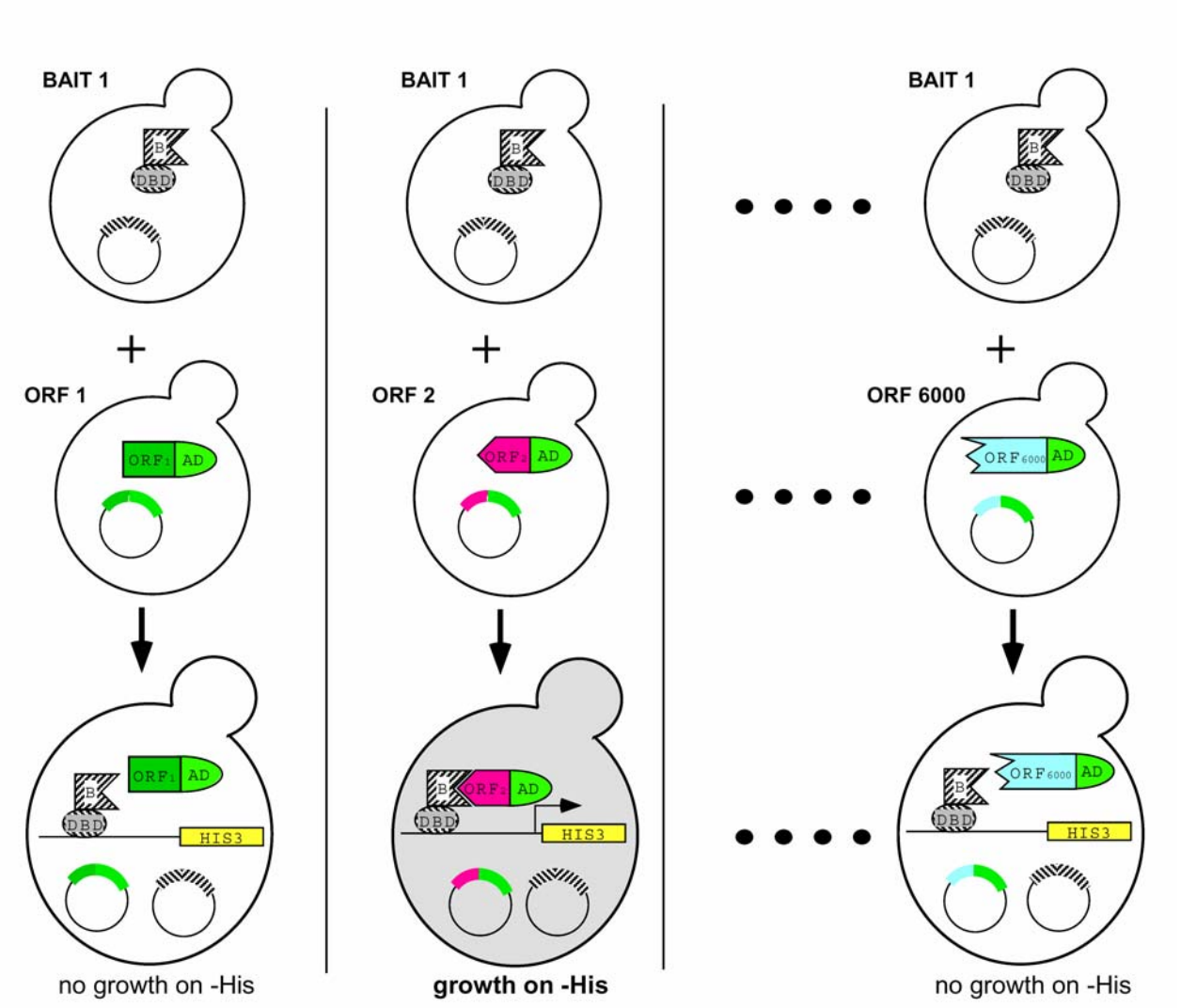
Figure 11: Scheme of mapping linear (A) and discontinuous (B) protein-protein interaction epitopes

Since protein-protein interactions are usually mediated by relatively large contact interfaces (Jones and Thornton 1996), in most cases proteins have more than one binding site to interact with their binding partners. In such discontinuous (conformational) binding sites the key residues contributing to the binding affinity are normally separated in the primary structure, but closer in the conformation on the surface of a folded protein to form a conjunct binding epitope (Fig. 11, B). The mapping of discontinuous epitopes using fragments of protein either generated chemically or biologically suffers from the drawback that peptides comprising only a single binding region of a discontinuous binding site generally have very low affinities for the binding partner in solution, even if the complete binding site mediates a high affinity interaction. Thus, the mapping of discontinuous epitopes with overlapping peptides on cellulose might be very difficult. However, the SPOT approach has the unique advantage that the density of the immobilized peptides on membranes can be as high as 50 nmol/cm^2 or even higher, which corresponds to a high local peptide concentration (mM level or higher, estimated) and thus increases the screening sensitivity. In this way, the inherent defect that peptides comprising only the individual binding regions normally having low affinities for the binding partner can be overcome to a great extent by the SPOT method. The scheme of mapping discontinuous epitopes is shown in Figure 11 (B), where the sequences coloured red and green are separated in primary protein sequence individually with detectable signals in the binding assay.

Experimental procedures

Yeast two-hybrid assay high-throughput screening

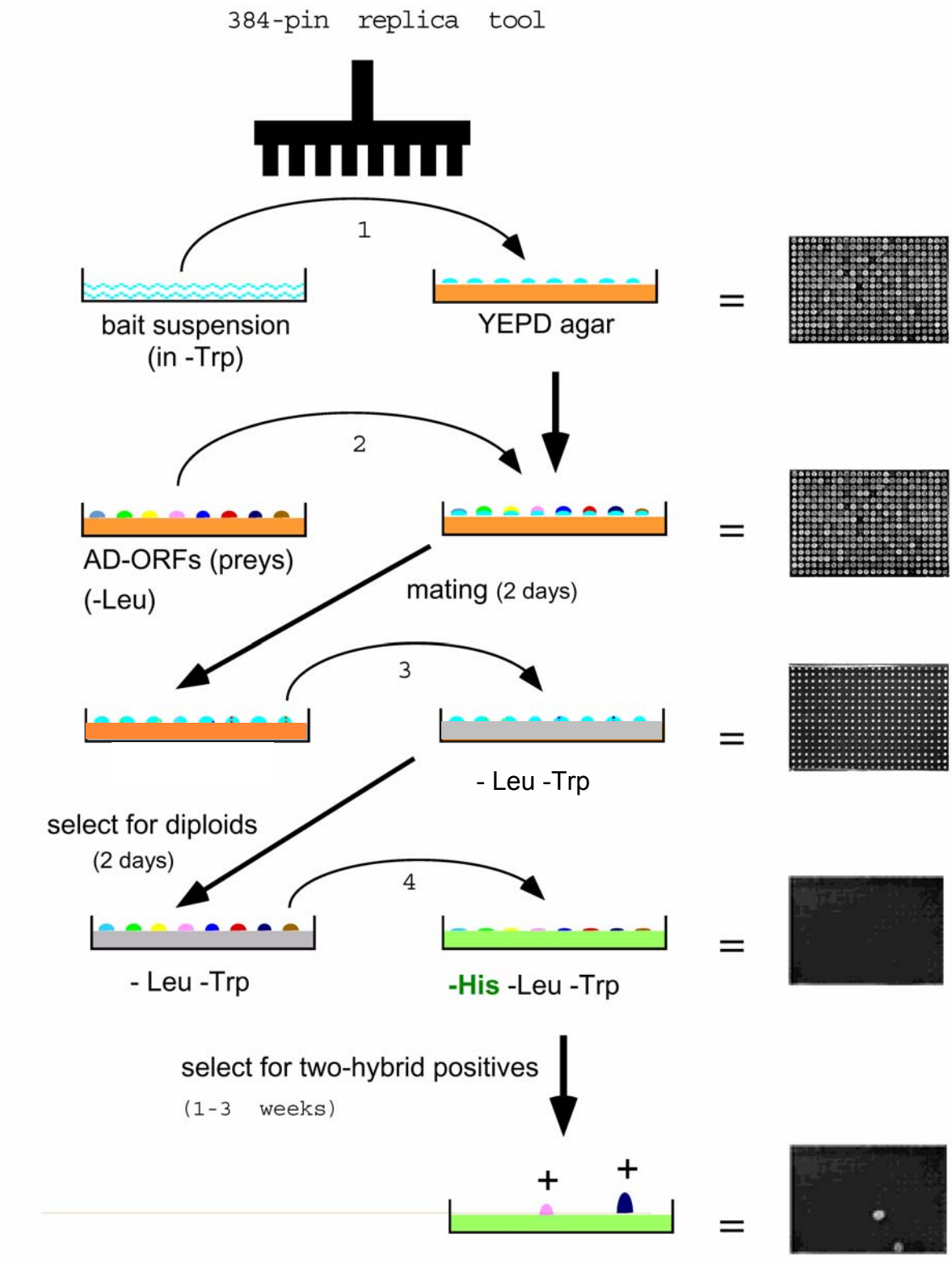
Schematic overview:



A strain expressing a Gal4 DNA-binding domain-ORF fusion protein (top row) is mated to 6000 different yeast strains of the opposite mating type that expresses one of the yeast ORFs fused to the Gal4 activation domain (second row). If the hybrid proteins interact, a transcriptional activator is reconstituted and a reporter gene (HIS3) is expressed. This expression allows the diploid cell to grow on selective media lacking histidine. Each column represents one individual two-hybrid test.

Interaction mating using robotic procedures (Cagney et al. 2000)

Methodical Scheme:



1. Haploid yeast expressing the DNA-binding domain-ORF fusion protein is transferred from a liquid culture to solid medium.
2. Haploid cells from the activation domain array are transferred with the same tool onto the DBD-ORF fusion-expressing cells for mating.
3. After 2 days of growth, the colonies are transferred to –Leu-Trp medium to allow growth of diploids.
4. After 2 days the diploid cells are transferred in step to two-hybrid selective plates (-Trp –Leu –His), on which visible colonies start to grow after a few days. Weak two-hybrid positives may, however, take up to 3 weeks or more to form colonies.

Required material:

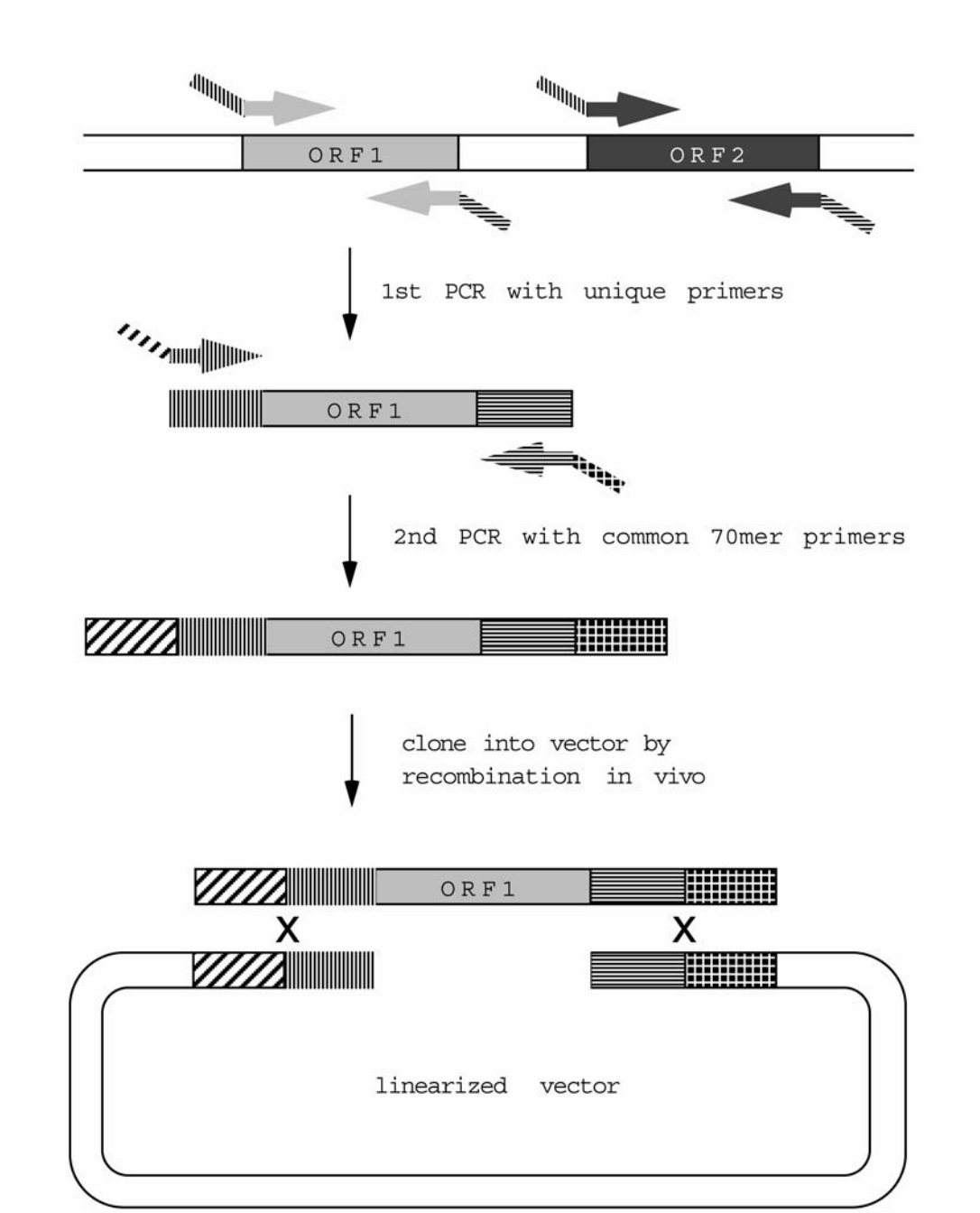
- Yeast synthetic media lacking appropriate nutrients
- 384-pin replicating tool: Biomek 2000 Laboratory Automation Workstation (Beckman-Coulter)
- Yeast strains expressing Gal4p AD-ORF fusion proteins arrayed in 384-colony format (Prey)
- Yeast strain expressing Gal4p DBD-ORF fusion (Bait)

Procedure:

- Inoculate 20ml of complete yeast medium (YEPD) with a 0.3ml liquid pre-culture of the strain expressing Gal4p DBD-ORF fusion into a 250ml conical flask. The culture is suitable for use for several days if stored at 4°C.
- Grow overnight at 30°C.
- Transfer the DBD-ORF culture onto OmniTrays containing solid YEPD medium using a 384-pin replicating tool. Allow the yeast to dry onto the plates for 10-20 minutes.
- Transfer the yeast AD-ORF array elements directly onto the dry DBD-ORF cultures using the same tool.
- Incubate for 1-2 days at 30°C to allow mating of the strains.
- Transfer the colonies to OmniTrays containing medium lacking leucine and tryptophane.
- Incubate for 2-4 days at 30°C to allow diploid cells to grow.
- Transfer the colonies to OmniTrays containing selective media lacking leucine, tryptophane and histidine, which had been supplemented with an appropriate amount of 3AT (for nonactivating DBD-ORF strains routinely 3mM 3AT is used).
- Incubate for 1-3 weeks at 30°C. Score and document the screen (take photos and Filemaker Database).

Bait and Prey construction

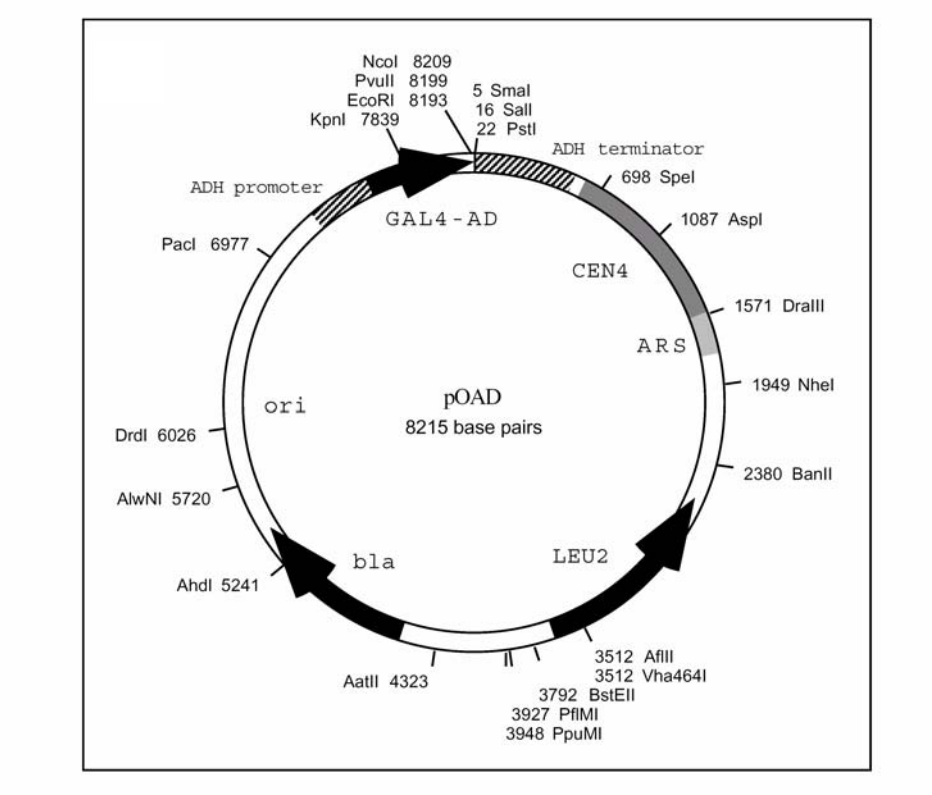
Strategy:



Yeast ORFs are amplified (first PCR) with specific primers that generated products with common 5' and common 3' 20-nucleotide tails. A second PCR generates products with common 5' and common 3' ~70-nucleotide tails. The common ~70-nucleotide ends allow cloning into linearized two-hybrid expression vectors by recombination.

Vectors

Low copy prey vector: pOAD (Cagney et al. 2000)



Sequence features:

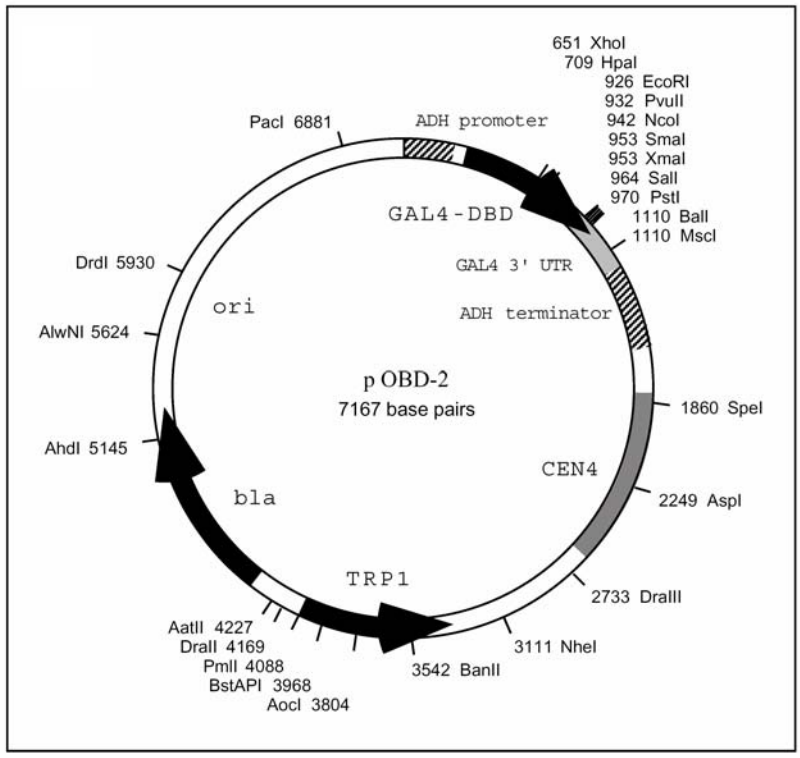
• **LEU2 (leucine auxotrophy)**: 4045-2954
• **bla: beta-lactamase** (ampicillin resistance): 4673-5527
• **CEN4** (centromere element for stable maintenance of the plasmid): 866-1719
• **ARS1**: 1748-1964 (corresponds to sequence between TRP1 and GAL3 on yeast chromosome 4)
• **GAL4**-AD (activation domain): 7996-8429 (first 66 bp not from Gal4)

Cloning site (position 8358-8370):

C TAT CTA TTC GAT GAT GAA GAT ACC CCA CCA AAC CCA AAA AAA GAG ATC GAA TTC CAG CTG ACC ACC ATG GCA ATT CCC GGG GAT CCG TCG ACC TGC AGA GAT CTA TGA ATC GTA GAT ACT GAA AAA C[CC CGC AAG]

This sequence corresponds to the two re-PCR primers (green + blue = forward primer, pink + black = reverse primer) except for the very 5' 8 bp of the reverse primer which are shown in brackets. The two restriction sites for linearizing pOAD (PvuII = CAGCTG and NcoI = CCATGG) are underlined. In the 4 bp-region of mixed color (blue and pink) the two primer sequences overlap). The primers and vectors are designed in a way that a single re-PCR product can be used for both the bait (pOBD2) and prey (pOAD) clones.

Low copy bait vector 1: pOBD-2 (Cagney et al. 2000)



Sequence features:

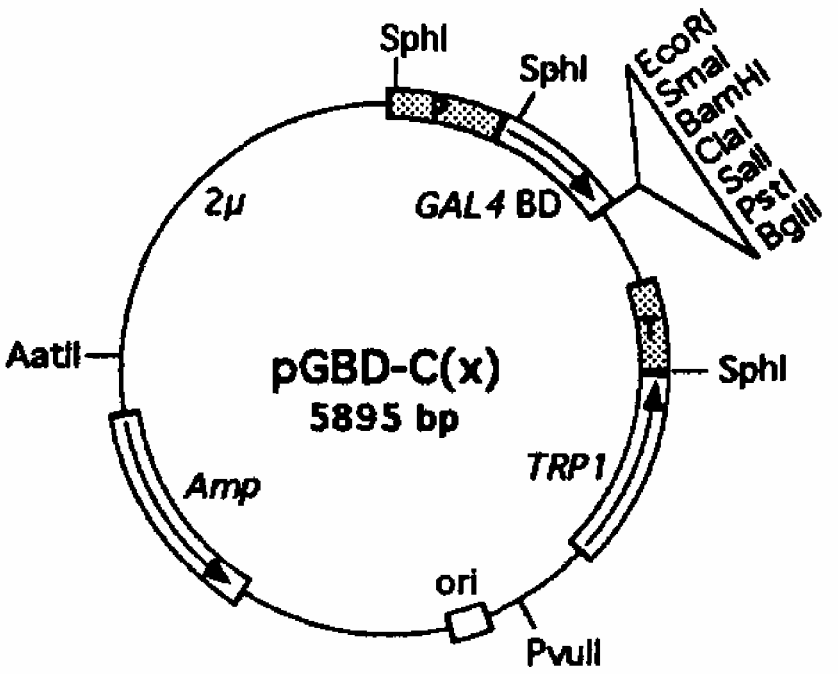
• **ADH1** (alcohol dehydrogenase 1) **promoter**: 1-298 (at least; region of homology when searched against SGD)
• **GAL4-DBD**: 434-982
• **ADH1 terminator**: 1221-1687 (at least; region of homology to SGD genom. seq.)
• **beta lactamase** (ampicillin resistance): 4363-5220
• **TRP1** (tryptophane auxotrophy): 3379-4050
• **CEN4** (centromere element for stable maintenance of the plasmid):: 1814-2667

Cloning site (position 877-1012):

C TAT CTA TTC GAT GAT GAA GAT ACC CCA CCA AAC CCA AAA AAA GAG ATC GAA TTC CAG CTG ACC ACC ATG GCA ATT CCC GGG GAT CCG TCG ACC TGC AGA GAT CTA TGA ATC GTA GAT ACT GAA AAA CCC CGC AAG

This sequence corresponds exactly to the two re-PCR primers (green + blue = forward primer, pink + black = reverse primer). The two restriction sites for linearizing pOBD2 (PvuII = CAGCTG and NcoI = CCATGG) are underlined. In the 4 bp-region of mixed color (blue and pink) the two primer sequences overlap).

High copy bait vector : pGBD (James et al. 1996)



Sequence features:

Stippled regions indicate the ADH1 (alcohol dehydrogenase) promoter (P) and transcription termination (T) elements.
GAL4 BD (DNA binding domain) encodes amino acids 1-147
2μ: High copy yeast plasmid
TRP1: Tryptophane auxotrophy
Amp: Ampicillin resistance

1st Round PCR:

PCR conditions for amplification of ORFs from Genomic DNA

1	µl	genomic DNA
1	µl	dNTP mix (each 10 mM)
10	µl	5x Pfu Polymerase Buffer
1,5	µl	$MgCl_2$ (50mM)
1	µl	F Primer (10 pmol)
1	µl	R Primer
1	µl	Taq Polymerase
0,4	µl	Pfu Polymerase
33,1	µl	Water
50	µl	total

1st Round PCR programm:

5 min 95°C

40 sec 94°C
1 min 54°C x 30
2 min 72°C

10 min 72°C

Hold on 4°C

2nd Round PCR:

1	µl	1st round PCR product
1	µl	dNTP mix (each 10 mM)
10	µl	5x Pfu Polymerase Buffer
1,5	µl	MgCl2 (50mM)
1	µl	F Primer (10 pmol)
1	µl	R Primer
1	µl	Pfu Polymerase
33,5	µl	Water
50	µl	total

2nd Round PCR programm:

5 min 95°C

40 sec 94°C
1 min 54°C x 30
2 min 72°C

10 min 72°C

Hold on 4°C

Primers:

First round PCR

Forward primer:

5' AA TTC CAG CTG ACC ACC ATG xxx xxx xxx xxx xxx xxx xxx 3'

where the xxx's represent the ORF-specific sequences and the ATG represents the actual start codon of the yeast ORF.

Reverse primer:

3' xxx xxx xxx xxx xxx xxx *** GTA CCG TTA AGG GCC CCT AG 5'
where the ***'s represent one of the 3 stop codons.

Second round PCR

Forward primer:

5' C TAT CTA TTC GAT GAT GAA GAT ACC CCA CCA AAC CCA AAA AAA GAG ATC
GAA TTC CAG CTG ACC ACC ATG 3'

Reverse primer:

5’ GTA CCG TTA AGG GCC CCT AGG CAG CTG GAC GTC TCT AGA TAC TTA GCA
TCT ATG ACT TTT TGG GGC GTT C 5'

Scoring of yeast two-hybrid high-throughput PX interactions

- Positive prey clones from a first-round screen were re-arrayed as quadruplicate copies and tested again for reproducibility.
- All retests were carried out at least 8 times and their results were used to assign “quality” scores to obtained positives:

  Score 3: Positives which were reproducible in at least 8 out of 12 or more tests

  Score 2: Positives which were found at least 4 times out of 12 (or more) tests

  Score 1: Postitives that were found two to three times. Some highly reproducible positives were classified as “1” if the results were difficult to interpret due to high background or other reasons.
- Two-hybrid positives were classified by Gene Ontology descriptors as provided by the *Saccharomyces Genome Database* (http://www.yeastgenome.org).

Yeast genomic DNA preparation

Required material:

- Lysis Buffer
- Phenol
- Chloroform and chlorform/Isoamylalcohol
- Tris-EDTA (TE), pH 7.6
- 100% ethanol
- 70% ethanol
- Glass beads (acid glas beads 425-600 µm, Sigma G-9268)

Lysis Buffer

1 % SDS
2 % Triton X-100
0.1 M NaCl
10 mM Tris-HCl, pH 8.0
1 mM EDTA

Procedure:

- Centrifuge at 3500 rpm for 3 min 2ml of an over night yeast culture in complete yeast medium (YEPD)
- Wash the pellet with 1ml of water and centrifuge 3 min at 3500 rpm
- Add 200µl lysis buffer and 0,3g of glass beads
- Add 200µl of phenol and vortex shortly
- Add 200µl of chloroform and vortex shortly
- Add 200µl TE buffer, vortex and centrifuge 5 min at 8000 rpm
- In the meantime prepare 400µl chlorform/Isoamylalcohol in microfuge tubes
- Add the supernatant to the chlorform/Isoamylalcohol and vortex
- Centrifuge 5 min at 8000 rpm
- In the meantime prepare microfuge tubes with 800µl abs. ethanol
- Add the supernatant to the ethanol and vortex
- Centrifuge 15 min at 13000 rpm
- Discard the supernatant
- Wash the pellet with 70% ethanol
- Let the DNA pellet dry at RT
- Solve the pellet in 50µl TE buffer

Yeast Strains

We use two strains which are identical except for being of opposite mating type:

Bait:
PJ69-4α (James et al. 1996) *MATα, trp1-901, leu2–3, ura3–52, his3–200, gal4Δ, gal80Δ, GAL2-ADE2,*
LYS::GAL1-HIS3, met2::GAL7-lacZ

Prey:
PJ69-4a (Hudson et al. 1997) *MAT* a*, trp1-901, leu2–3, ura3–52, his3–200, gal4Δ, gal80Δ, GAL2-ADE2, LYS::GAL1-HIS3, met2::GAL7-lacZ*

Cloning by recombination (Ma et al. 1987) and Yeast transformation (Ito et al. 1983)

Required material:

- Host strains: PJ69-4a or PJ69-4α
- Yeast synthetic media lacking appropriate nutrients
- AD Vector DNA 200ng of NcoI- and PvuII-linearized pOAD
- DBD Vector DNA 200ng of NcoI- and PvuI-linearized pOBD2
- Insert DNA 3µl of Second Round PCR products
- Carrier DNA: Dissolve 7.75mg/ml salmon sperm DNA (Sigma Cat. No. D1626) in water and store at –20°C following a 15 min 121°C autoclave cycle
- DMSO
- 0.1M Lithium acetate (LiOAc)
- '96PEG' solution: 45.6 g PEG (Sigma P3640) make up to 94,8 ml with water

  6.1 ml 2M LiOAc

  1.14 ml 1M Tris.HCl pH 7.5

  232 µl 0.5M EDTA.

Procedure:

- Inoculate 50ml of complete yeast medium (YEPD) with 0.3ml of host strain (for activation-domain fusions we use a and alpha for baits) liquid pre-culture in a 250ml conical flask and grow overnight at 30°C (minimum 15 h max. 24h).
- Harvest the yeast by centrifugation (3500 rpm, 3 min, RT), pour off the supernatant, resuspend the yeast in 2ml of 0.1M LiOAc and transfer into 2 microfuge tubes. Spin out yeast and resuspend all this yeast in a total volume of 1,8ml (incl. yeast)
- Boil 0.58ml of carrier DNA for 5min, and plunge it into ice water.
- Add the following reagents in order to a 50ml tube and immediately shake for 30s followed by vortexing for 1min: 20.73 ml of '96PEG'
  0.58 ml carrier DNA
  200 ng vector DNA
  2.62 ml DMSO
- Add the 1,8ml yeast suspension to the mixture and mix well by hand for 1 min
- Prepare 96 well plates or individual microfuge tubes with 3µl rePCR (2nd Round PCR) products or 1µl control plasmid (e.g. pOAD)
- Add to each tube/well 245µl of the mixture and vortex for 4 min
- Incubate for 30 min at 42°C
- Recover the yeast by centrifugation (2000 rpm, 7min, room temperature on a benchtop centrifuge) and aspirate the supernatant
- Add 220µl of water to each well and resuspend the yeast
- Plate the yeast suspensions singly on to 35mm –Leu or (–Trp) plates and allow to grow for 2-3 days

Yeast Media

YEPD-Medium

For liquid medium:

10g Yeast extract

20g Bactopepton

20g Glucose

Add to 1 liter and autoclave

Store up to 3 months at RT

For solid medium:

Add 16g agar before autoclaving.

Cool to 45°C and pour into appropriate plates. Wrap plates and store up to 6 month at 4°C

YEPD+Ade medium

Prepare solid medium and add after autoclaving 4ml sterile filtered 1% adenine solution in 0,1M NaOH

Selective media

For 1 Liter:

Autoclave 16g agar in 800ml dest. water

After autoclaving add 200ml sterile filtered medium concentrate

According to the wished selective media you have to add the missing amino acids or 3AT (3-amino-1,2,4-triazol):

- T Plates: 8,3 ml Leucine solution (7,2 g/l sterile filtered)
8,3 ml Histidine solution (2,4 g/l sterile filtered)

- **L Plates:** 8,3 ml Tryptophane solution (4,8 g/l sterile filtered)
8,3 ml Histidine solution (2,4 g/l sterile filtered)

- LT Plates: 8,3 ml Histidine solution (2,4 g/l sterile filtered)

- LTH plates: 6 ml 3AT- Solution (0,5M, sterile filtered)

Medium concentrate

For 1 Liter

8,5	g	Yeast Nitrogen Base
25	g	Ammoniumsulfate
100	g	Glucose
7	g	Dropout Powder

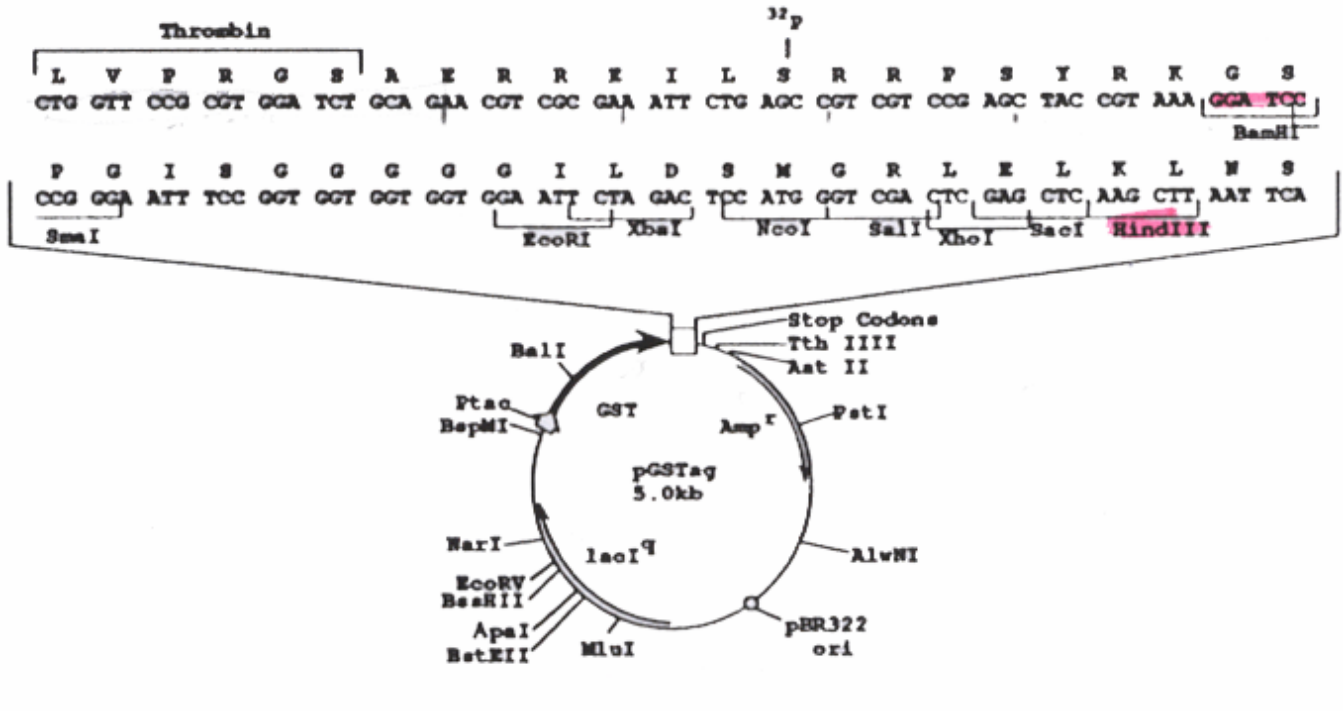
Fill up to 1l with dest. water and sterile filter

Droput powder

Methionine	1	g
Arginine	1	g
Phenylalanine	2,5	g
Lysine	3	g
Tyrosine	3	g
Isoleucine	4	g
Glutamic acid	5	g
Aspartic acid	5	g
Valine	7,5	g
Threonine	10	g
Serine	20	g
Adenine	1	g
Uracil	1	g

GST-PX Domain fusion proteins

All PX domains were cloned at the BamHI and HindIII sites of the MCS (multiple cloning site) (Yu and Lemmon 2001) of the plasmid pGSTag (Ron and Dressler 1992):



Map of the pGSTag plasmid. The sequence of the high affinity PK-A site follows the thrombin cleavage site and is followed by a polylinker. The restriction sites present in the map are unique to the polylinker.

GST-Fusion Proteins Expression

Required material:

- BL21 *E.coli* cells containing the respective cloned pGSTag plasmid
- Ampicillin (Stock solution 100 mg/ml dH_2O)
- IPTG (Stock solution : 1M in dH_2O)
- Reduced Glutathione (10mM in 50mM Tris-HCl pH8.0)
- Glutathion Sepharose (GS) 4 Fast Flow (Amersham Biosciences)

  50% slurry preparation:

  1 Gently shake the bottle of GS to resuspend the mix
  2 Use a pipet to remove sufficient slurry
  3 Dispense 1,33ml of the original GS slurry per ml of bed volume required
  4 Wash the GS by adding 10ml of cold (4°C) 1x PBS per 1,33ml of the orifinal slurry. Invert to mix
  5 Sediment the matrix by centrifugation at 500 x g for 5 min
  6 Decant the supernatant
  7 For each 1,33ml of the original slurry of GS add 1 ml of 1xPBS (50% slurry)
  8 Mix well prior to subsequent pipetting steps

PBS

NaH_2PO	40.23 g
Na_2HPO	41.15 g
NaCl	8.75 g

Add dH_2O to 900ml

Adjust pH to between 7.2 and 7.4 using 1M NaOH or 1M HCl
Adjust volume to 1 liter with dH_2O

LB Media

Bacto Tryptone	10.0 g
Yeast Extract	5.0 g
NaCl	5.0 g
Bacto Agar	15.0 g

Add to 1 liter dH_20

Lysis Buffer

50 mM Tris pH 7.6
150 mM NaCl
3 mM EDTA
Proteinase Inhibitor

Procedure:

- Add 20ml overnight culture to 1 liter of LB +Ampicillin (100mg/l final concentration)
- Shake at 37°C approx. 2 hrs until they grow at a OD_{600} of ~0,6
- Induce fusion protein expression by adding 0,5mM IPTG
- Continue growth by shaking 4 hrs at 37° C
- Centrifuge bacteria at 3500 rpm for 10 min (pellet can be frozen at -20)
- For 1l probe resuspend Pellets in ice cold 50ml lysis buffer
- Lyse cells on ice using a probe sonicator: (2 x 15 pulses hold 4, 40 volts)
- Add 1 % Triton x-100 and incubate 10 min on ice
- Centrifuge at 10 000 rpm at 4 °C for 10 min
- To the 50ml probe add 2-4 ml of 50% slurry sepharose beads
- Rotate 1 h at 4°C
- Wash beads 3x with PBS
- Freeze beads at –20°C with 10% Triton/ or elute with 10mM reduced glutathione

Preparation and Transformation of "Ultra Competent" *E.coli* Cells

This protocol reproducibly generates competent cultures of *E.coli* that yield $1x10^8$ to $3x10^8$ transformed colonies/mg of plasmid DNA. The protocol works optimally when the bacterial culture is grown at 18°C.

Required material:

- DMSO
- Plasmid DNA
- Inoue transformation buffer:

  A) Prepare 0,5M pipes (pH6.7) (piperazine-1,2-bis[ethanesulfonic acid])
  Dissolve 15,1g of PIPES in 80ml dH2O
  Adjust the pH to 6.7 with %M KOH
  Add to 100ml and sterile filter
  Divide into aliquots and store at -20°C

  B)

$MnCl_2.4H_2O$	10,88	g	(55 mM)
$CaCl_2.2H_2O$	2,20	g	(15 mM)
KCl	18,65	g	(250 mM)
PIPES	20	ml	(10 mM)
Sterile filter			

- **LB Media**

Bacto Tryptone	10 g
Yeast Extract	5 g
NaCl	10 g
Bacto Agar	16 g
Add to 1 liter dH_20	

- LB plates with appropriate antibiotic

Procedure:

Preparation

- Pick a single bacterial colony from a plate that has been incubated for 16-20 hrs. at 37°C and transfer it to 25ml of LB media
- Incubate the culture for 6-8 hrs at 37°C with vigorous shaking (250-300 rpm)
- At about 6 am use this starter culture to inoculate 3 1-liter flasks, each containing 250ml of LB. The first flask receives 10ml of starter culture, the second 4ml and the third 2ml.
- Incubate all 3 flasks overnight at 18-22°C with moderate shaking
- The following morning, read the OD_{600} of all 3 cultures. Continue to monitor every 45 min
- When the OD_{600} of one of the cultures reaches 0,55, transfer the culture vessel to an ice-water bath for 10 min. Discard the other 2 cultures.

- Harvest the cells by centrifugation at 2500 g (3900 rpm in a Sorvall GSA rotor) for 10 min at 4°C
- Pour off the medium and store the open centrifuge tube on a stack of paper towels for 2 min. Use a vacuum aspirator to remove any drops of remaining medium adhering to the walls of the centrifuge tube
- Resuspend the cells gently in 20ml of ice-cold Inoue buffer
- Add 1,5ml of DMSO
- Mix the bacterial suspension by swirling and store it on ice for 10 min
- Working quickly, dispense the aliquots of the suspensions into chilled, sterile microfuge tubes. Immediately snap-freeze the competent cells by immersing the tubes in a bath of liquid nitrogen
- Store tubes at -70 °C until needed

Transformation

- Remove a tube of competent cells form the -70 °C freezer
- Thaw the cells by holding the tube in the palm of the hand and transfer after thaw on ice
- Add the transforming DNA (up to 25ng per 50µl competent cells), swirl to mix and incubate on ice for 30 min.
- Transfer the tubes into a 42°C heat block for exactly 90 sec.
- Rapidly transfer the tubes to ice for 1-2 min
- Add 800µl of LB medium and shake the cultures at 37°C for 1 h
- Centrifuge the cells at 13000 rpm for 1 min and resuspend in 200µl H_2O
- Transfer the suspension on to a LB plate with appropriate antibiotic
- Let the plates dry at RT and incubate them at 37°C
- Colonies should appear in 12-16 hrs

SDS PAGE

Acrylamide Gels (for 2 gels 10cm x 8cm with 1mm spacers)

Required material:

- 1 M Tris-HCl pH 8.8 (Roth 3029.1)
- 1 M Tris-HCl pH 6.8
- 0,5 M EDTA
- 20 % SDS
- 30 % Acrylamid solution
- 10 % Ammonium persulfate (APS)
- TEMED
- Gel Chambers (2 gels 10 cm x 8cm with 1mm spacers)
- **2X protein sample buffer**

  For 100ml

Glycerol (20%)	20 ml
SDS (20%)	20 ml
β-mercaptoethanol (10%)	10 ml
1M Tris-HCl pH 6.8	12,5 ml
H_2O	5 ml
Bromphenol blue	a small spatula tip

- Molecular weight marker

Procedure:

Resolution Gel (15% acrylamid)

H_2O	3,33 ml
Tris-HCl pH 8.8	2,53 ml
Acrylamid	4 ml
SDS 20%	50 µl
Temed	13,33 µl
APS (10%)	50 µl

- Pour into Gel chamber (so that it fills up 2/3 of the chamber) and cover with water using a Pasteur pipette (for the removal of air bubbles) and let solidify

Stacking Gel

H_2O	4,35 ml
Tris-HCl pH 6.8	750 µl
Acrylamid	900 µl
SDS 20%	30 µl
Temed	11, 25 µl
APS (10%)	30 µl

- Remove water and pour stacking. Insert immediately the combs and let solidify

10 x SDS-Running Buffer

Glycin	150.17	g
Tris base	60.57	g
SDS (20%)	50	ml

Add to 1 liter

Coomassie Blue Staining of Acrylamide Gels

Required Material:

Coomassie Blue stain:

Methanol	50	%
Acetic acid	10	%
Coomassie Brilliant blue R250	0.2	%

Destain:

Methanol	30 %
Acetic acid	10 %

Procedure:

- Incubate on a shaker with an appropriate amount of coomassie blue stain for 30 min
- Recover Coomassie stain (can be used several times)
- Incubate in destain (change several times the solution) until the background is sufficiently reduced
- Rinse in water and dry the gel

In vitro transcription and translation using the TNT T7 Quick for PCR DNA (promega)

1-TNT PCR

Primer design:

Forward Primer
5´
3´
GGATCC TAATACGACTCACTATAGGG AACAG CCACC**ATG** **gene sequence (18bp)**

Spacer	**T7 promoter**	**Spacer**	**Start codon**

Reverse Primer

5´ TAA **(stop codon) - gene sequence (20bp) 3´**

Primers used:

Yip1p-forward: GGA TCC TAA TAC GAC TCA CTA TAG GGA ACA GCC ACC ATG TCT TTC TAC AAT ACT AGT

Yip1p-reverse: TCA GAC AAA AAT TAC CAT GAG

Yip1p-N-Terminus-reverse: GAT CCC CGG GAA TTG CCA TGC CCA GCC AAA TCT GAA TCG TT

Yif1p-forward: GGA TCC TAA TAC GAC TCA CTA TAG GGA ACA GCC ACC ATG TCT TAT AAT CCG TAC GCA,

Yif1p-reverse: TCA ACC CAT TAA CCA CAT TAG

Yif1p-N-Terminus-reverse: GAT CCC CGG GAA TTG CCA TGG TCC ATA ATT CGT TGC CAG TT

Yip4p-forward: GGA TCC TAA TAC GAC TCA CTA TAG GGA ACA GCC ACC ATG TCA TAC GGA AGA GAA GAC

Yip4p-reverse: TCA GAA CTT TCT GCC GTG GCT

PCR conditions for TNT PCR

1 µl	plasmid DNA encoding appropriate gene
1 µl	dNTP mix (each 10 mM)
10 µl	5x Pfu Polymerase Buffer
1 µl	F Primer (10 pmol)
1 µl	R Primer (10 pmol)
1 µl	Pfu Polymerase
35 µl	Water
50 µl	total

TNT PCR programm:

5 min 95°C

40 sec 94°C 1 min 54°C 4 min 72°C	x 30

10 min 72°C

Hold on 4°C

2- *In vitro* transcription and translation

- Remove the reagents from -70°C. Rapidly thaw the TnT T7 PCR Quick Master Mix by hand-warming and place on ice. The other components can be thawed at room temperature.
- Freeze immediately with liquid nitrogen unused Master Mix to minimize loss of translational activity
- Assemble the reaction components in a 1,5 ml microcentrifuge tube (safe lock):

• TNT T7 PCR Quick Master Mix	40 µl
• [35S] methionine (1,000Ci/mmol at 10mCi/ml)	4 µl
• PCR-generated DNA template	5 µl
• Nuclease-free water	1 µl
Total Volume	50 µl

- After addition of all the components, gently mix by pipetting
- Centrifuge briefly and incubate the reaction at 30°C for 60-90 min
- Analyze translation results by means of SDS-PAGE and autoradiography

GST Pull-Down

Required material:

- GST-fusion protein bound to sepharose beads
- **Pull-Down (PD) Buffer**

  20 mM Tris pH 7,5-8.0
  1 mM ß-mercaptoethanol
  3 mM EDTA
  150 mM Na_2Cl
  1 % NP40 (leave also one fraction without detergent!)

Procedure:

- Take between 10-100µl GST-protein (according to the amount of protein) bound to the slurry sepharose beads
- Add 200µl PD buffer + 4-8µl radioactive labelled in vitro translation product
- Rotate for 2 hrs at 4°C

Wash

- **1 x** PD buffer
- **4 x** PD buffer with **0,1% NP40**
- **1 x** PD buffer with **no detergent**
- Between each step rotate 10 min at 4°C and centrifuge at 1500 rpm at 4°C
- Add 40µl of 1x protein sample buffer
- Freeze at -20 °C or load 10-20µl on a SDS PAGE
- Analyze with autoradiography

Site directed mutagenesis using the QuickChange Method (Statagene)

Methodical Scheme:

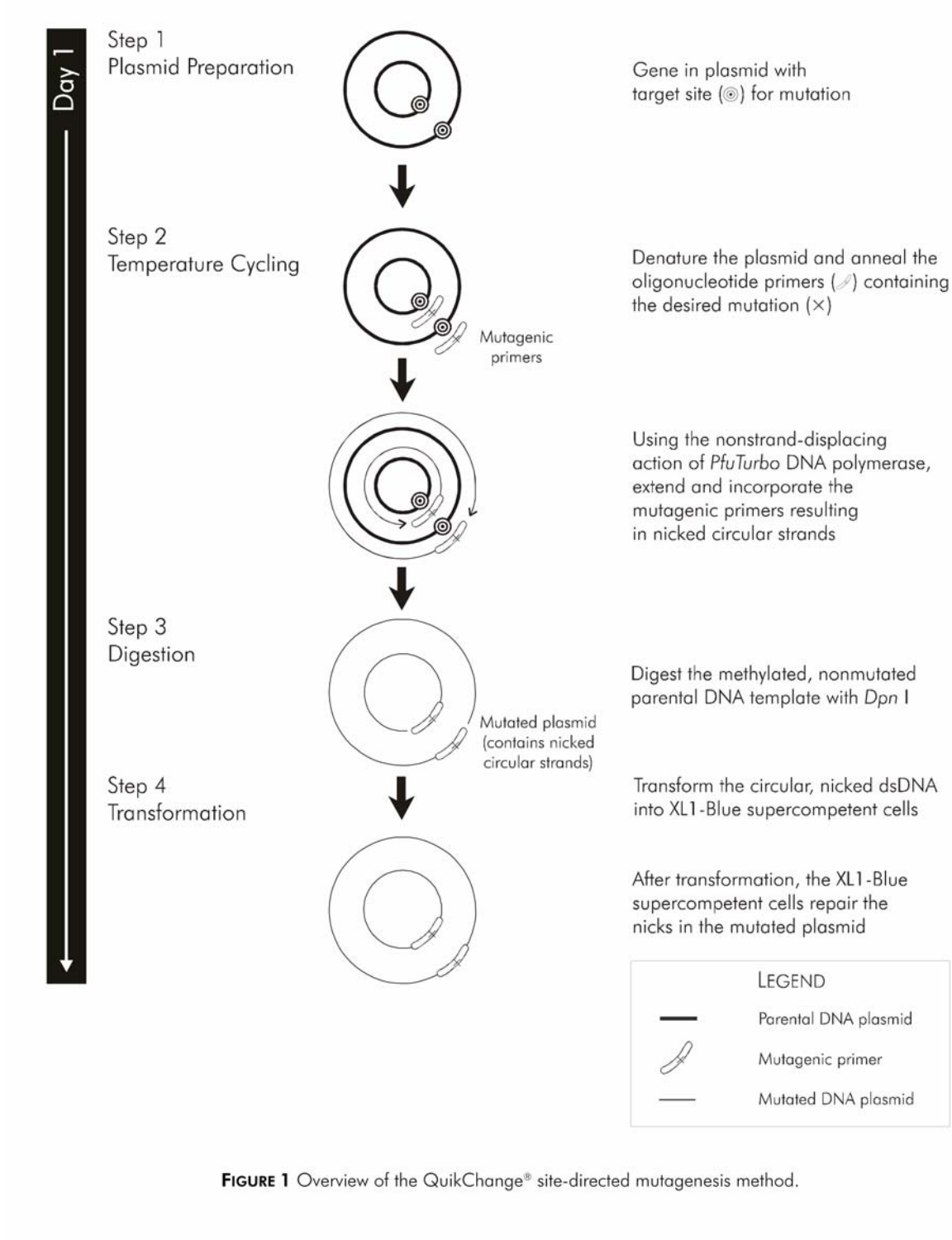


FIGURE 1 Overview of the QuikChange® site-directed mutagenesis method.

Primer Design:

The mutagenic oligonucleotide primers for use in this protocol must be designed individually according to the desired mutation. The following considerations should be made for designing mutagenic primers:

- Both of the mutagenic primers must contain the desired mutation and anneal to the same sequence on opposite strands of the plasmid.
- Primers should be between 25 and 45 bases in length, with a melting temperature (*T*m) of ≥78°C. Primers longer than 45 bases may be used, but using longer primers increases the likelihood of secondary structure formation, which may affect the efficiency of the mutagenesis reaction.
  The following formula is commonly used for estimating the *T*m of primers:

  Tm =m 81.5 +0. 41(%GC) - 675/ *N* -% mismatch

  • *N* is the primer length in bases
  • values for **%GC** and **% mismatch** are whole numbers

- The desired mutation (deletion or insertion) should be in the middle of the primer with ~10–15 bases of correct sequence on both sides.
- The primers optimally should have a minimum GC content of 40% and should terminate in one or more C or G bases.

Primers used:

Ypt35-PX Y123G mutation:

Forward :

CCA AAC CTA TAA ACG A**GC** CTC CGA TTT CGT CAG

Tm=81,5 + 0,41 * 48,5%GC-675/33 -6% mismatch = 74,9°C

Reverse :

CTG ACG AAA TCG GAG GCT **CG**T TTA TAG GTT TGG

Tm=81,5 + 0,41 * 48,5%GC-675/33 -6% mismatch = 74,9°C

Yip1*1*-2 mutation:

Forward :

5’ CAG CGT CCA TCT TAG **A**AT ACT GCT TTT TGC C 3’

Tm=81,5 + 0,41 * 45%GC-675/31 -3,2% mismatch = 75°C

Reverse :

5’G GCA AAA AGC AGT AT**T** CTA AGA TGG ACG CTG 3’

Tm=81,5 + 0,41 * 45%GC-675/31 -3,2% mismatch = 75°C

Yip1 K87A T88A K89A mutation:

Forward :

5´C GAT CAT ATC ATA ACG **GC**A **G**CT **GC**A ATG GTT CTT ATA CC 3´

Tm= 81,5 + 0.41 * 43,6% GC -675/39- 12,8% mismatch = 69,27

Reverse :

5 CG TAT TGG TAT AAG AAC CAT T**GC** AG**C** T**GC** CGT TAT GAT ATG ATC 3

Tm= 81,5 + 0.41 * 42.2% GC -675/45- 11,1% mismatch = 72.8

→ mutated bases shown in **bold**

PCR

Cycling Parameters :

Segment	Cycles	Temperature	Time
1	1	95°C	30 seconds
2	12–18	95°C	30 seconds
		55°C	1 minute
		68°C	1 minute/kb of plasmid length

Type of mutation	desired Number of cycles
Point mutations	12
Single amino acid changes	16
Multiple amino acid deletions or insertions	18

Procedure:

- Add 1µl of the *Dpn* I restriction enzyme (10 U/µl) directly to each amplification reaction
- Gently and thoroughly mix each reaction mixture by pipetting the solution up and down several times. Spin down the reaction mixtures in a microcentrifuge for 1 minute and immediately incubate each reaction at 37°C for 1 h to digest the parental (i.e., the nonmutated) supercoiled dsDNA
- Transformation of XL1-Blue Supercompetent Cells (see protocol Preparation and Transformation of “Ultra Competent” E.coli Cells)
- Gently thaw the XL1-Blue 50µl aliquots of supercompetent cells on ice. For each sample reaction to be transformed add1µl of the *Dpn* I-treated DNA from each sample reaction
- Swirl the transformation reactions gently to mix and incubate the reactions on ice for 30 min
- Heat pulse the transformation reactions for 90 sec at 42°C and then place the reactions on ice for 2 min
- Add 0.5ml LB media preheated to 42°C and incubate the transformation reactions at 37°C for 1 h with shaking at 225–250 rpm.

- Centrifuge at 13 000 rpm for 1 min
- Resuspend the cells in 200µl H_2O and spread cells on LB plates containing the appropriate antibiotic
- Incubate the transformation plates at 37°C for >16 hrs

Lipid overlay assay

Required Material:

Phospholipids (Cell Signals)
Hybond-C extra membrane (Amersham Biosciences)
Fatty acid-free BSA
Eluted GST-fusion protein
Chloroform
Methanol

TBS 20x → + 0,1%Tween 20 **= TBST**

Tris 60 g
NaCl 160 g
KCl 4 g
pH 7,4 (HCl)

→ add to 1 L

- Spot 1µl of lipid solution (containing 200, 100, 50, 25, 12,5 and 6,25 pmol of phospholipids) in a mixture of chloroform/methanol/water (1:2:0.8) on to a Hybond-C extra membrane (only this type of membrane works!) and allow to dry at RT for 1 h
- Block the membrane in TBST containing 3% (w/v) fatty acid-free BSA for 1h
- Incubate the membrane overnight at 4 °C with gentle stirring in the same solution (blocking solution) containing 2µg of the indicated GST-fusion protein
- Wash the membranes six times over 30 min in TBST
- Incubate for 1h the 1st antibody (mouse anti-GST 1:1000) in blocking solution
- Wash the membranes as before and then incubate for 1h with the 2nd antibody-HRP (goat anti-mouse 1: 5000) in blocking solution
- Wash the membranes 12 times for 5 min in TBST
- **ECL**: - Mix 1 vol. of solution A and 1 vol. of solution B
  - Pipette the mixture on to the blot and let activate for 1 min
  - Put between blot and film a cling film and expose

Peptide Synthesis

Required Materials:

Whatman paper (hardened low ash Nr. 1450 917)
DMF (Dimethylfromamide)
NMP (N-methyl pyrrolidone)
DIC (Diisopropyl carbodiimide)
Piperidine (20% in DMF)
HOBt (Hydroxy Benzotriazole)
Fmoc-AA-OH
Fmoc-β–Ala-Opfp
Acetic Anhydride
TFA (Trifluoroacetic acid)
Ethanol
TIBS (Triisobutylsilane)
dH_2O
NMI (1-Methylimidazole)
Triisoporpylsilan

β–Alanine membranes

- Cut 10x15 cm Whatman paper
- Incubate in a metal dish following solution:

β-alanine OH	2,56	g
DMF	40	ml
DIC	1496	µl
NMI	1270	µl

- Incubate 20ml pro membrane overnight shaking gently
- Wash 3 x with DMF
- Incubate membranes 20 min with 20% piperidine (removal of F-moc group)
- Wash 5 x in DMF
- Wash 2 x in EtOH
- Let dry overnight

Amino Acids (AA) Aliquots

- Weigh 0,5mmol AA
- Add for each AA 1ml of HOBt (2.3 g in 20 ml NMP)
- Solve AA and fill up to 1.5ml with NMP
- Store at -20°C

Peptide Synthesis

Day 1

- Thaw AA overnight at RT

Day2

- Activate AA with 240µl **Activation Stock Solution:**
  0.4 ml DIC
  2.0 ml NMP
  2.8 ml DMF
- Mix AA and activate for 30 min at RT
- Centrifuge 3 min at 4000 rpm
- Pipet solution (avoiding precipitated AA) to the synthesizer vials in correct order
- Fill up:

  DMF

  Ethanol

  20% Piperidine solution

  Capping solution: 0.3ml Acetic Anhydride in 15ml DMF
- Activate membrane in DMF
- Start Program
- For β-alanine membranes spacer spotting solve 0,0573g Fmoc.β.Ala-Opfp in 400µl DMSO
- Thaw ON AA aliquots

Day 3

- Activate AA
- Discard old vials and pipet AA into new ones
- Fill up Capping and Piperidine solutions
- Thaw AA overnight at RT

Day 4

- Activate AA
- Discard old vials and pipet AA into new ones
- Fill up Capping and Piperidine solutions
- Thaw AA overnight at RT
- Let membrane dry overnight

TFA removal of all side chain protecting groups (for β-Alanine membranes)

- Incubate the membranes without shaking in 90% TFA solution

  0.5 g Phenol
  1 ml dH_2O
  1.5 ml TIBS
  2.5 ml DCM
  45 ml TFA

- Wash 4x 3 min with DCM
- Wash 3x 3 min with DMF
- Wash 3x 3 min with EtOH
- Let Dry

- Incubate the membranes without shaking in 50% TFA solution

  0.5 g Phenol
  1 ml dH_2O
  1.5 ml TIBS
  22.5 ml DCM
  25 ml TFA

- Wash 4x 3 min with DCM
- Wash 3x 3 min with DMF
- Wash 3x 3 min with EtOH
- Let Dry

TFA removal of all side chain protecting groups (for PEG membranes = Intavis membranes)

- Pro membrane: 9 mlTFA
  0.5 ml Triisoporpylsilan
  0.5 ml dH_2O

- Incubate for 1 h in the mixed solution, shake gently in between

- Wash 4x 3 min with DCM
- Wash 3x 3 min with DMF
- Wash 3x 3 min with EtOH
- Let Dry

Positive control peptides

- Three control peptides that bind to the anti-GST antibody were used:
  1. QRALAKDLIVPRRP
  2. LAKDLIVPRRPEWN
  3. DLVIRPPRPPKVLGL

Protein Incubation

- Activate the membrane 10 min with methanol
- Wash 3 x 5 minwith 1x TBS

10x TBS

Tris base 24.2 g
NaCl 292.2 g
pH to 7.5 with HCl

- Block 3 hrs with **Blocking Solution**

1 g skim powder milk
2.5 g Saccharose
In 50ml 1 x TBS

- Incubate protein 10 µg/ml in blocking solution (- Tween) overnight rotating at 4°C
- Wash 3 x 5 min with 1x TBS
- Incubate 3 hrs at RT with 1st antibody in blocking solution + 0.05% Tween
- Wash 3 x 5 min with 1x TBS
- Incubate 1.5 hrs at RT with 2nd antibody in blocking solution + 0.05% Tween
- Wash 3 x 5 min with 1x TBS
- **ECL**: - Mix 1 vol. of solution A and 1 vol. of solution B
  - Pipete the mixture on to the blot and let activate for 1 min
  - Put between blot and film a cling film and expose

Pulse + Chase Immunoprecipitation

Required Material:

50x Chase Mix

50 mM Methionine
5 mM Cysteine
in dH_2O
Filter sterilize and store up to 1 year at -20°C in small aliquots

Detergent IP buffer

50 mM Tris-HCl, pH7.5
0.1 mM EDTA
150 mM NaCl
0.5 % (w/v) Tween 20 (polyoxyehtylenesorbitan monolaurate)
Store up to 3 months at 4°C

Detergent/Urea Buffer

100 mM Tris-HCl, pH7.5
200 mM NaCl
0.5 % (w/v) Tween 20 (polyoxyehtylenesorbitan monolaurate)
2 M Urea
Store up to 3 months at 4°C

SDS/Urea Buffer

50 mM Tris-HCl, pH7.5
1 mM EDTA
1 % SDS
6 M Urea
Store up to 1 month at RT

Procedure:

- Grow strains overnight in 5ml YEPD at 30°C. In the morning back-dilute cultures to ~0.25ODs/ml in 5ml media and again place at 30°C until cells reach an OD600 of ~1.0ODs/ml
- Harvest cell (~1.5 ODs for each time point of chase) and wash 1 time with 5ml sterile dH2O
- Resuspend in enough SC-Methionine media so that you have 1ml for each time point
- Place at 30°C for 20 min
- Add ~10µl per ml of ^{32}S-Methionine. Place at 30°C for 5 min (a short pulse in necessary)
- After 5 min pulse, add 20µl per ml of 50X Chase Mix to each tube
- Immediately transfer 1ml of each to tubes containing 250µl 50% TCA (trichloroacetic acid) and keep on ice (this is the 0 time point)

- Place the remaining culture at 30°C for desired time (CPY is maturated by 10 min in a WT strain). At each desired time point transfer 1ml of each to tubes containing 250µl of 50% TCA and keep on ice
- Incubate TCA mixtures on ice for at least 20 min
- Centrifuge at 14000 rpm for 10 min
- Aspirate supernatants into radioactive waste trap and wash pellets 1x with 1ml ice cold acetone
- Centrifuge 14000, 10 min
- Aspirate supernatants and allow pellets to air dry (~5 min)
- Resuspend the pellets in 100µl SDS Urea Buffer and incubate at RT for 15 min
- Add glas beads (0.1- 0.25 mm) to 80%-90% (v/v) of the sample volume
- Vortex samples for 1min
- Heat at 95°C for 5 min and vortex again for 30 sec
- Add 900µl detergent IP buffer, vortex and put samples on ice for minimum 20 min
- Centrifuge samples at 14 000 rpm for 15 min and transfer ~850µl of the supernatants 8lysate) to fresh tubes being careful not to disturb the glass beads or any insoluble material pelleted.
- Add 2µl of CPY/Kex2 or any other antibody to each sample and incubate at 4°C with rocking overnight
- Add 30µl Protein-G-Sepharose (protein G has much higher affinity to mouse IgG monoclonal antibodies than protein A) to each and incubate at 4°C for 2 hrs
- Pellet immune complexes by centrifuging at 14 000 rpm
- Transfer the supernatant to new labelled tubes (these supernatants can be used to immunoprecipitate a different protein, for eg. ALP, PrA etc)
- Wash the pellets (beads) 2 x with 1ml detergent/urea buffer and 1x with detergent IP buffer
- Resuspend the beads in 50µl 1x SDS sample buffer and heat at 95°C for 10 min
- Load half the sample on to a 10% SDS-PAGE gel (run the gels as usual).
- Save the remainder of the samples at 20°C in case the electrophoresis has to be done again
- Soak the gels for 20 min in 1M Salicylic Acid with 2% glycerol
- Dry gels at 80°C for 2 hrs
- Expose gels to film. Usually a ~3 day exposure is sufficient for a nice signal

Yeast protein extracts

Required material:

- Yeast overnight culture
- Liquid nitrogen
- **2X protein sample buffer**

  For 100ml

Glycerol (20%)	20	ml
SDS (20%)	20	ml
β-mercaptoethanol (10%)	10	ml
1M Tris-HCl pH 6.8	12,5	ml
H_2O	5	ml
Bromphenol blue	a small spatula tip	

Procedure:

- Collect 4ml of yeast overnight culture (in YEPD) at 3500 rpm for 5 min
- Wash the cells with 1ml H2O
- Resuspend the cells in 200µl 2x protein sample buffer
- Heat resuspended cells for 3 min at 95°C
- Vortex 45 sec
- Freeze immediately in liquid nitrogen

} Repeat procedure 4 times

- Load 5µl on a SDS-PAGE or store at -70°C

Western Blot Transfer (semi-dry)

Required material:

- **Transfer buffer**

  Tris Base 3.03 g
  Glycine 14.27 g
  Methanol 200 ml

  → add to 1 l with dH_20

- Ponceau S (commercial)
- Blot Chamber
- Whatman paper
- Nitrocellulose membrane (Immobilon-P, Millipore)

Procedure:

- Cut blot paper (8 sheets of Whatman paper per gel) and to the size of your gel and equilibrate in transfer buffer
- Cut blotting membrane to the size of your gel,shortly activate in methanol and equilibrate in transfer buffer
- Disassemble electrophoresis chamber, lift short plate form saucer plate and carefully transger gel (cut off stacking gel) into a tray with transfer buffer, shortly equilibrate
- Build up blot: To bottom platinum anode of Trans-Blot-Semi-Dry-place:
  Pre-wet filter paper (4 sheets of Whatman paper)
  Pre-wet membrane
  Gel
  Pre-wet filter paper (4 sheets of Whatman paper)
  Roll out air bubbles
- Secure safety cover and connect to power supply
- Run 1 gel at 15V for 45 min, 2 gels at 20 V for 45 min
- Stop transfer, discard filter paper and briefly wash blot in dH_2O
- Check transfer by staining blot in Ponceau S solution and destain in dH_2O

Western Blot Detection

Required material:

10x TBS

Tris base 24.2 g
NaCl 292.2 g

pH to 7.5 with HCl

→ add to 1 liter dH2O and autoclave

TBST

1xTBS with 0,05% Tween-20

1%-, 2%,-3% TBSTM

Dissolve skim milk powder to 1%,2% and 3% in TBST, prepare fresh

- Block the membrane (in a Falcon tube and roll, or in a dish and shake gently) in 10ml 3%TBSTM for 30 min at RT
- Wash 3 times with 1%TBSTM for 5 min each wash
- Replace 1%TBSTM with 2%-TBSTM and add primary antibody
- Incubate the membrane overnight at 4 °C with gentle shaking
- Wash the blot once with 1%TBSTM for 10 min
- Wash 2 times with 1%TBSTM for 5 min each wash
- Replace 1%TBSTM with 2%-TBSTM and add secondary antibody
- Incubate for 1h at RT (rolling or gently shaking)
- Wash 3 times in 10ml TBST for 10 min each wash
- **ECL**: - Mix 1 vol. of solution A and 1 vol. of solution B
  - Pipete the mixture on to the blot and let activate for 1 min
  - Put between blot and film a cling film and expose

Lucifer yellow assay

Required materials:

- Lucifer Yellow CH Dilithium salt (Fluka 62642)
- Low melting agarose
- **Succinate Buffer**

  50 mM succinate-NaOH, pH 5.0
  20 mM NaN_3

Procedure:

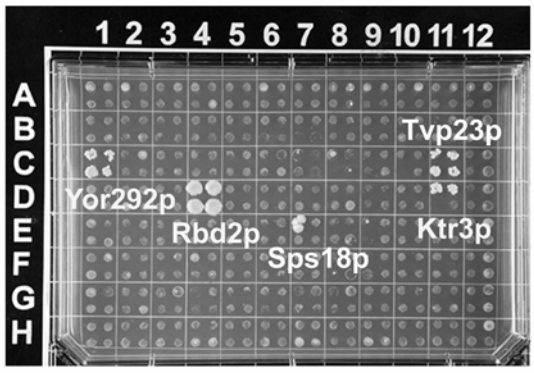
- Grow yeast cell to stationary phase on YEPD media at 30°C during 2 days
- Inoculate 5-10µl of this overnight culture to 50 ml YEPD and shake at 30°C
- After ~15 hrs they should have reached an OD of 1.5
- Collect 1ml of cell suspension (3 min at 3500 rpm) and resuspend in 80µl YEPD
- Add 20 µl of Lucifer Yellow solution (8 mg/100µl H_2O solved 5 min at 30°C)
- Incubate at 30 °C for 1 hr
- Wash the cells 3 x in 1ml ice-cold succinate buffer
- An equal volume of cells (usually 3-4µl) is mixed with a 1.6% low melt agarose (precooled to 45°C) on a microscope slide that can be kept on ice until observation under the microscope
- Lucifer Yellow CH can be visualized by fluorescence microscopy using FITC optics
- Filters: exitation 450-490nm, FT510, LP520
- A defect in accumulation of LY in the vacuole can only be proven in cells that contain vacuoles visualized by Nomarski optics

Results

Yeast two-hybrid data

Previous two-hybrid data (Uetz et al. 2000) suggested that PX domains may act as protein interaction domains, although it should be noted that even the yeast 'PX domain only' proteins Grd19p and Ypt35p contain some additional sequences in addition to the PX domain itself. To extend previous findings and to investigate a possible role of the PX domain as a bona fide interaction module, all yeast PX domain-containing proteins and their isolated PX domains were screened against a *S. cerevisiae* whole-genome two-hybrid array which contains most yeast open reading frames as Gal4 activation domain fusions (Uetz et al. 2000). Since two-hybrid interactions often suffer from low reproducibility all screens were performed twice and only double positives were considered as positive interactions. Double positive interactions and selected single interactions of interesting candidates were reanalyzed several times in a retest plate, and finally quadruplicate colonies were screened to ensure that none of them had arisen by random activation events. Representative results of these screens are shown in Figures 13 and 14 for the baits Ypt35p and Grd19p respectively. A summary of all interactions is shown in Figure 15 and Tables 6 and 7. In order to reflect various degrees of reproducibility, one of three scores (S) was assigned to the interactions in our datasets (Table 6, see Experimental Procedures for details). We found 27 highly reproducible interactions, denoted by S values of 3 in Table 2, 18 well-reproducible interactions (S=2), and 30 interactions that were reproduced several times but not consistently (S=1). This classification serves as a rough indication for the reliability of our interactions and may roughly correlate with the strength of the interactions. A representative result of this classification is shown in Figure 12.

Bait: Ypt35p



Prey	Score
Rbd2p	3
Yor292p	2
Tvp23p	2
Ktr3p	1
Spsp18p	1

Figure 12: Quadruplicate retest plate with Ypt35p as a bait
Representative result for the reliability and possible interaction strength correlation of obtained interactions. Rbd2p (S=3), Yor292p (S=2), Tvp23 (S=2), Ktr3p (S=1) and Sps18p (S=1). See text for details.

Out of the 75 interactions listed in Table 6, 17 have been published before (Table 6). The majority of those, 14, belong to scores 2 and 3, which confirms their validity.

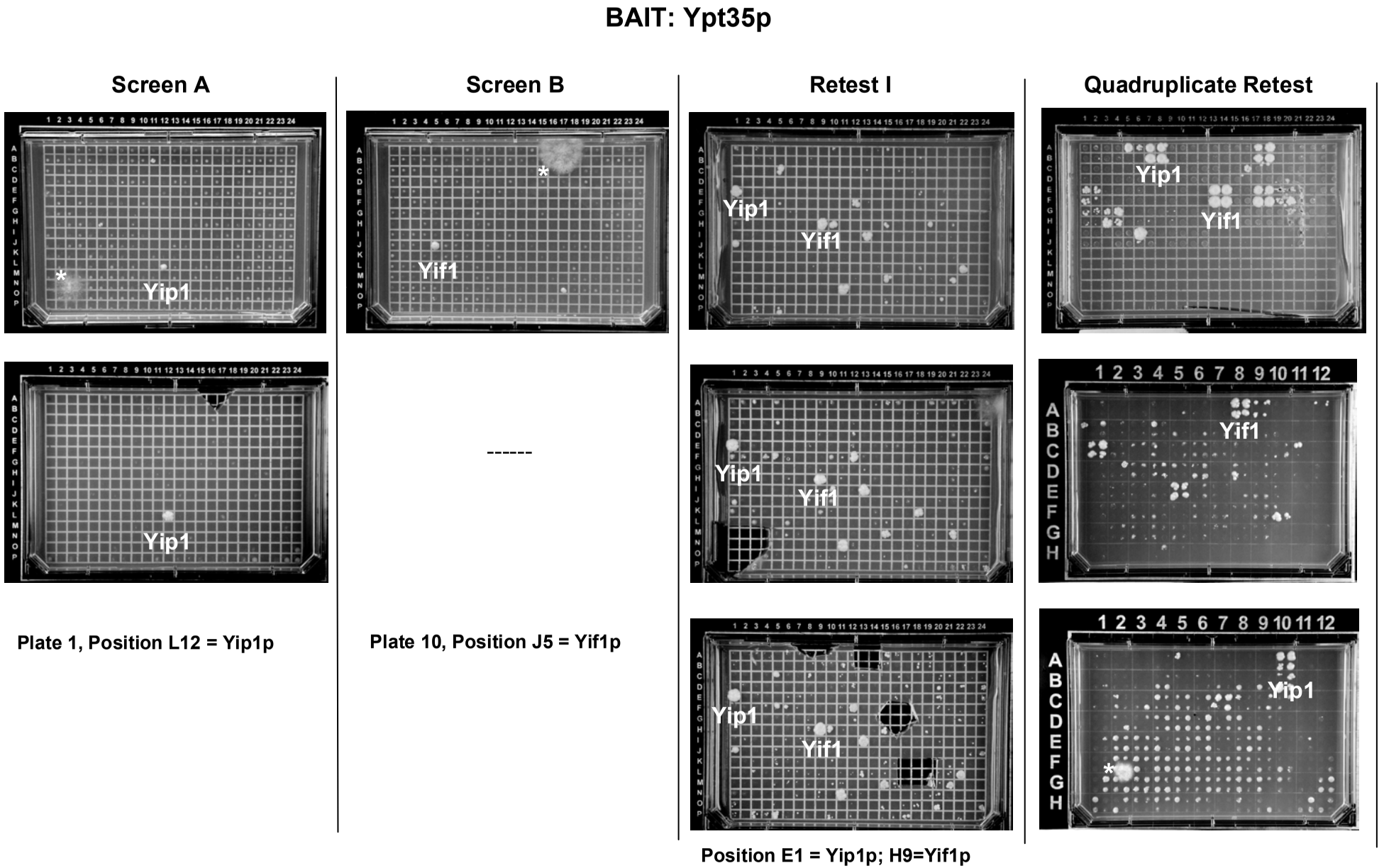


Figure 12: Two-hybrid array screens and retests using Ypt35p as bait

Screen: Shown is only plate 1 and 10 (out of 16), negative plates are not shown; **Retest I:** plate with selected double or single positive interactions performed 3 times; **Quadruplicate retest:** Each prey in quadruplicate. *: contaminations.

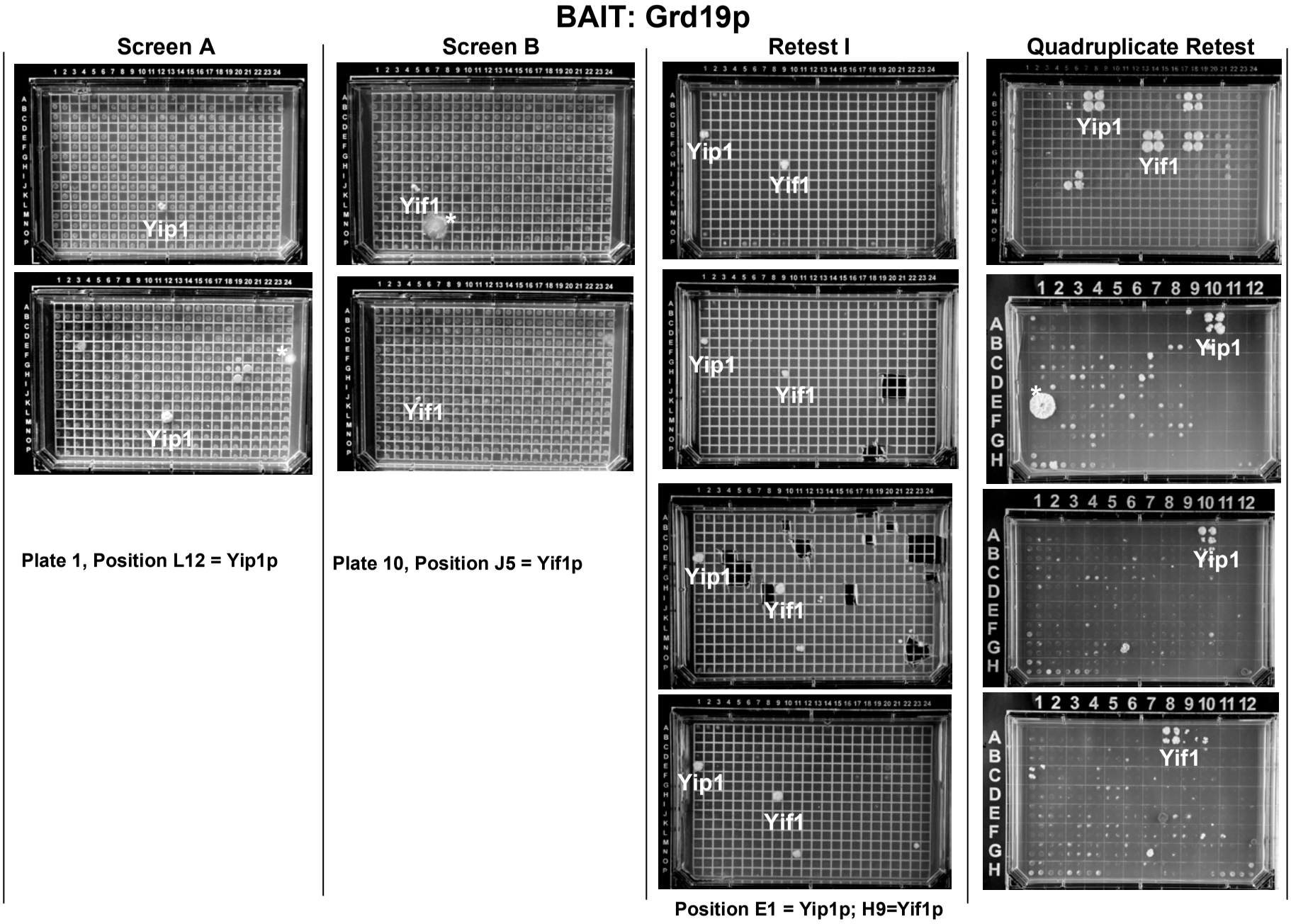


Figure 13: Two-hybrid array screens and retests using Grd19p as bait

Screen: Shown is only plate 1 and 10 (out of 16), negative plates are not shown; **Retest I:** plate with selected double or single positive interactions performed 4 times; **Quadruplicate retest:** Each prey in quadruplicate. *: contaminations.

	Prey	S	GO keyword	R
	Bem1p			
	Cdc24p	2	cell polarity *	I
	Grd19p			
	Adh1p	1	fermentation	
	Akr2p	1	unknown	
	Apg17p	1	autophagy	
	Rbd2p	3	unknown	
	Rvs167	1	endocytosis*	
	Sec15p	1	cell polarity*	
	Yif1p	3	ER-Golgi transport	
	Yip1p	3	vesicular transport	
	Yip5p	3	vesicular transport	
	Ylr031p	1	unknown	
	Ylr424p	1	splicing	
	Ypt53p	2	vacuolar targeting*	
	Mvp1p			
p	Std1p	1	Pol II transcript.*	
p	Yra2p	2	mRNA export	
	Snx42p			
p	Rrn10p	2	Pol I transcription	U
	Snx4p	3	Vesicular transport*	U
	Yip1p	1	Vesicular transport	
	Snx4p			
	Apg17p	1	autophagy	I
	Ask10p	2	transcription	
	Cog2p	3	ER to Golgi transport*	
	Cog6p	3	intra-Golgi transport	
	Dam1p	1	spindle assembly	
	Ecm11p	3	cell wall biogenesis	
	Lcp5p	1	rRNA modification*	
	Nip100p	3	mitotic anaphase B	
	Sds3p	3	histone deacetylation*	
	Sec15p	3	cell polarity *	
	Sgt1p	1	complex assembly*	
	Snx41p	3	protein transport	I
	Snx42p	3	vacuolar targeting*	
	Swf3p	1	mitochondria	
	Vma8p	1	vacuolar acidification	
	Vps17p	3	Endosome-Golgi tr.	
	Ylr031p	3	unknown	
	Ylr424p	1	mRNA splicing	
	Ynl086p	3	unknown	
	Ypl201p	1	unknown	

P	Prey	S	GO keyword	R
	Vam7p			
o	Akr2p	1	unknown	
	Avt2p	2	transport	
o	Bsp1p	3	actin cytoskeleton*	
	Ecm11p	2	cell wall biogenesis	
o	Rbd2p	1	unknown	U
	Rpt5p	2	protein catabolism	
	Spc72p	2	Chromos.	U
	Vps21p	1	vacuolar targeting*	
	Ygr223p	3	unknown	
o	Yif1p	3	ER to Golgi transport	U
o	Yip1p	3	vesicular transport	
	Yip5p	2	vesicular transport	U
	Vps17p			
p	Akr2p	1	unknown	
p	Bsp1p	1	actin cytoskeleton*	
	Vps5p	3	Golgi retention*	U
p	Yif1p	2	ER to Golgi transport	
p	Yip1p	2	vesicular transport	
p	Ypt53p	1	vacuolar targeting*	
	Vps5p			
	Bub1p	1	Protein phosphoryl.*	
	Vps17p	2	endosome -> Golgi	U
	Vps35p	2	Golgi retention*	S
	Ynl086p	1	unknown	
	Ypt35p			
	Akr2p	3	unknown	U
	Atr1p	1	multidrug transport	
	Bet1p	1	ER to Golgi transport*	
	Bos1p	2	ER to Golgi transport	
	Ktr3p	1	cell wall biogenesis*	U
	Pep12p	1	Golgi-vacuole	
	Rbd2p	3	unknown	U
	Sps18p	1	sporulation	
	Tvp23p	2	unknown	
	Yif1p	3	ER to Golgi transport	U
	Yip1p	3	vesicular transport	
	Yip4p	3	vesicular transport	
	Yip5p	3	vesicular transport	U
	Ymd8p	2	nucleotide-sugar	
	Yor292p	2	unknown	

Table 6:

Two-hybrid interactions of the yeast PX proteins

P = PX domain, **[no symbol]**: interaction has been obtained only with the full length protein but note that Grd19p and Ypt35p are essentially PX-only proteins, **[o]:** interaction obtained with both full length protein and its PX domain, **[px]:** interaction has been obtained only with its PX domain**. S** = two-hybrid quality score: **[3]** highly reproducible with strong signal, **[2]** reproducible, **[1]** reproduced several times but not consistently. Asterisks (*) indicate proteins with multiple functions. **R**: Published interactions and their references [I =(Ito et al. 2001), U = (Uetz et al. 2000), S = (Seaman et al. 1998)].

Preys	Baits								
	Bem1	Grd19	Mvp1	Snx4p	Snx42	Vam7	Vps5p	Vps17	Ypt35
Adh1p		1							
Akr2p		1				1-1		0-1	3
Apg17p		1		1-0					
Ask10p				2-0					
Atr1p									1
Avt2p						2-0			
Bet1p									1
Bos1p									2
Bsp1p						3-1		0-1	
Bub1p							1-0		
Cdc24p	2-0								
Cog2p				3-0					
Cog6p				3-0					
Dam1p				1-0					
Ecm11				3-0		2-0			
Ktr3p									1
Lcp5p				1-0					
Nip100				3-0					
Pep12p									1
Rbd2p		3				1-1			3
Rpt5p						2-0			
Rrn10p					2-0				
Rvs167		1							
Sds3p				3-0					
Sec15p		1		3-0					
Sgt1p				1-0					
Snx41p				3-0					
Snx42p				3-0					
Snx4p					3-0				
Spc72p						2-0			
Sps18p									1
Std1p			0-1						
Swf3p				1-0					
Tvp23p									2
Vma8p				1-0					
Vps17p				3-0			2-0		
Vps21p						1-0			
Vps35p							2-0		
Vps5p								3-0	
Ygr223						3-0			
Yif1p		3				3-3		0-2	3
Yip1p		3			1-0	3-3		0-2	3
Yip4p									3
Yip5p		3				2-0			3
Ylr031p		1		3-0					
Ylr424p		1		1-0					
Ymd8p									2
Ynl086				3-0			1-0		
Yor292									2
Ypl201				1-0					
Ypt53p		2						0-1	
Yra2p			0-2						

Table 7: All PX protein interactions in a matrix format

Numbers indicate involvement of the PX domain in interactions; e.g. 3-0 = score 3 with full length protein and score 0 (= no interaction) with PX domain; only one number is provided for PX-only proteins (Grd19p and Ypt35p).

Further comparison of obtained interactions were done by looking at the several large-scale projects which have attempted the systematic isolation and identification of protein complexes in yeast (Gavin et al. 2002; Ho et al. 2002; Hazbun et al. 2003). Surprisingly few of them recovered any PX domain proteins (see appendix Table I) and their interactions did not turn out to be very informative for those complexes of which PX proteins have been demonstrated to be involved in, such as Vps17p and Vps5p which form part of the retromere complex (Fig.52), were not isolated by these methods.

A number of interactions have been reported for PX proteins that we failed to identify (false negatives, see appendix Table II). It is difficult to explain this discrepancy but it has been shown that differences in strains, plasmids, or reporter genes may account for these results.

More detailed information on baits and preys with localization and GO (Gene Ontology) annotations, links to SGD, Interpro, and PDB are provided at http://itgmv1.fzk.de/www/itg/uetz/domains/PX/.

Full-length vs. domain interactions

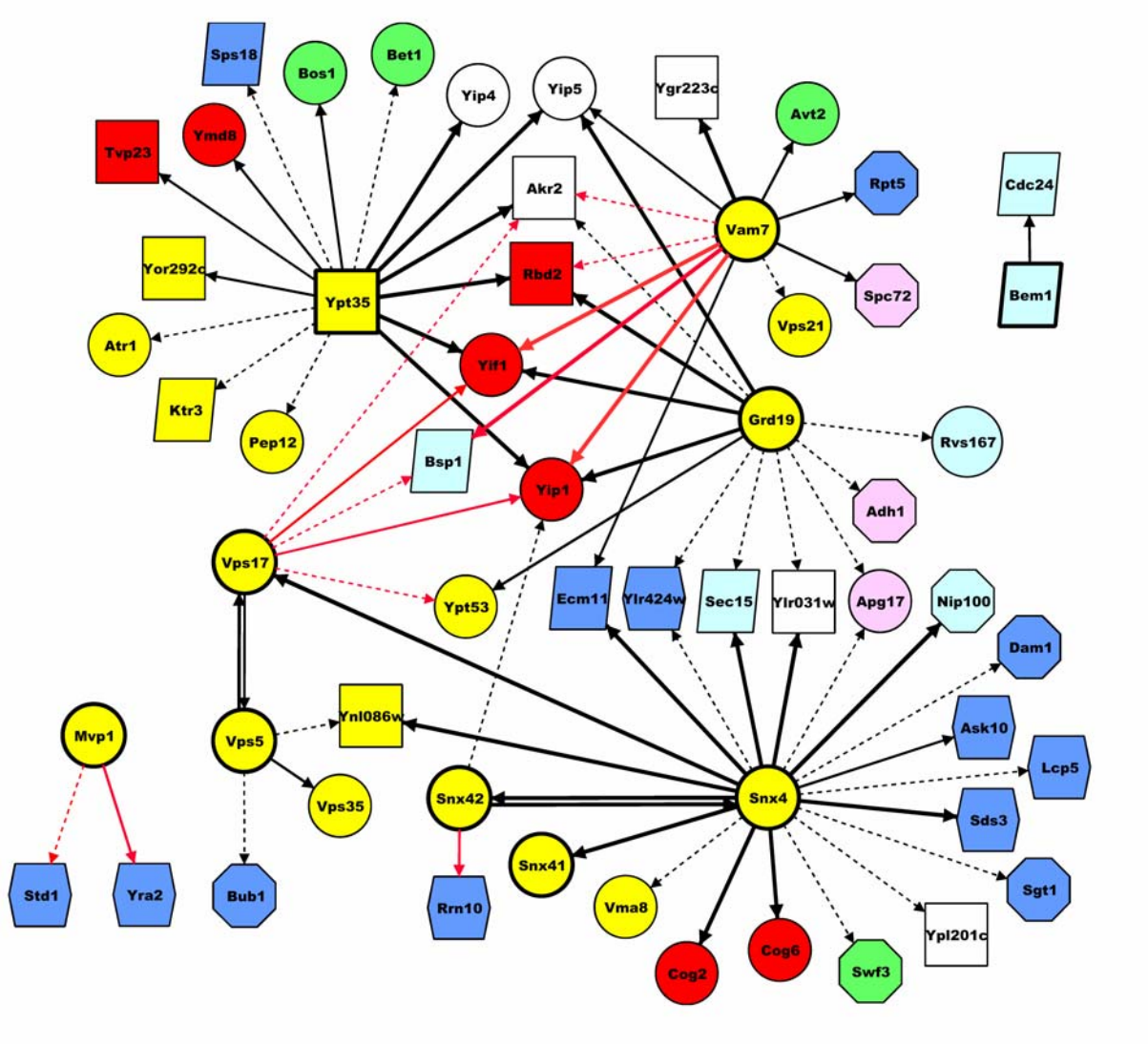
Of the 15 PX domain proteins in yeast, 9 showed a total of 67 two-hybrid interactions when they were screened as full-length proteins (Table 6). No reproducible interactions were found for 5 proteins (Bem3p, Mdm1p, Spo14p, Ykr078p, and Ypr097p). Snx41p was not investigated further because it turned out to be a very strong transcriptional activator, even under high 3AT concentrations. 3-aminotriazole (3AT) acts as a competitive inhibitor of the *HIS3* encoded gene (the reporter gene used in our two-hybrid system) and can be used to increase the growth threshold of the host strain. Under these conditions it is expected that even if the bait (DBD-ORF) activates *HIS3* to some extend, the interaction with a prey (AD-ORF) leads to more expression to overcome the growth threshold imposed by 3AT. This strategy is not always successful, especially for strong self-activators.

When the isolated PX domains were used as bait, only those from Mvp1p, Snx42p, Vam7p, and Vps17p showed a total of 13 reproducible interactions (Table 6).

Bem1p, Snx4p, and Vps5p interacted in the assays only as full-length proteins, that is, their PX domain did not interact with any other protein in my two-hybrid array. This indicates that other parts/domains of these proteins mediate their interaction. Grd19p and Ypt35p were tested only as full-length proteins as they consist almost exclusively of their PX domains. However, their two-hybrid interactions were confirmed with PX-only constructs in vitro (see below). In case of Mvp1p no interactions were found for the full-length protein, while its PX domain surprisingly interacted with two nuclear proteins, Std1p and Yra2p.

Finally, for Snx42p, Vam7p, and Vps17p different, but specific, interactions with both the full-length protein and the isolated domain were obtained. Interestingly, only Vam7p yielded interactions that were found with both the full-length protein and its PX domain.

Since only 8 interactions were found with the PX domain but not the corresponding full-length protein, I conclude that full-length proteins often prevent interactions, possibly due to their fusion with the Gal4 domain. Whatever the mechanistic reason may be, this indicates that I may have missed a significant number of interactions despite my comprehensive approach. Such false negatives may be prevented by more extensive usage of domains as baits.



ER
Cytosol
Golgi & ER to Golgi
Unknown
Endosome and Vacuole
Nucleus
Bud/Budneck & Actin

Energy generation, Metabolism, Protein modification, Protein degradation & Mitochondrion organization and biogenesis

Chromosome/Chromatin structure, Mitosis, Pol I & Pol II transcription, RNA metabolism, RNA splicing & Nuclear cytoplasmic transport

Cell growth and/or mantainance, Cell wall organisation and biogenesis & Cell polarity

Vesicular transport, Endocytosis, Autophagy &Vacuolar organization and biogenesis

Unknown

Figure 15: The PX domain interaction network in yeast

PX-domain containing proteins (*thick border*) and isolated PX-domain interactions (red arrows) in yeast. Thickness of the arrows represents the reproducibility of the interactions (i.e. scores 1-3) in Table 6. Fill colors of boxes represent the localization of the proteins and the shape their Gene Ontology (GO) class (see Experimental Procedures).

The nature of PX protein interactors

As expected from the role of many PX domain-containing proteins such as sorting nexins, the identified preys were enriched in proteins known to be involved in vesicular transport (36% of preys as opposed to 5% in the whole genome). Interestingly, a relatively large fraction of interactors were uncharacterized proteins (20% vs. 30.5%). All other functional classes combined made up 44% of all preys (whole genome: 64%) (Fig.16).

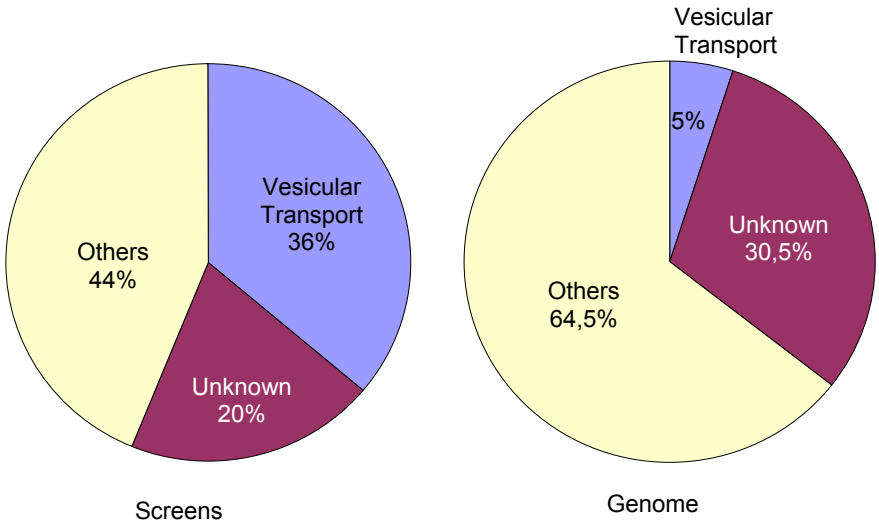


Figure 16: Gene Ontology distribution of interacting partners vs. whole yeast genome

GO (biological function) terms provided from SGD (Dwight et al. 2004).

Similarly, when localization data was analyzed in contrast to functional annotation, the majority (50%) of preys has been localized to the secretory or endocytic pathway, that is, to the ER, Golgi, endosomes, or the vacuole. Hence 53% of preys represented membrane or membrane-associated proteins which was somewhat unexpected because membrane proteins are often said to resist detection in two-hybrid screens. Another unexpected finding was that Snx4p, Snx42p, and Mvp1p all interacted with a significant number of nuclear proteins.

No single domain or motif was common among our preys. However, some domains were significantly enriched. Four PX domain-containing proteins bind to another PX domain protein, resulting in 4 pairs: Vps17p-Vps5p, Vps17p-Snx4p, Snx4p-Snx42p, and Snx42p-Snx41p. However, since the PX domain-only baits do not reproduce these interactions, this reflects characteristics of other parts of these sorting nexins - particularly their coiled-coil regions, which have been shown to drive homo- and hetero-oligomerization in other studies. This finding has been confirmed independently in a study of the Vps17p-Vps5p interaction (Seaman and Williams 2002).

A notable class of preys consists of the Yip1 family of proteins (Calero et al. 2002). Five out of the 9 productive PX proteins (Ypt35p, Vam7p, Grd19p, Vps17p, Snx42p) interacted with at least one member of the Yip1 family, and these interactions were defined by the PX domain as assessed using PX domain-only baits and/or by *in vitro* interaction studies (see below).

Other domains that were found repeatedly among PX protein interactors were the SNARE domain (in Ypt35p interactions with Bet1p, Bos1p, and Pep12p), the Rab

domain (Vps17p-Ypt53p, Grd19p-Ypt53p, Vam7p-Vps21p), and PH domains (Snx4p-Ask10p, Bem1p-Cdc24p). However, none of these interactions seems to involve the PX domain as only the full-length baits but not the PX-only baits detect those preys.

In vitro data

PX domains interact with members of the Yip1 family *in vitro*

Given the surprisingly strong interactions of PX domains with the highly hydrophobic Yip1 family members in the two-hybrid screens, I wanted to verify these results by *in vitro* binding assays. All yeast PX domains that were positive in the two-hybrid screens were expressed as GST fusion proteins in *E. coli* and purified on Glutathione-Sepharose beads. Note that the Mvp1p and Vam7p PX domains could not be tested because they were either unstable or insoluble. Yip1p, Yip4p, and Yif1p were translated and ^{35}S-labelled *in vitro*, and incubated with the GST-PX fusion proteins. Yip5p could not be tested because its *in vitro* translation repeatedly failed. The *in vitro* binding assays clearly show that Yip1 family members bind specifically to certain PX domains (Fig. 17). The strongest interaction was shown for Ypt35-PX which interacted strongly with Yif1p, and somewhat less with Yip1p and Yip4p. Vps17-PX showed a similar binding pattern, although the overall binding strength appears to be weaker. Snx4-PX only bound significantly to Yip4p. The PX domains from Snx42p preferred Yip1p over Yif1p, while the Vps5p PX domain appeared to bind most strongly to Yif1p. The *in vitro* interactions of isolated PX domains with Yip1 family members are summarized in Table 8.

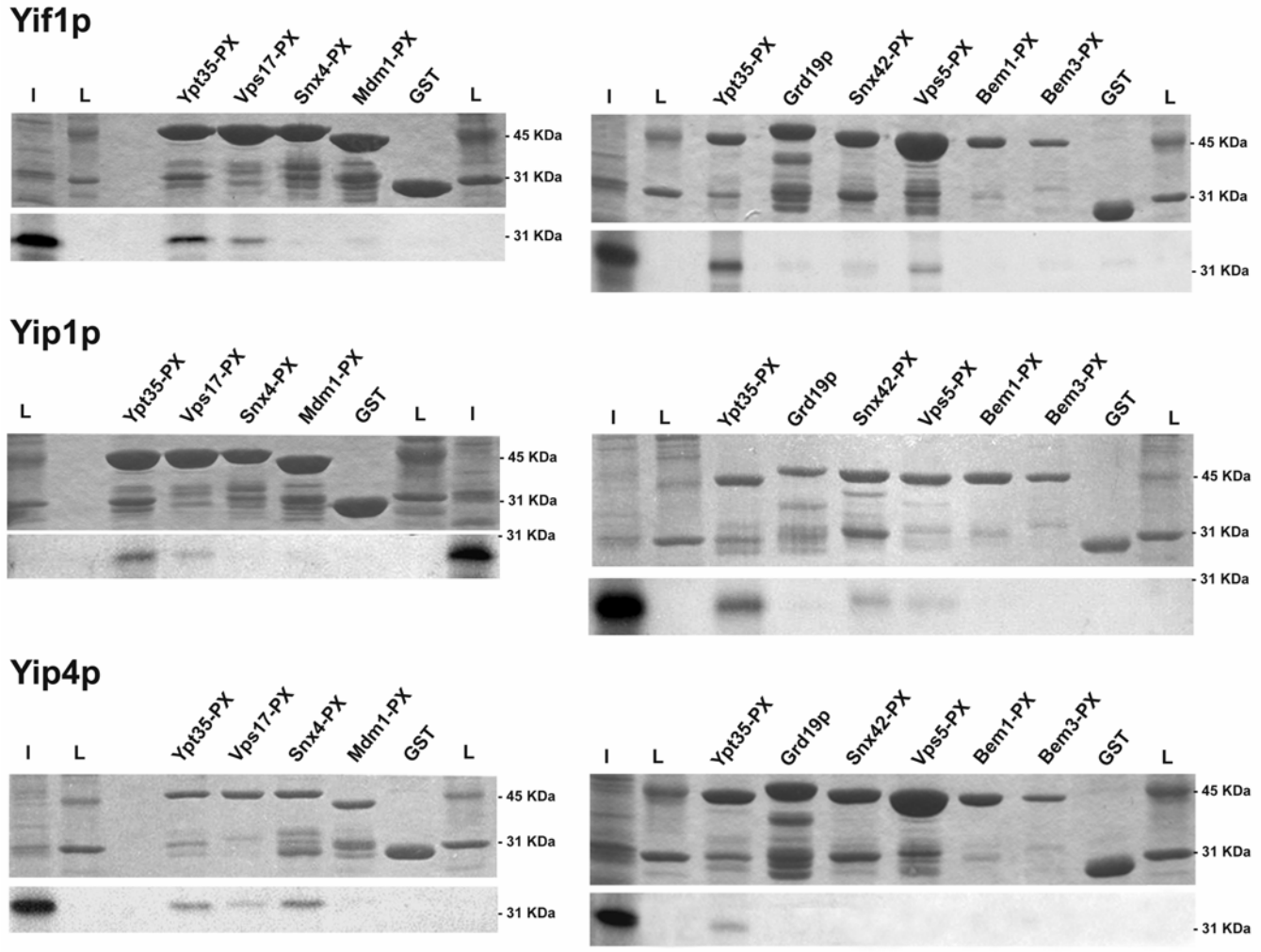


Figure 17: PX domains bind to Yip family members *in vitro*

Various GST-PX domain fusion proteins were tested for Yip1p, Yif1p and Yip4p binding *in vitro.* Pictures on top of each panel show Coomassie Blue-stained GST fusion proteins while bottom rows show autoradiography of bound Yip proteins (see Experimental Procedures for details). Yip1p and Yif1p bound equally strong to the Ypt35-PX domain while Yip4p bound only weakly. Yif1p and Yip1p were also bound by Vps17p and Vps5p PX domains and weakly by Snx42 PX domain. Yip4p was additionally bound by the PX domains Snx4p and Vps17p. **I:** Input (1µl of *in vitro* translated protein), **L:** Ladder (molecular mass marker). Only ¼ of each pull-down probe was loaded on to the gel.

	Preys		
GST	***Yip1p***	***Yip4p***	***Yif1p***
Ypt35-PX	++	+	++
Vps17-PX	+	(+)	+
Snx4-PX	-	++ *	-
Mdm1-PX	-	-	-
Grd19p	- 2H	-	(-/+) 2H
Snx42-PX	+ **	-	(+) *
Vps5-PX	(+)	-	+
Bem1-PX	-	-	-
Bem3-PX	-	-	-

Table 8: Results of GST-pull-down experiments

++: Very strong interaction; +: Strong interaction; (+): Weak interaction; -: no interaction
2H: Two-hybrid positive interaction; ***:** No two-hybrid interaction; ****:** No two-hybrid interaction with PX bait.

Comparison of two-hybrid and in vitro binding data

The in vitro binding data presented in Figure 17 largely supported our two-hybrid results. However, there were a few notable differences: Yif1p clearly bound to Vps5-PX in vitro, whereas our two-hybrid screens did not identify any Yip1 family interactions with Vps5p as bait. Similarly, Snx4-PX appeared to interact with Yip4 in vitro, but not in our two-hybrid screen.

By contrast, Grd19p interacted strongly with Yif1p, Yip1p, and Yip5p in a two-hybrid assay, but binding was not detectable in vitro (except perhaps Yif1p).

Finally, we observed one two-hybrid interaction, Snx42p-Yip1p, only with the full-length bait (but not the PX-only bait), although Snx42-PX interacted weakly with Yip1p and Yif1p *in vitro* (Fig. 17, Table 8).

Mapping of the interacting regions

Ypt35-PX binds to Yip1 and Yif1 N-Terminus

Yip1 family members are extremely hydrophobic proteins with 2-5 predicted transmembrane domains. The only significant cytoplasmic part is an N-terminal domain of approximately 100-150 amino acids that is conserved among Yip proteins (Calero et al. 2002), (Fig.18).

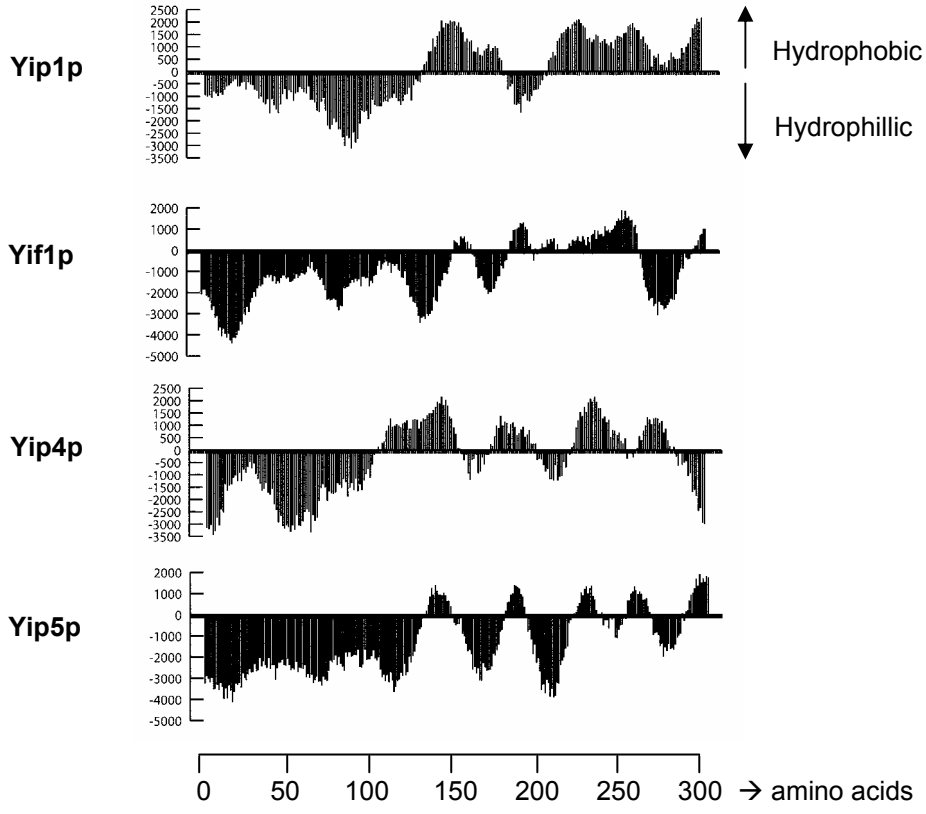


Figure 18: Prediction of membrane spanning regions and their orientations for Yip1p, Yif1p, Yip4p and Yip5p

TMpred plots were generated using the program TMpred with a 17 residue minimal and 33 residue maximal length of the hydrophobic part of the transmembrane helix. The TMpred plot shows the relative location of the hydrophobic/hydrophilic segments of the protein Sequence data indicate a cytoplasmically oriented N-terminus and a hydrophobic C-terminal domain with several potential membrane-spanning/insertion segments (Calero et al. 2002).

Since the N-Terminal domain of Yip1p and Yif1p is facing the cytosol (Yang et al. 1998; Matern et al. 2000) and therefore accessible to the PX-domain, I wondered if it interacts with PX domains. *In vitro* translated Yif1p and Yip1p N-terminus indeed bound to GST-Ypt35-PX (Fig. 19). Binding assays with the remaining C-terminal half of Yip1p also showed some weak binding (Fig. 20). It may be possible that the small cytoplasmic loops in the C-terminus of Yip1p contribute to PX binding.

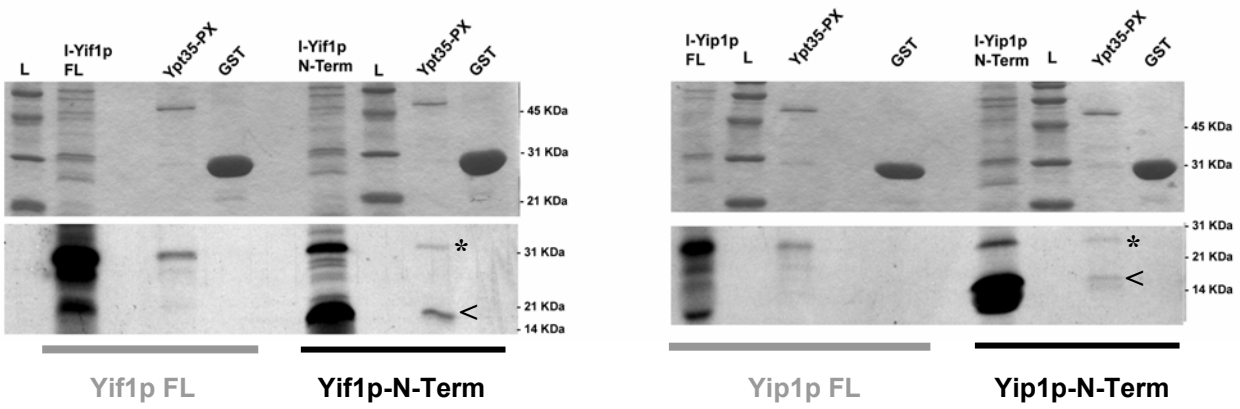


Figure 19: Yip1p and Yif1p N-Terminal fragments bind *in vitro* to Ypt35-PX

Ypt35p binds *in vitro* to the N-Terminal domains of Yip1p (1-112aa) and Yif1p (1-138aa). **I:** Input, **L:** Ladder (molecular mass marker), **FL:** Full-length protein, **N-Term**: N-terminal fragment. <: N-terminal fragment. *: Full-length protein which was additionally (to the N-terminal PCR fragment) *in vitro* translated due to some remaining contaminating full-length PCR products.

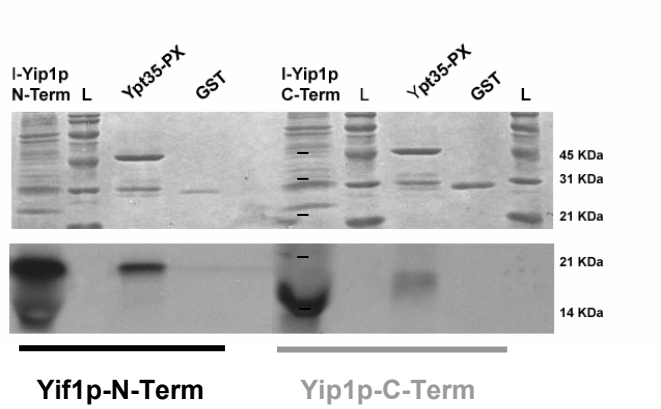


Figure 20: Ypt35-PX binds *in vitro* to Yip1p N-terminus and weakly to Yip1p C-Terminus

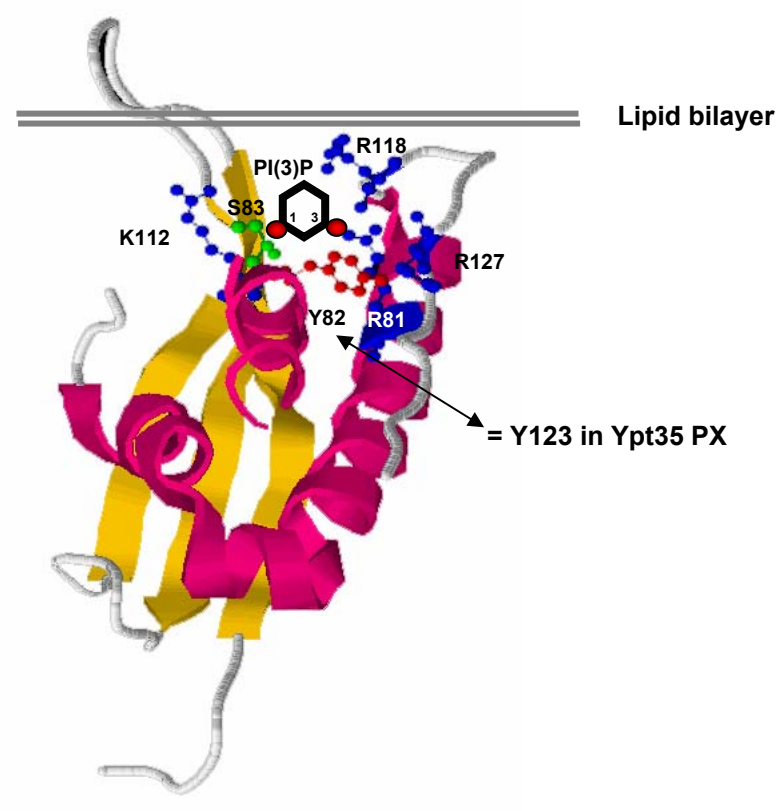
Ypt35p binds *in vitro* to the N-Terminal domain of Yip1p (1-112aa) and weakly to its C-terminus (112-248aa). **I:** Input, **L:** Ladder (molecular mass marker), **N-Term**: N-terminal fragment, **C-Term**: C-terminal fragment.

PI-binding site is different from putative protein-protein interaction sites

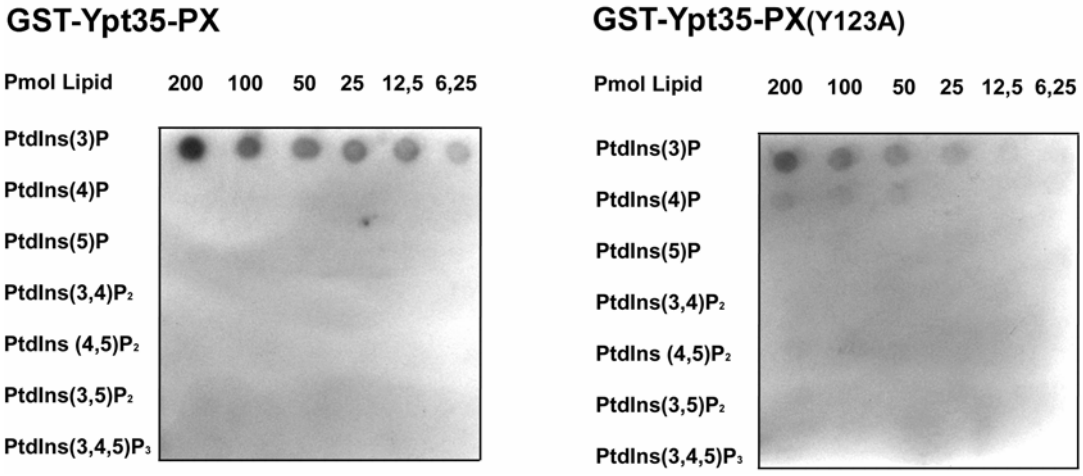
The observation that PX domains are involved in protein-protein interactions possess the question if the PtdIns(3)P binding site also contributes to these interactions. Do lipids and proteins compete for binding to the same sites in PX domains? I addressed this possibility by introducing a point mutation (Y123A) into the lipid-binding pocket of Ypt35-PX which I predicted should impair PtdIns(3)P-binding based on results with an analogous mutation in Vam7p, Snx3p, Cvt20p and Cvt13p (Sato et al. 1998; Cheever et al. 2001; Xu et al. 2001; Nice et al. 2002). As shown by the crystal

structure of Grd19p´s PX domain (Fig 21 A), this strongly conserved residue forms the floor of the phospholipid binding pocket (Zhou et al. 2003). The Y123A mutation did not completely abolish PtdIns(3)P binding by Ypt35p as shown by a protein-lipid overlay assay, although its binding affinity was significantly reduced (Fig. 21 B). Importantly, this mutation did not prevent *in vitro* binding of Ypt35-PX to any of the Yip1 family members (Fig. 21 C), although the amount of bound protein appeared to be slightly reduced compared with that seen for wild-type protein. Thus, lipid binding does probably not interfere with protein-protein interactions in PX domains (although the two ligands may cooperate). In addition, this suggests that interacting proteins also use a different interaction surface than lipids or at least do not require the critical Tyrosine-123 that is involved in binding the phosphate head group of phosphoinositides.

A)



B)



C)

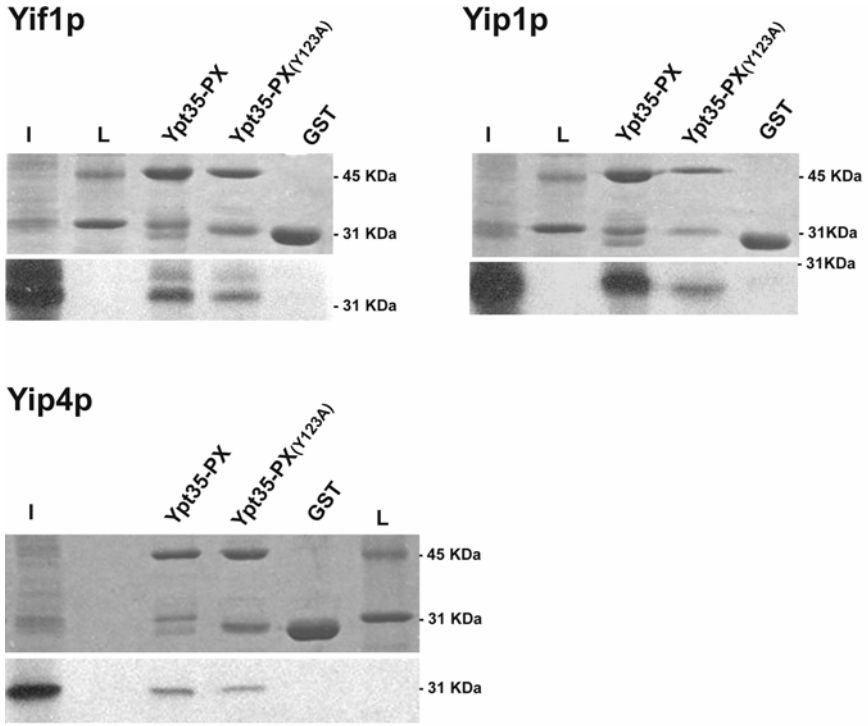


Figure 21: Protein-protein interaction is independent of PX-domain phospholipid binding

(A) Crystal Structure of the PX domain of Grd19p. Important conserved residues for lipid binding pocket formation are shown in ball and stick display: R81 (arginine 81:main specific interaction with the 3-phosphate group), Y82 (tyrosine 82: floor of the pocket, the face of the inositol ring rests on it, analogous to mutation Y127A in Ypt35-PX), S83 (serine 83: stabilizes hydrogen bond of 3-phosphate group), R118 (arginine 118: forms a second salt bridge with the 3-phosphate group), R127 (arginine 127: forms hydrogen bonds with the 4- and 5-OH groups of PI(3)P), K112 (lysine 112: close to the non-bridging oxygen of the 1-phosphate group) (Zhou et al. 2003). Cartoon represented with the Rasmol program (Sayle and Milner-White 1995).

(B) The affinity of Ypt35-PX and its mutant Ypt35p^{Y123A}-PX for PtdIns(3)P were assessed by a protein-lipid overlay assay. PtdIns were spotted onto a nitrocellulose membrane at different concentrations and incubated with 5µg of GST-fusion protein as well as GST alone (data not shown). Binding of the proteins was detected with anti-GST antibodies. The Ypt35p^{Y123A}-PX mutant exhibits significantly reduced binding affinity as no binding was detected at a lipid concentration of 12.5 pmol when compared to the wild-type protein which still bound at a concentration of 6.25 pmol. The overall binding to PtdIns(3)P of the mutant was therefore reduced by about 3-fold.

(C) Mutant GST-Ypt35p^{Y123A}-PX with partially abolished PtdIns(3)P binding still interacts with Yip1p, Yif1p and Yip4p. **I:** Input, **L:** Ladder (molecular mass marker).

Peptide arrays

To narrow down the interacting region of the Yip1 family members with the PX- only protein Grd19p and the PX domain of Ypt35p to the level of the single amino acid, peptide arrays have been used. Additionally, other PX domains and non Yip1p family members were analysed in order to compare and depict an interacting motif.
For this purpose peptides were synthesised on cellulose membranes either coupled with β-alanine OH as spacer or directly on to commercially available membranes from Intavis Bioanalytical Instruments AG (Germany) with incorporated spacer (see experimental procedures). Peptides were synthesized with 3 amino acids offsets

along the sequence. The membranes were then incubated over night with eluted GST-PX fusion proteins or GST alone, and binding was detected via α–GST antibodies and chemiluminescence (see experimental procedures).

Yip1 family members

Experimental setup

To test the reproducibility and reliability of this method we synthesised peptides of all Yip1 family members on different membranes. We started to test those two PX domains which interacted either with all family members (Ypt35-PX) or with 3 of them (Grd19p). We repeated these experiments 3 times for each protein and all family members. GST was used as a negative control. These membranes are represented in figures 22, 24, 26 and 28. Membranes are positioned on a grid were positive peptides are identified by their position.

Recognizing interacting regions

To have a clear representation of the distribution of positive interacting peptides on the predicted structure of Yip1 family members, spots representing the peptides were positioned on a schematic representation of Yip1 family proteins (Figures 23, 25, 27 and 29). The different colours identify the different PX-domains fused to GST. As expected from my *in vitro* results, I find binding peptides on the cytosolic N-termiuns of Yip1 family members but also at the C-terminal loops. Yip1p shows the clearest binding pattern concentrating its binding at the N-terminal motifs PVGILHALSTKG and ITK. It is possible to restrict this interacting motif to 3 amino acids which are shared by all 5 positive peptides (Fig. 22 23). I also observe binding at the loop between TM1 and TM2 (Spots B23, 24) which according to the prediction, should not be accessible to PX domains for they are facing the Golgi lumen (Fig. 23). Further, the binding peptide C24 positioned at the C-terminus of Yip1p should be facing the cytosol. It should be kept in mind that the spots B23, 24 and C24 showed also strong binding to GST and may be therefore unspecific binding motifs due to the GST fusion of PX domains.
To verify these binding motifs and those of Yif1p, Yip4p, and Yip5p, alanine scanning of selected peptides was performed (see mapping to the single amino acid with alanine scans).
PX domain binding peptides of Yif1p, Yip4p and Yip5p are more promiscuous and distributed quite uniformly throughout the whole protein. It is therefore more difficult to assign a particular PX domain binding motif for those proteins (Figures 25, 27, 29).

Mapping to the single amino acid with alanine scans

For the analysis of the amino acid residues which are essential for PX domain binding, selected peptides were subjected to mutational anlysis by successively replacing all 15 amino acids by alanine (alanine walk), (Liu et al. 1999; Bluthner et al. 2000), (Fig. 23).

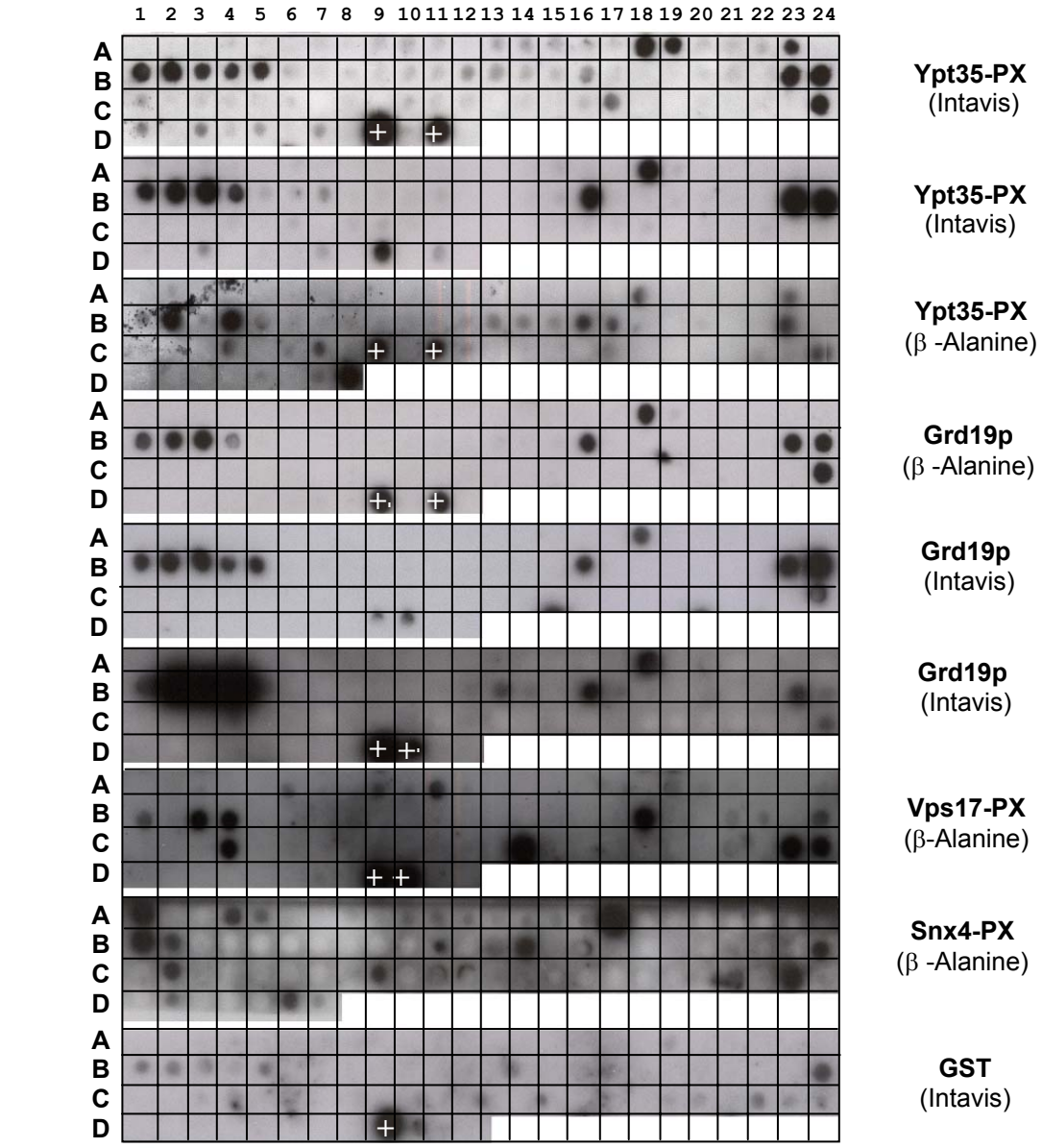


Figure 22: PX-Domain mapping on Yip1p peptides

Yip1p peptide arrays (the complete sequence of Yip1p synthesized as 79 15meres with 3 amnio acid offsets) were incubated overnight with GST-PX fusion protein or GST alone respectively (see right margin). Positive interactions were visualized using α-GST antibodies and chemiluminescence (see experimental procedures). Membranes used are indicated in parentheses on the right margin. +: Positive Controls (see experimental procedures).

Yip1p

We tested here the N-terminal binding peptides A18 and B3 (Fig. 23). Binding of A18 to Ypt35-PX and Grd19p was reproducible (Table 9). Residues KG are essential for PX domain binding as the replacements with alanines abolish the interaction (no black spot on the membrane, Fig 23). In the case of the B3 peptide binding was narrowed down to the ITK sequence. I expected to obtain a loss of interaction upon ITK alanine replacement. Instead KTK was obtained, i.e. only an overlap of the first lysine in KTK (Fig. 23). To further analyse this discrepancy, mutational analysis and peptide competition assays were performed (see *Yip1p HIITKTKM motif analysis on Ypt35-PX interaction*).

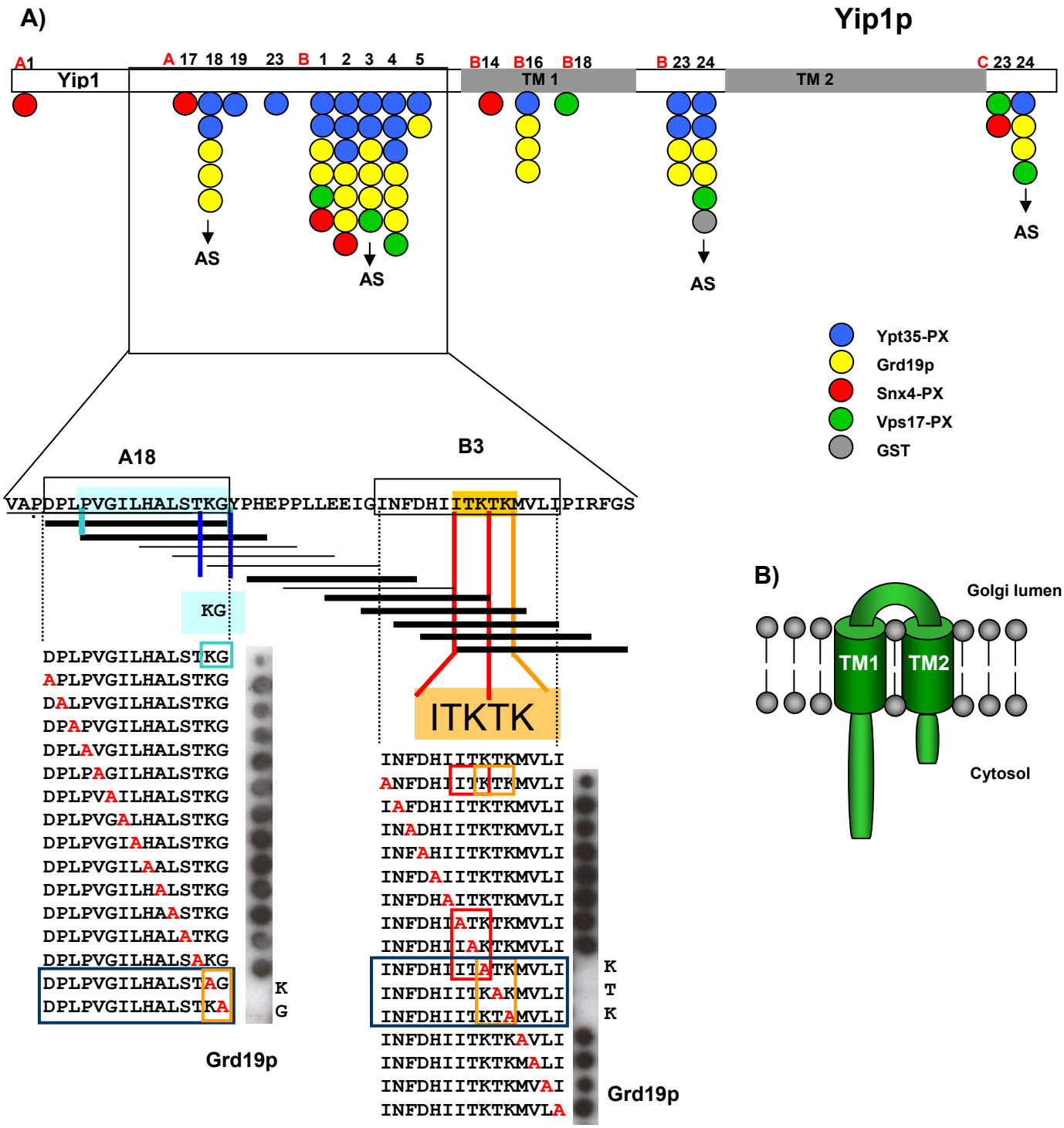


Figure 23: Schematic representation of PX domain binding sequences on Yip1p

A) Amino acid sequence of Yip1p represented as a rectangle (TM: predicted transmembrane domains). Coloured spots represent positive interaction of distinct PX domains (see legend) with particular peptides (peptides are indicated above, eg. A18 etc). **AS**: Indicates peptides were alanine scans were performed. **Box**: zoom in section with amino acid sequence. Bars represent peptides shifted by 3 amino acids. Thick bars represent positive interactions. Rows of interacting peptides indicate interacting region (represented in coloured boxes). Alanine scan information of Yip1p peptides A18 and B3 for Grd19p. Replacement for KG with alanine at peptide A18 abolished the interaction. For peptide B3 replacement of amino acids KTK abolished Grd19p interaction. This region does not overlap exactly with the one obtained from the 5 in a row positive shifted spots (ITK).
B) Schematic presentation of Yip1p at the membrane with two predicted transmembrane domains. The N-terminus has been shown to face the cytosol (Yang et al. 1998). Accordingly the loop between TM1 and TM2 would be facing the lumen of the Golgi and therefore not accessible to the PX domain.

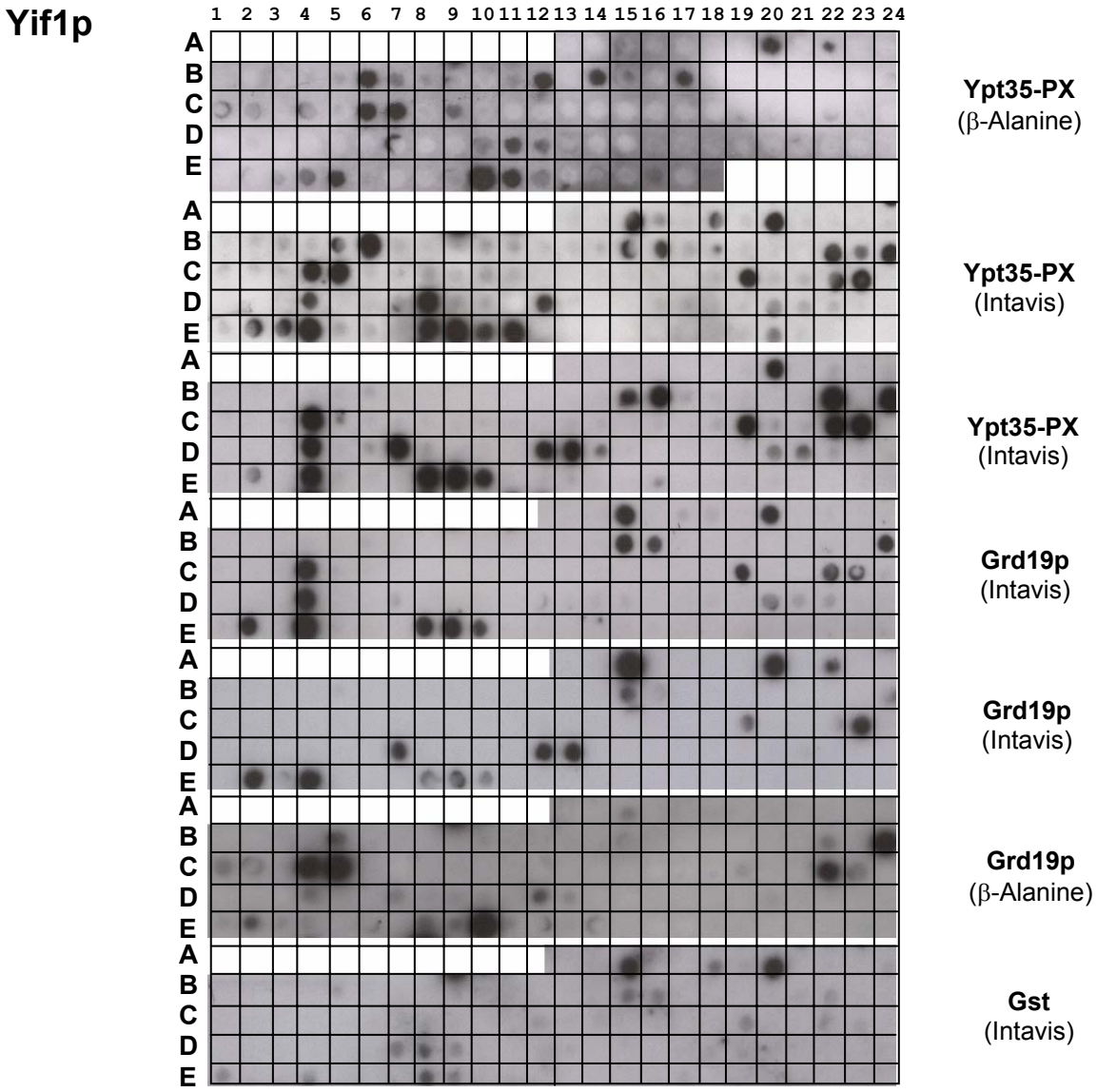


Figure 24: PX-Domain mapping on Yif1p peptides

Yif1p peptide arrays (118 15meres covering the complete sequence of Yif1p synthesized with 3 amnio acid offsets) were incubated overnight with GST-PX fusion protein or GST alone respectively (see right margin). Positive interactions were visualized using α-GST antibodies and chemiluminescence (see experimental procedures). Different types of membranes are indicated in parentheses on the right margin.

Yif1p

Here alanine scans of several peptides were performed (Fig. 25) with relative contradictory results (Table 9, Fig. 25). As an example, the alanine scans of peptides B24 and C4, which overlap only by 3 amino acids, are shown in Fig. 25. B24 shows abolishment of the interaction upon mutation of the last two lysines. These two lysins overlap at the beginning of the C4 peptide (shown in green) but the interaction still takes place when replaced with alanine. The spatial position of the peptide relative to the membrane may be responsible for these discrepancies. C4 on the other hand, loses its interaction with both Ypt35-PX and Grd19p upon replacement of the last lysine. This motif can be narrowed down to 6 amino acids (blue rectangle in Fig. 25 B) by means of the four positive peptides obtained peptide arrays (C4 to C7, Fig 24). The E10 peptide shows a strong binding and a clear alanine scan with the last two lysines being important for interaction (Fig. 25 B) although it should be facing the lumen of the Golgi according to the predicted topology (Fig. 25 A) and therefore should not be accessible to the PX domain.

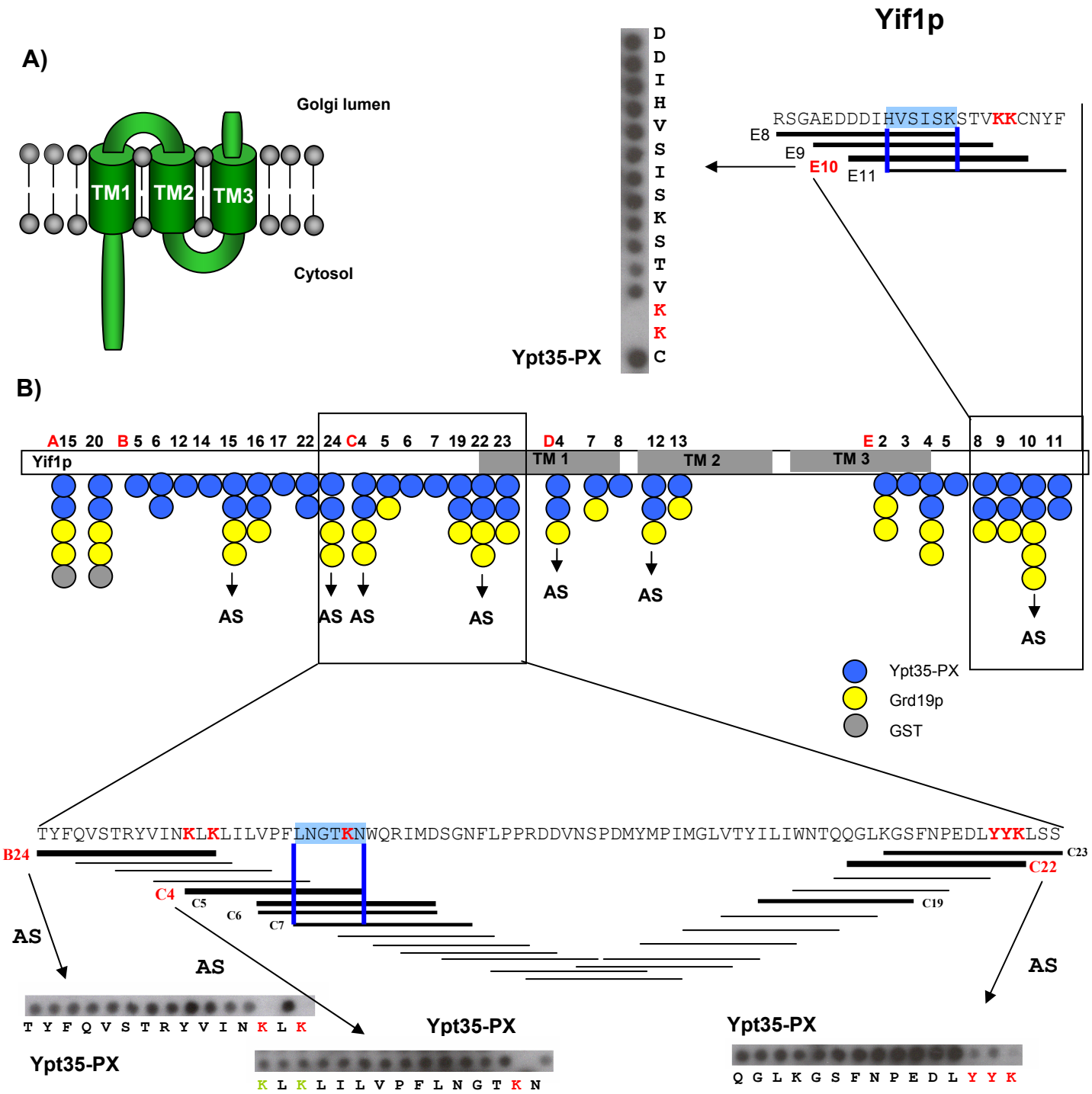


Figure 25: Schematic representation of PX domain binding sequences on Yif1p

A) Schematic figure of Yif1p at the membrane according to its predicted transmembrane domains. The N-terminus has been shown to face the cytosol (Matern et al. 2000). Only the 2nd loop would be facing the cytosol and therefore be accessible to the PX domain. **B)** Amino acid sequence of Yip1p represented as a rectangle (TM: predicted transmembrane domains). Coloured spots represent positive interactions of distinct PX domains (see legend) with particular peptides (peptides are indicated above, eg. A15 etc). **AS**: Indicates peptides were alanine scans were performed.
Boxes: zoom in sections with amino acid sequences. Bars represent peptides shifted by 3 amino acids. Thick bars represent positive interactions, peptide numbers are on the side of the bars. Rows of interacting peptides indicate interacting region (represented in coloured boxes).
Peptides marked in red: alanine scan with amino acids in red where interaction has been abolished by alanine replacement. Peptides in green, expected abolishment of interaction by replacement with alanine.

Yip4p

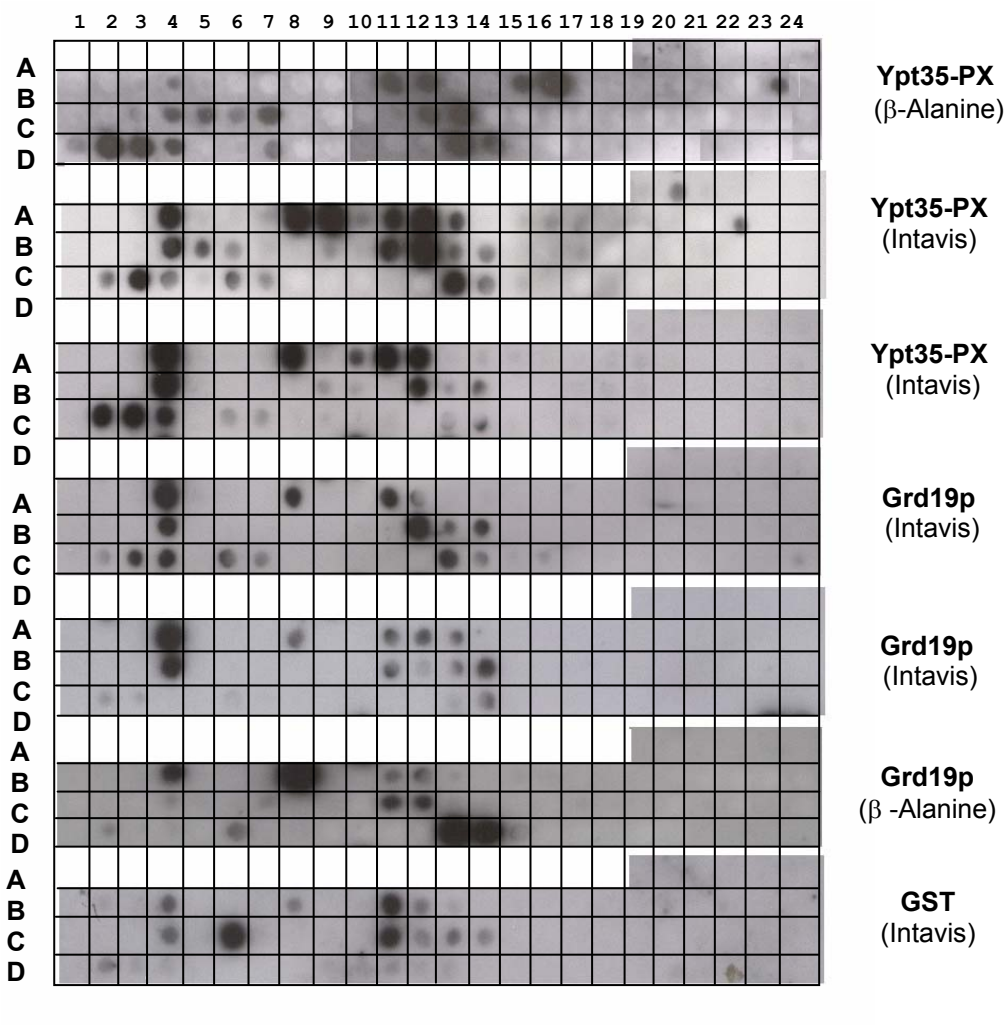


Figure 26: PX-Domain mapping on Yip4p peptides

Yip4p peptide arrays (the complete sequence of Yip4p synthesized as 78 15meres with 3 amnio acid offsets) were incubated overnight with GST-PX fusion protein or GST alone respectively (see right margin). Positive interactions were visualized using α-GST antibodies and chemiluminescence (see experimental procedures). Different types of membranes are indicated in parentheses on the right margin.

Yip4p

Yip4p peptides exhibit similar discrepancies as Yif1p (Fig. 26, 27). B4 and B8 overlap by 3 amino acids and notably in B4, arginine replacement abolished PX domain interaction not in B8 (Fig. 27 A). Instead, B8 showed loss of interaction with mutated C-terminal lysine, but this amino acid does not affect binding when mutated in an N-terminal positon of peptide B12. The positioning of basic residues at the C-terminal seems to play a role.

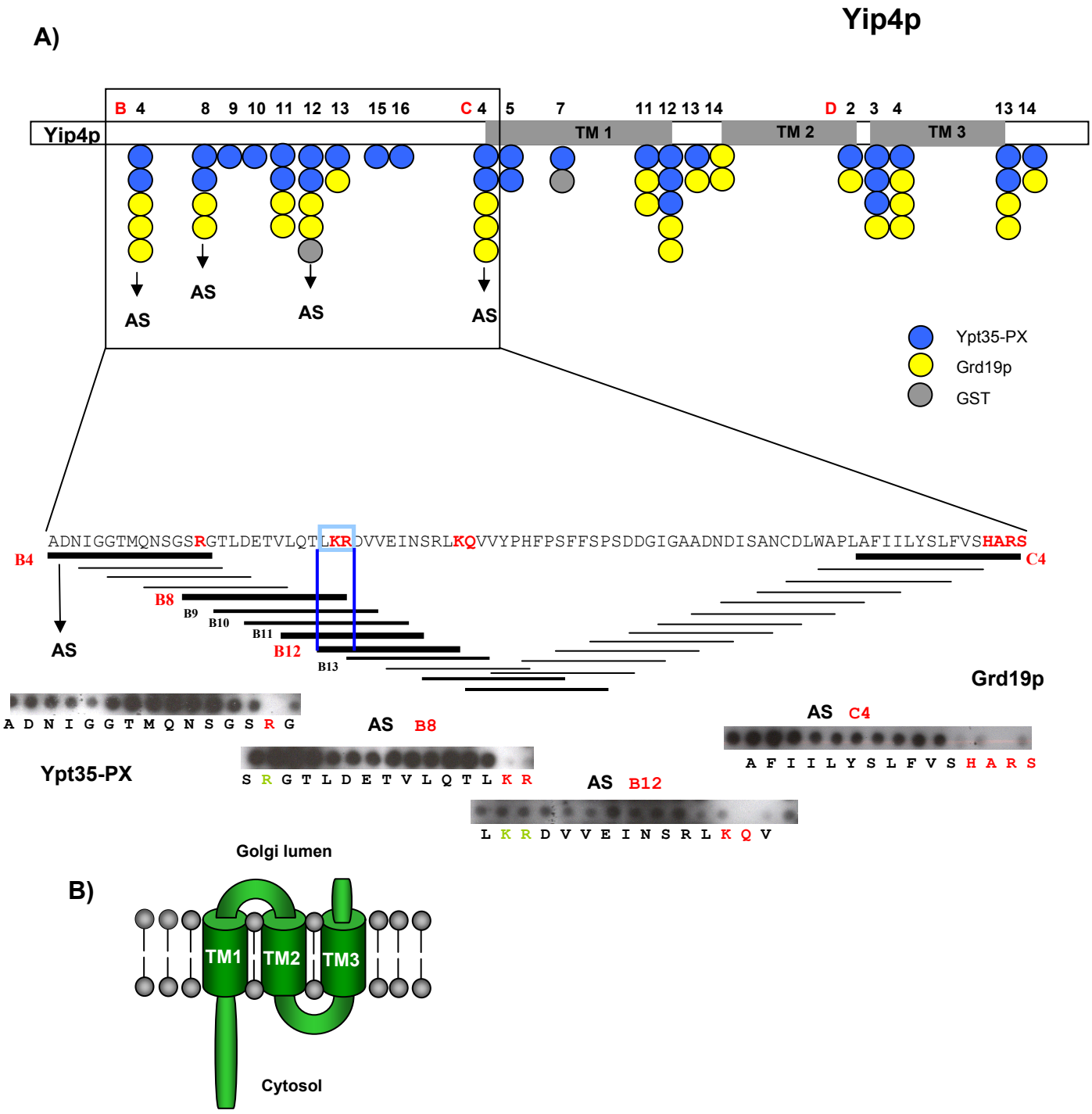


Figure 27: Schematic representation of PX domain binding sequences on Yip4p

A) Amino acid sequence of Yip4p represented as a rectangle (TM: predicted transmembrane domains). Coloured spots represent positive interactions of distinct PX domains (see legend) with particular peptides (peptides are indicated above, eg. B4 etc). AS: Indicates peptides were alanine scans were performed. Box: zoom in section with amino acid sequence. Bars represent peptides shifted by 3 amino acids. Thick bars represent positive interactions, peptide numbers are on the side of the bars. Rows of interacting peptides indicate interacting region (represented in coloured box).
Peptides marked in red: alanine scan with aminoacids in red were interaction has been abolished upon alanine replacement. Peptides in green, expected abolishment of interaction on replacement with alanine. **B)** Schematic figure of Yip4p at the membrane according to its predicted transmembrane domains. The N-terminus is predicted to face the cytosol based on homology to Yip1p and Yif1p (Matern et al. 2000; Calero et al. 2002). Only the 2nd loop would be facing the cytosol and therefore be accessible to the PX domain.

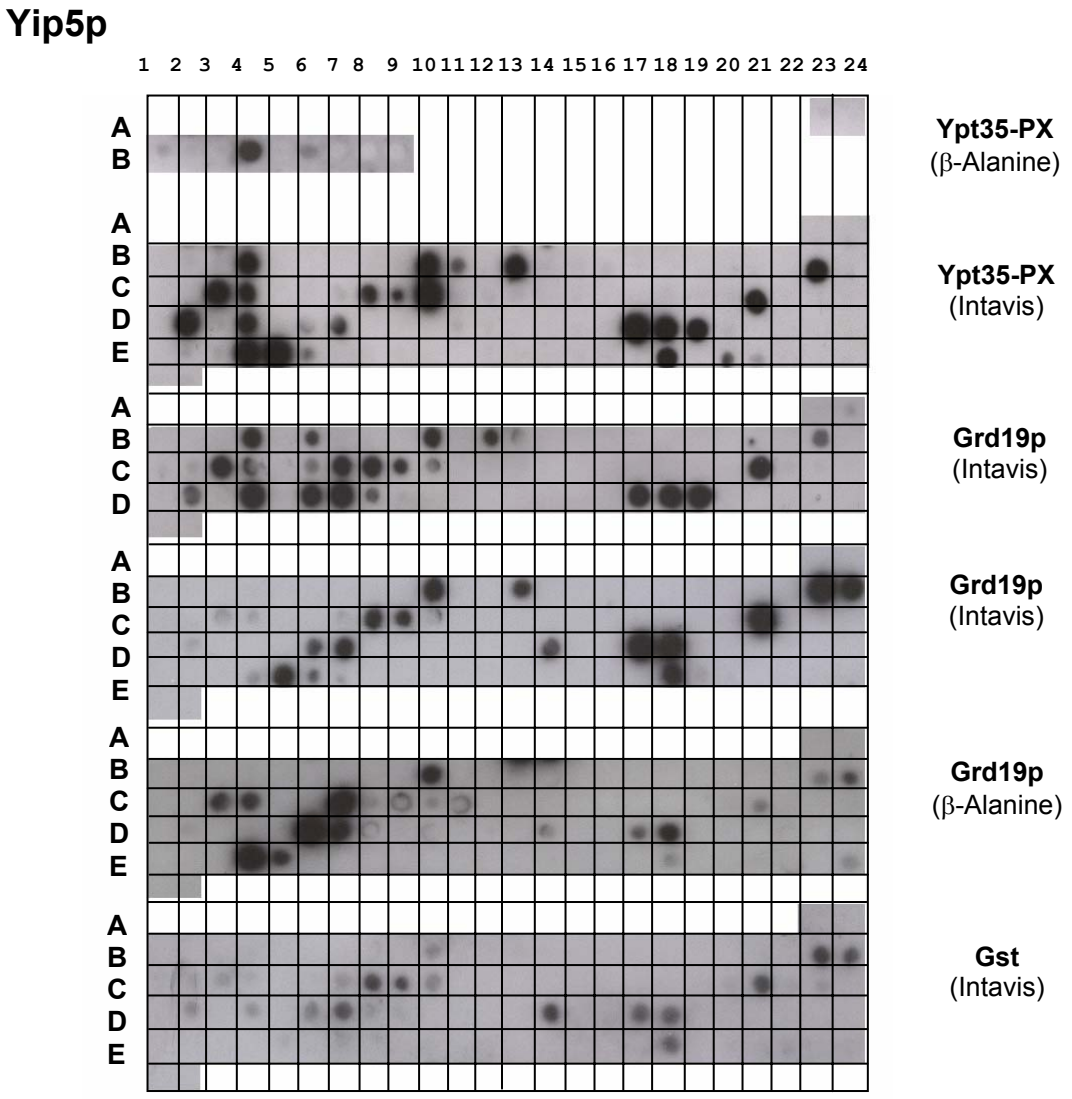


Figure 28: PX-Domain mapping on Yip5p peptides

Yip5p peptide arrays (116 15meres covering the complete sequence of Yif1p synthesized with 3 amnio acid offsets) were incubated overnight with GST-PX fusion protein or GST alone respectively (see right margin). Positive interactions were visualized using α-GST antibodies and chemiluminescence (see experimental procedures). Different types of membranes are indicated in parentheses on the right margin

Yip5p

Yip5p is a case where overlapping amino acids such as in B10 and B13 causes loss of the interaction when mutated (C-terminal K in B10 and N-terminal K in B13, Fig. 29 A). Clear alanine scans are also obtained at the C-terminus of the protein, i.e. in the loops between the hydrophobic regions.

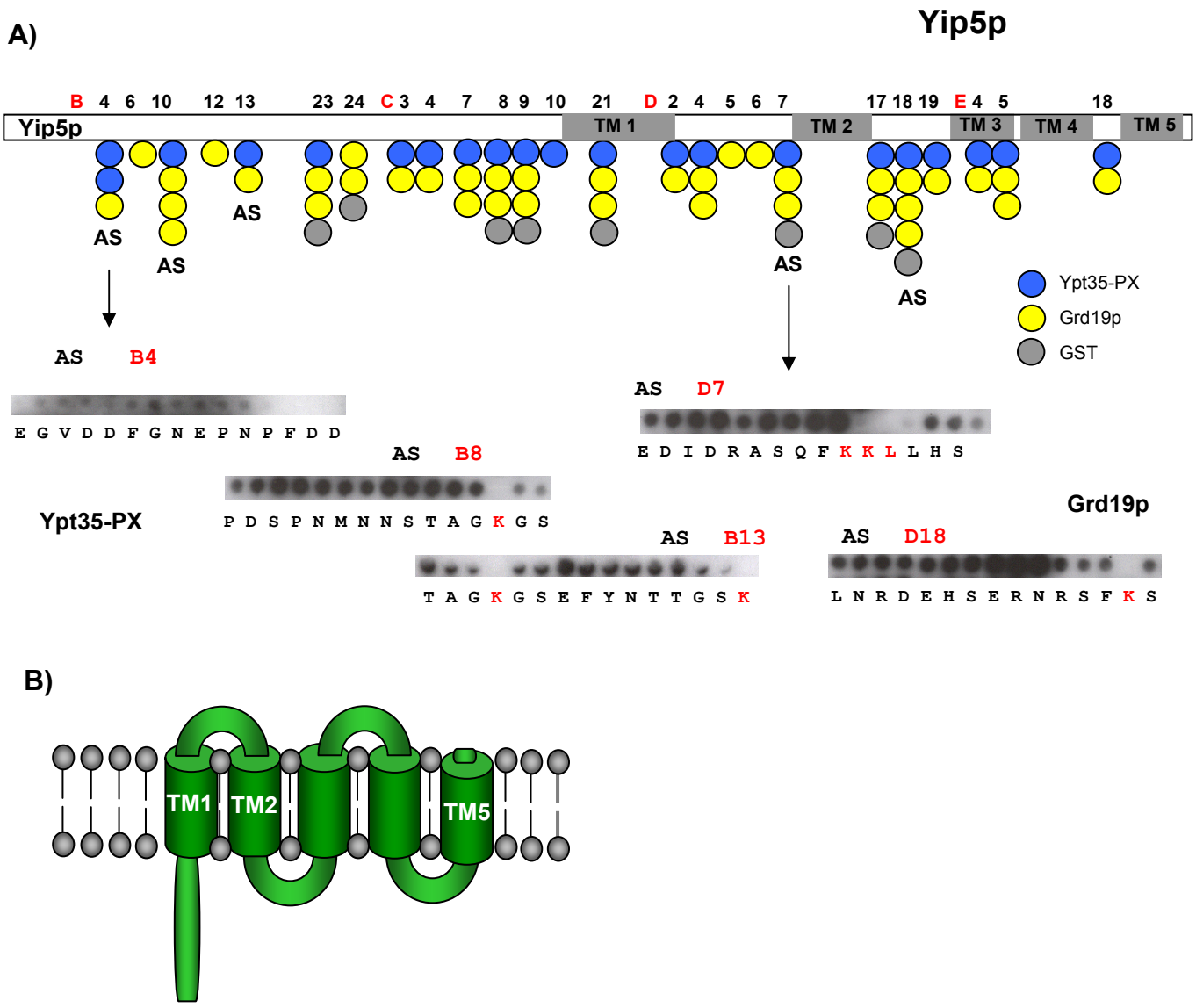


Figure 29: Schematic representation of PX domain binding sequences on Yip5p

A) Amino acid sequence of Yip5p represented as a rectangle (TM: predicted transmembrane domains). Coloured spots represent positive interaction of distinct PX domains (see legend) with particular peptides (peptides are indicated above, eg. B4 etc). AS: Indicates peptides were alanine scans were performed. Red amin oacids indicate were the interaction has been abolished through alanine replacements. **B)** Schematic figure of Yip5p at the membrane according to its predicted transmembrane domains. The N-terminus is predicted to face the cytosol based on homology to Yip1p and Yif1p (Matern et al. 2000; Calero et al. 2002). Loops 2 and 4 would be facing the cytosol and therefore be accessible to the PX domain.

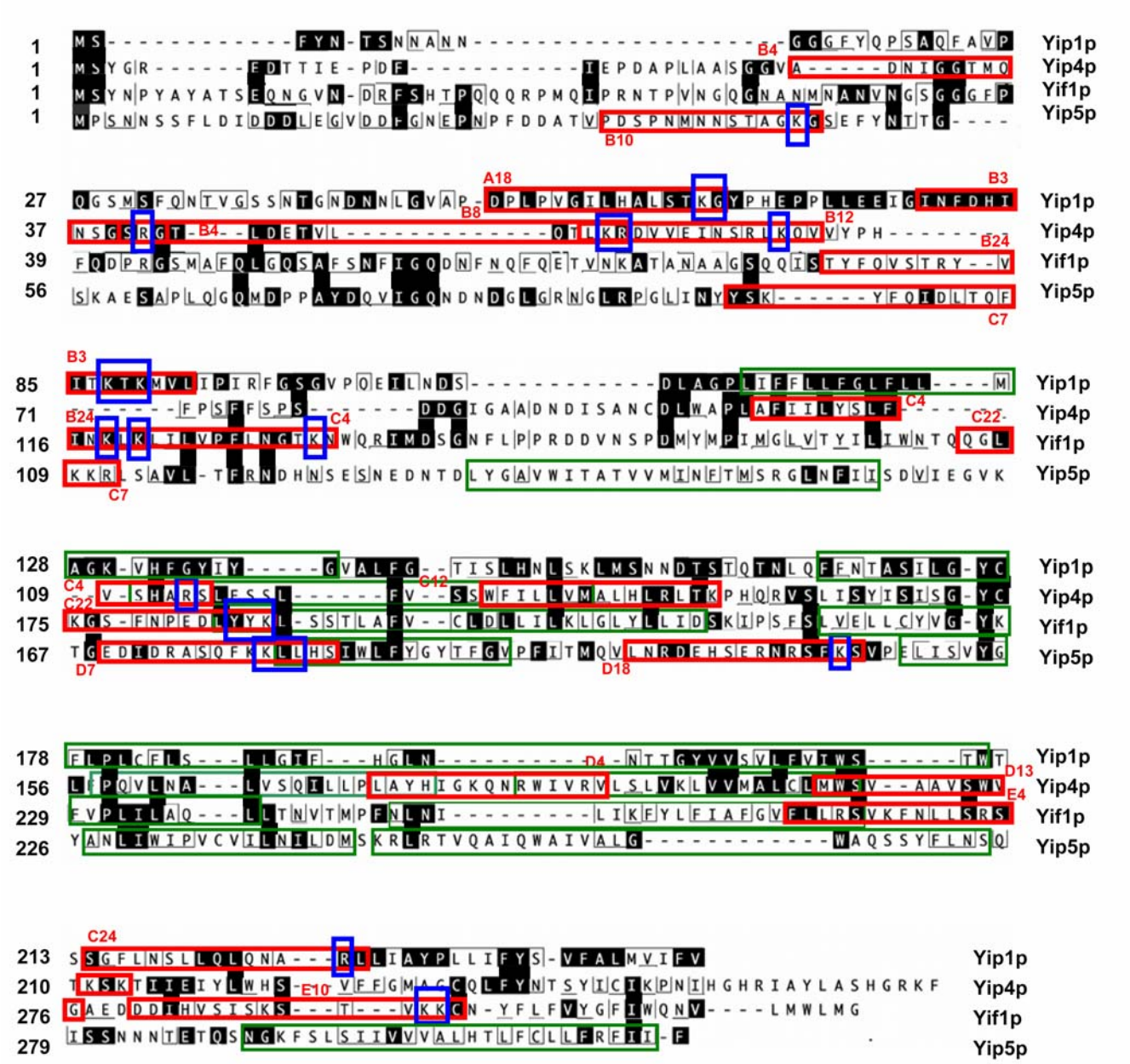


Figure 30: Peptide array information on yeast Yip1 family multiple alignment

C24:	Peptide position
	Surrounding peptide sequence
	Surrounding amino acids whose replacement by alanine abolished the interaction with Grd19p and Ypt35-PX
	Transmembrane domain

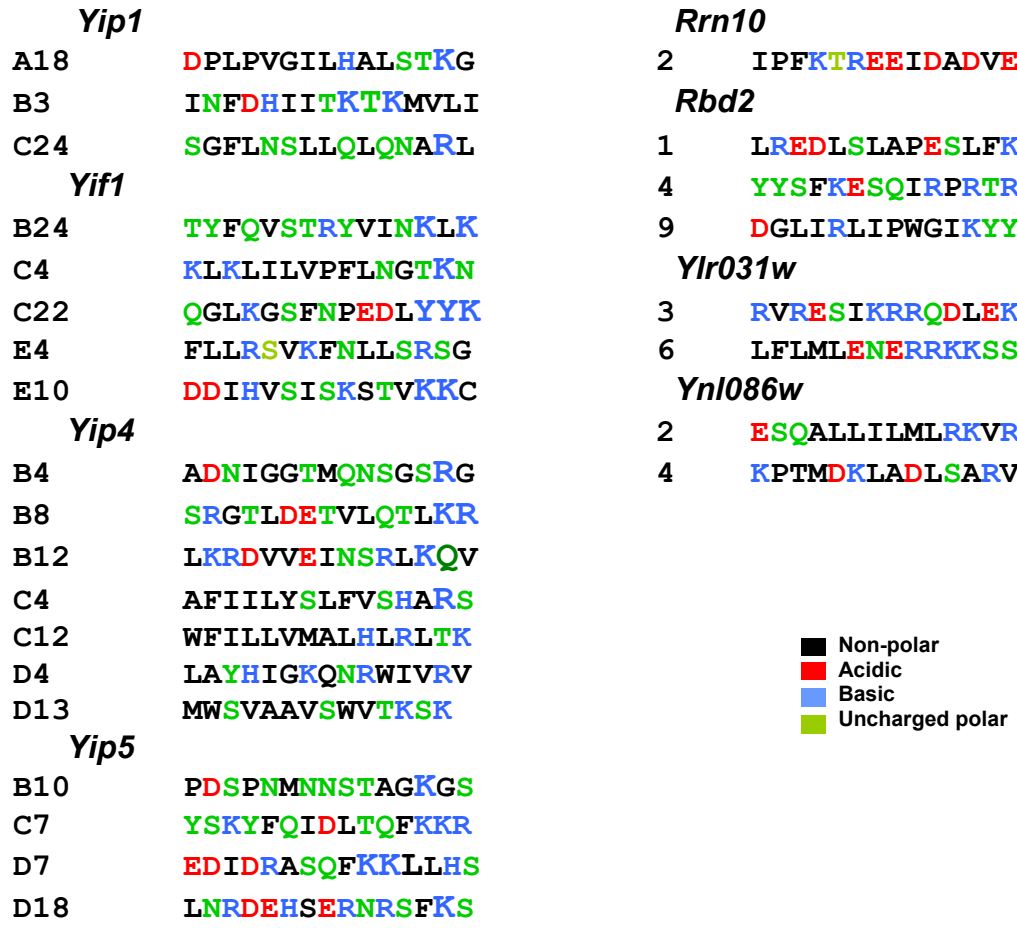


Figure 31: PX domains bind peptides with basic C-termini of Yip1 family members

Peptides which interacted several times with different PX domains (incl. alanine scans in table 9) have been selected. Amino acids were coloured according to their properties, **red**: acidic, **green**: uncharged polar, **blue**: basic and **black**: non-polar. Amino acids bigger in size are essential for binding: when changed to alanine the interaction is lost. When Yip1 family peptides are compared to non-Yip1 family peptides (these only reproduced once) we clearly see a preference for peptides with basic residues at their C-terminus. The peptides additionally contain few acidic residues or none at all.

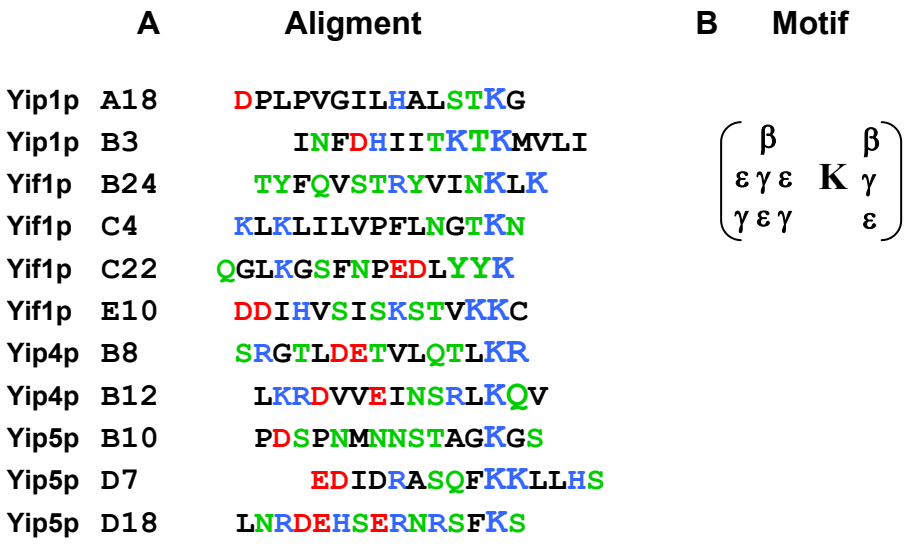


Figure 32: Aligment of essential K´s depicting an interacting motif

Depiction of the general context in which the essential K´s are embeded (A) defining it as in (B) were β represents any basic residue, ε any hydrophobic residue and γ any uncharged polar residue.

PX domains bind to basic peptides at the N-terminus of Yip1 family members and their intermembrane loops.

Reproducibly interacting peptides as well as reproducible alanine scans were selected (Table 9 and Fig. 31). Amino acids of these peptides were coloured according to their properties (acidic, basic etc., Fig. 31). As already observed from the alanine scans, we clearly see a tendency for binding peptides which are composed of basic residues at their C-terminus. Compared to those of non-Yip1 family members (although these were not reproduced several times) they also contain a reduced number of acidic residues or none at all. These peptides are concentrated at the N-terminus of Yip1 family members but are also located at their hydrophobic C-terminal half which may contribute to PX binding.

When I aligned the peptides that carry the essential lysines for binding, that is, those that were indispensable for the interaction according to the alanine scan, I was able to observe general context in which the positive lysines are embedded (Fig. 32). I defined the binding motif as follows:

$$\begin{pmatrix} \beta & & \beta \\ \varepsilon\,\gamma\,\varepsilon & \mathbf{K} & \gamma \\ \gamma\,\varepsilon\,\gamma & & \varepsilon \end{pmatrix}$$

where β represents any basic residue, ε any hydrophobic residue and γ an uncharged polar residue.

Binding site conservation among Yip1 family members

I wondered if Yip1 family interacting peptides have conserved amino acids (Fig. 30). For this purpose, reproducible peptides are shown (boxes) in an alignment adapted from Calero et al. (2002). I do observe some overlap but not between all members of the family. The strongest conservation is observed for the peptides B3 for Yip1p and B24 for Yif1p. Here the lysins at positions K87, K89 and K118, K120 respectively, which were identified in alanine scans and considered as important for interaction, are conserved within these two members. Yip4p and Yip5p may have other interacting regions.

Differences between PX domains

To test if we are able to see the same difference between the PX domains in the spot-array as in the two-hybrid analysis (i.e. PX domains that interact with Yip1 family members and those which do not) peptide arrays of Yip1p were incubated with Yip1p binding PX domain Vps17p (additionally from Grd19p and Ypt35-PX) and the non-Yip1p-binding Snx4-PX domain. Vps17-PX and Snx4-PX showed binding at the expected interacting peptides at positions B1-5. Snx4-PX shows only 2 interactions of the 5 expected peptides were as Vps17-PX only 3, but these variations were also obtained when incubating β–ala membranes with Ypt35-PX (Fig.22). Snx4 had additional interacting peptides not found with other PX-domains but these were single spots and possible false positive interactions. I was not able to observe the expected differences from the two-hybrid results which may be due to structural differences which are lost when using this method. We must also consider that peptide arrays of Vps17-PX and Snx4-PX were only performed once and on β-ala membranes which seem to exhibit stronger spot pattern variations than the commercially available Intavis membranes.

Peptide Position	Sequence	Protein: positves/total	Protein: Alanine scan	Result Alanine scan
		Yip1p	*Figures 22-23*	
A1	MSFYNTSNNANNGGG	**Snx4-PX:** 1/1	--	--
A17	VAPDPLPVGILHALS	**Snx4-PX:** 1/1	--	--
A18	DPLPVGILHALST**KG**	**Grd19p:** 3/3 **Ypt35-PX:** 2/3	**Grd19:** 2/2 **Ypt35-PX:** 2/3	**Grd19:** KG/ K **Ypt35-PX:** KG
A19	PVGILHALSTKGYPH	**Ypt35-PX:** 1/3	--	--
A23	YPHEPPLLEEIGINF	**Ypt35-PX:** 1/3	--	--
B1	LLEEIGINFDHIITK	**Grd19p:** 2/3 **Ypt35-PX:** 2/3 **Vps17-PX:** 1/1 **Snx4-PX:** 1/1	--	--
B2	EIGINFDHIITKTKM	**Grd19p:** 3/3 **Ypt35-PX:** 3/3 **Snx4-PX:** 1/1	--	--
B3	INFDHIIT**KTK**MVLI	**Grd19p:** 3/3 **Ypt35-PX:** 2/3 **Vps17-PX:** 1/1	**Grd19:** 3/4 **Ypt35-PX:** 1/4	**Grd19:** KTK **Ypt35-PX: --**
B4	DHIITKTKMVLIPIR	**Grd19p:** 3/3 **Ypt35-PX:** 3/3 **Vps17-PX:** 1/1	--	--
B5	ITKTKMVLIPIRFGS	**Grd19p:** 1/3 **Ypt35-PX:** 1/3	--	--
B14	AGPLIFFLLFGLFLL	**Snx4-PX:** 1/1	--	--
B16	FLLFGLFLLMAGKVH	**Grd19p:** 3/3 **Ypt35-PX:** 1/3	--	--
B18	FLLMAGKVHFGYIYG	**Vps17-PX:** 1/1	--	--
B23	VALFGTISLHNLSKL	**Grd19p:** 2/3 **Ypt35-PX:** 2/3	--	--
B24	FGTISLHNLSKLMSN	**Grd19p:** 2/3 **Ypt35-PX:** 2/3 **Vps17-PX:** 1/1 **GST:** 1/1	**Grd19:** 0/1 **Ypt35-PX:** 0/2	**Grd19:** -- **Ypt35-PX: --**
C23	WTSSGFLNSLLQLQN	**Vps17-PX:** 1/1 **Snx4-PX:** 1/1	--	--
C24	SGFLNSLLQLQNA**R**L	**Grd19p:** 2/3 **Ypt35-PX:** 1/3 **Vps17-PX:** 1/1	**Grd19:** 0/1 **Ypt35-PX:** 1/2	**Grd19:** -- **Ypt35-PX:** R
		Yif1p	*Figures 24-25*	
A15	AYATSEQNGVNDRFS	**Grd19p:** 2/3 **Ypt35-PX:** 2/3 **GST:** 1/1	--	--
A20	HTPQQQRPMQIPRNT	**Grd19p:** 2/3 **Ypt35-PX:** 2/3 **GST:** 1/1	--	--
B5	NVNGSGGGFPFQDPR	**Ypt35-PX:** 1/3	--	--
B6	GSGGGFPFQDPRGSM	**Ypt35-PX:** 2/3	--	--
B6	GSGGGFPFQDPRGSM	**Ypt35-PX:** 1/3	--	--
B12	LGQSAFSNFIGQDNF	**Ypt35-PX:** 1/3	--	--
B14	SNFIGQDNFNQFQET	**Ypt35-PX:** 1/3	--	--
B15	IGQDNFNQFQETVNK	**Grd19p:** 2/3 **Ypt35-PX:** 2/3	**Grd19:** 0/1 **Ypt35-PX:** 0/1	**Grd19:** -- **Ypt35-PX: --**
B16	DNFNQFQETVNKATA	**Grd19p:** 1/3 **Ypt35-PX:** 2/3	--	--
B22	GSQQISTYFQVSTRY	**Ypt35-PX:** 2/3	--	--
B24	TYFQVSTRYVIN**K**L**K**	**Grd19p:** 2/3 **Ypt35-PX:** 2/3	**Grd19:** 1/1 **Ypt35-PX:** 1/1	**Grd19:** K, K **Ypt35-PX:** K, K
C4	KLKLILVPFLNGT**K**N	**Grd19p:** 2/3 **Ypt35-PX:** 2/3	**Grd19:** 1/1 **Ypt35-PX:** 1/1	**Grd19:** K **Ypt35-PX:** K
C5	LILVPFLNGTKNWQR	**Grd19p:** 1/3 **Ypt35-PX:** 1/3	--	--
C6	VPFLNGTKNWQRIMD	**Ypt35-PX:** 1/3	--	--

Peptide Position	Sequence	Protein: positves/total	Protein: Alanine scan	Result Alanine scan
C7	LNGTKNWQRIMDSGN	**Ypt35-PX:** 1/3	--	--
C19	TYILIWNTQQGLKGS	**Grd19p:** 1/3 **Ypt35-PX:** 2/3	--	--
C22	QGLKGSFNPEDL**YYK**	**Grd19p:** 2/3 **Ypt35-PX:** 2/3	**Grd19:** 1/1 **Ypt35-PX:** 1/1	**Grd19:** YYK **Ypt35-PX:** YYK
C23	KGSFNPEDLYYKLSS	**Grd19p:** 1/3 **Ypt35-PX:** 2/3	--	--
D4	TLAFVCLDLLILKLG	**Grd19p:** 1/3 **Ypt35-PX:** 2/3	**Grd19:** 0/1 **Ypt35-PX:** 0/1	**Grd19:** -- **Ypt35-PX: --**
D7	LILKLGLYLLIDSKI	**Grd19p:** 1/3 **Ypt35-PX:** 1/3	--	--
D8	KLGLYLLIDSKIPSF	**Ypt35-PX:** 1/3	--	--
D12	PSFSLVELLCYVGYK	**Grd19p:** 1/3 **Ypt35-PX:** 2/3	**Grd19:** 0/2 **Ypt35-PX:** 0/1	**Grd19:** -- **Ypt35-PX: --**
D13	SLVELLCYVGYKFVP	**Grd19p:** 1/3 **Ypt35-PX:** 1/3	--	--
E2	FIAFGVFLLRSVKFN	**Grd19p:** 2/3 **Ypt35-PX:** 1/3	--	--
E3	FGVFLLRSVKFNLLS	**Ypt35-PX:** 1/3	--	--
E4	FLLRSVKFNLLSRSG	**Grd19p:** 2/3 **Ypt35-PX:** 2/3	--	--
E5	RSVKFNLLSRSGAED	**Ypt35-PX:** 1/3	--	--
E8	RSGAEDDDIHVSISK	**Grd19p:** 1/3 **Ypt35-PX:** 2/3	--	--
E10	DDIHVSISKSTV**KK**C	**Grd19p:** 3/3 **Ypt35-PX:** 2/3	**Grd19:** 1/1 **Ypt35-PX:** 1/1	**Grd19:** KK **Ypt35-PX:** KK
E11	HVSISKSTVKKCNYF	**Ypt35-PX:** 2/3	--	--
		Yip4p	*Figures 25-27*	
B4	ADNIGGTMQNSGS**R**G	**Grd19p:** 3/3 **Ypt35-PX:** 2/3	**Grd19:** 1/1 **Ypt35-PX:** 1/1	**Grd19:** R **Ypt35-PX:** R
B8	SRGTLDETVLQTL**KR**	**Grd19p:** 2/3 **Ypt35-PX:** 2/3	**Grd19:** 1/1 **Ypt35-PX:** 1/1	**Grd19:** KR **Ypt35-PX:** KR
B9	TLDETVLQTLKRDVV	**Ypt35-PX:** 1/3	--	--
B10	ETVLQTLKRDVVEIN	**Ypt35-PX:** 1/3	--	--
B11	LQTLKRDVVEINSRL	**Grd19p:** 2/3 **Ypt35-PX:** 2/3	--	--
B12	LKRDVVEINSRLKQV	**Grd19p:** 2/3 **Ypt35-PX:** 2/3 **GST:** 1/1	**Grd19:** 1/1 **Ypt35-PX:** 1/1	**Grd19:** KQ **Ypt35-PX:** KQ
B13	DVVEINSRLKQVVYP	**Grd19p:** 1/3 **Ypt35-PX:** 1/3	--	--
B15	SRLKQVVYPHFPSFF	**Ypt35-PX:** 1/3	--	--
B16	KQVVYPHFPSFFSPS	**Ypt35-PX:** 1/3	--	--
C4	AFIILYSLFVS**HARS**	**Grd19p:** 3/3 **Ypt35-PX:** 2/3	**Grd19:** 1/1 **Ypt35-PX:** 1/1	**Grd19:** R **Ypt35-PX:** HARS
C5	ILYSLFVSHARSLFS	**Ypt35-PX:** 2/3	--	--
C7	VSHARSLFSSLFVSS	**Grd19p:** 1/3 **Ypt35-PX:** 1/3	--	--
C11	VSSWFILLVMALHLR	**Grd19p:** 2/3 **Ypt35-PX:** 1/3	--	--
C12	WFILLVMALHLRLTK	**Grd19p:** 2/3 **Ypt35-PX:** 3/3	--	--
C13	LLVMALHLRLTKPHQ	**Grd19p:** 1/3 **Ypt35-PX:** 1/3	--	--
C14	MALHLRLTKPHQRVS	**Grd19p:** 2/3	--	--
D2	SQILLPLAYHIGKQN	**Grd19p:** 1/3 **Ypt35-PX:** 1/3	--	--
D3	LLPLAYHIGKQNRWI	**Grd19p:** 1/3 **Ypt35-PX:** 3/3	--	--
D4	LAYHIGKQNRWIVRV	**Grd19p:** 3/3 **Ypt35-PX:** 1/3	--	--

Peptide Position	Sequence	Protein: positves/total	Protein: Alanine scan	Result Alanine scan
D13	LMWSVAAVSWVTKSK	**Grd19p:** 2/3 **Ypt35-PX:** 2/3	--	--
D14	SVAAVSWVTKSKTII	**Grd19p:** 1/3 **Ypt35-PX:** 1/3	--	--
		Yip5p	*Figures 28-29*	
B4	EGVDDFGNEPNPFDD	**Grd19p:** 1/3 **Ypt35-PX:** 2/2	**Grd19:** 0/1 **Ypt35-PX:** 0/1	**Grd19:** -- **Ypt35-PX: --**
B6	GNEPNPFDDATVPDS	**Grd19p:** 1/3		
B10	PDSPNMNNSTAG**K**GS	**Grd19p:** 3/3 **Ypt35-PX:** 1/1	**Grd19:** 1/1 **Ypt35-PX:** 1/1	**Grd19:** K **Ypt35-PX:** K
B12	NNSTAGKGSEFYNTT	**Grd19p:** 1/3		
B13	TAG**K**GSEFYNTTGS**K**	**Grd19p:** 1/3 **Ypt35-PX:** 1/1	**Grd19:** 1/1	**Grd19:** K,K
B23	DQVIGQNDNDGLGRN	**Grd19p:** 2/3 **Ypt35-PX:** 1/1 **GST:** 1/1	--	--
B24	IGQNDNDGLGRNGLR	**Grd19p:** 2/3 **GST:** 1/1	--	--
C3	GRNGLRPGLINYYSK	**Grd19p:** 1/3 **Ypt35-PX:** 1/1	--	--
C4	GLRPGLINYYSKYFQ	**Grd19p:** 1/3 **Ypt35-PX:** 1/1	--	--
C5	PGLINYYSKYFQIDL	**Grd19p:** 1/3	--	--
C6	INYYSKYFQIDLTQF	**Grd19p:** 1/3	--	--
C7	YSKYFQIDLTQFKKR	**Grd19p:** 1/3 **Ypt35-PX:** 1/1	--	--
C8	YFQIDLTQFKKRLSA	**Grd19p:** 2/3 **Ypt35-PX:** 1/1 **GST:** 1/1	--	--
C9	IDLTQFKKRLSAVLT	**Grd19p:** 2/3 **Ypt35-PX:** 1/1 **GST:** 1/1	--	--
C10	TQFKKRLSAVLTFRN	**Ypt35-PX:** 1/1	--	--
C21	ITATVVMINFTMSRG	**Grd19p:** 2/3 **Ypt35-PX:** 1/1 **GST:** 1/1	--	--
D2	LNFIISDVIEGVKTG	**Grd19p:** 1/3 **Ypt35-PX:** 1/1	--	--
D4	DVIEGVKTGEDIDRA	**Grd19p:** 2/3 **Ypt35-PX:** 1/1	--	--
D5	EGVKTGEDIDRASQF	**Grd19p:** 1/3	--	--
D6	KTGEDIDRASQFKKL	**Grd19p:** 1/3		
D7	EDIDRASQF**KKL**LHS	**Grd19p:** 2/3 **Ypt35-PX:** 1/1	**Grd19:** 1/1 **Ypt35-PX:** 1/1	**Grd19:** KKL **Ypt35-PX:** KKL
D17	MQVLNRDEHSERNRS	**Grd19p:** 2/3 **Ypt35-PX:** 1/1	--	--
D18	LNRDEHSERNRSF**K**S	**Grd19p:** 3/3 **Ypt35-PX:** 1/1	**Grd19:** 1/1 **Ypt35-PX:** 1/1	**Grd19:** K **Ypt35-PX:** K
D19	DEHSERNRSFKSVPE	**Grd19p:** 1/3 **Ypt35-PX:** 1/1	--	--
E4	IPVCVILNILDMSKR	**Grd19p:** 1/3 **Ypt35-PX:** 1/1	--	--
E5	CVILNILDMSKRLRT	**Grd19p:** 2/3 **Ypt35-PX:** 1/1	--	--
E18	SSNNNTETQSNGKFS	**Grd19p:** 1/3 **Ypt35-PX:** 1/1	--	--

Non-Yip1 family members

Based on the two-hybrid results I tested other interesting PX domain interactors via peptide arrays. For example, Rbd2p, a transmembrane protein of unknown function, which interacted with the two PX-only proteins Ypt35p and Grd19p (Fig. 34). Unfortunately the alanine scans of the selected peptides for this protein were not very clear (Fig. 34), but still we observe several binding peptides with C-terminal basic residues. This is not the case for the nuclear protein Rrn10. This protein is interacting with the PX domain of Snx42p and most of the obtained peptides contained several acidic and uncharged polar residues (Fig. 33).
Peptides of the proteins of unknown function Ylr031w and Ynl086w were also probed with Grd19p. Here the obtained interacting peptides contain basic (Ylr031w) or non-polar (Ynl086w) amino acids in blocks (Figures 35, 36).

Rrn10p peptides incubated with Snx42-PX GST fusion protein

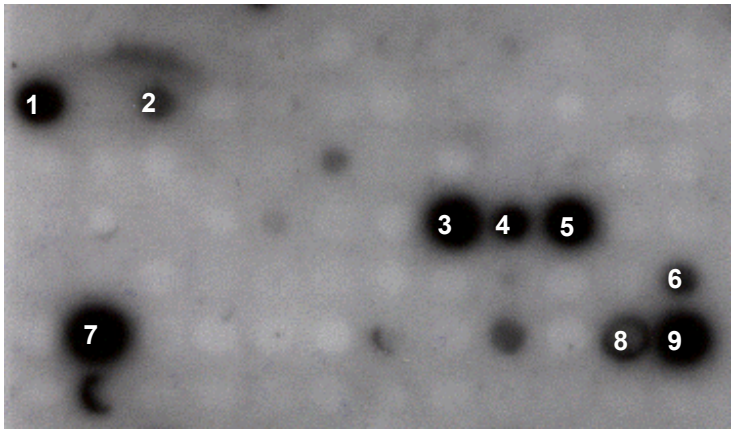
Spot	Sequence
1	AVPIPFKTREEIDAD
2	IPFKTREEIDADVEK
3	KTREEIDADVEKDRN
4	DLRVVHYYATQLCLN
5	VVHYYATQLCLNKYP
6	LLIEKWVKDYLTSIQ
7	SIQTEQGRQSKVIGK
8	GPCEFISKHIDYRHA

Figure 33: Snx42-PX-Domain mapping on Rrn10p peptides

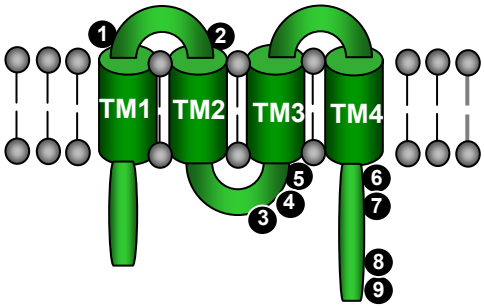
A peptide array covering the full length Rrn10p (synthesized as 15meres with 3 amino acid offsets) was incubated overnight with Snx42 GST-PX fusion protein. Positive interactions were visualized using α-GST antibodies and chemiluminescence (see experimental procedures). Sequences of interacting peptides are shown in the table below. Amino acids were coloured according to their properties, **red**: acidic, **green**: uncharged polar, **blue**: basic and **black** non-polar.

RBD2p peptides incubated with Grd19p GST fusion protein

A)



B)



Spot	Sequence
1	LREDLSLAPESLFKL
2	LAPESLFKLQMSRLS
3	LFAYYSFKESQIRPR
4	YYSFKESQIRPRTRI
5	FKESQIRPRTRIFRT
6	GHFFGLCVGYAIGYK
7	CVGYAIGYKESWFNK
8	KSLDGLIRLIPWGIK
9	DGLIRLIPWGIKYYR

C)

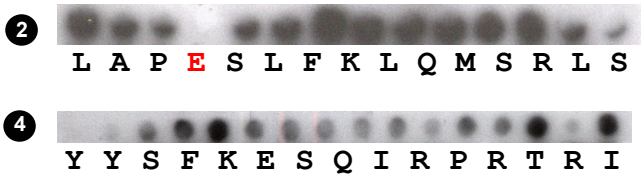
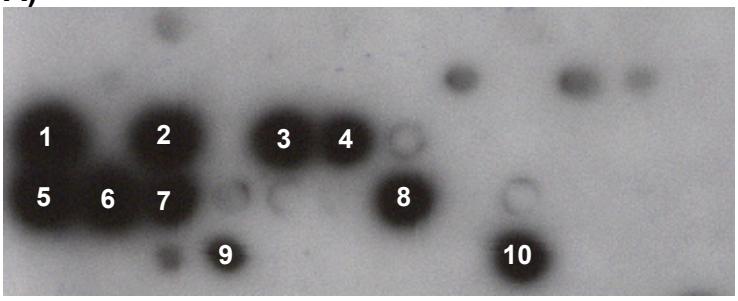


Figure 34: Grd19p mapping on Rbd2p peptides

A) Peptide array of the transmembrane protein Rbd2p. Peptides were analyzed as in Figure 33 with Grd19p-GST fusion protein. **B)** Schematic figure of Rbd2p at the membrane according to its predicted transmembrane domains. Positive interacting peptides are represented with black circles and respective peptide number. Sequences of interacting peptides are shown in the table below**.** Amino acids were coloured according to their properties, **red**: acidic, **green**: uncharged polar, **blue**: basic and **black** non-polar. **C)** Alanine scan for peptides 2 and 4. For peptide 2 the glutamic acid (E, red) seems to be important for interaction as its replacement by alanine abolishes the interaction. For peptide 4 results are not clear.

Ylr031w peptides incubated with Grd19p GST fusion protein

A)



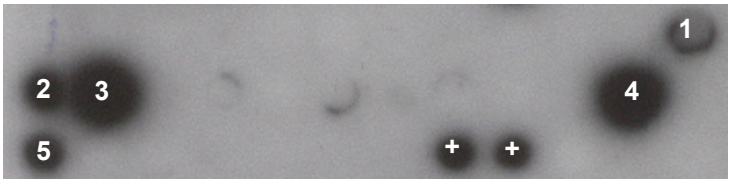
Spot	Sequence
1	NFQLAKLNNLCFRVR
2	LNNLCFRVRESIKRR
3	RVRESIKRRQDLEKK
4	ESIKRRQDLEKKLRT
5	NELLFLMLENERRKK
6	LFLMLENERRKKSSV
7	MLENERRKKSSVIIE
8	IIEFLSEIIREKSKR
9	EVKPLILDLSARINR
10	LNSILETKNTCIRRL

B)

Figure 35: Grd19p mapping on Ylr031w peptides

A) Peptide array of Ylr031w probed with GST-Grd19p fusion protein. Peptides were analyzed as in Figure 33. Sequences of interacting peptides are shown in the table below. Amino acids were coloured according to their properties, **red**: acidic, **green**: uncharged polar, **blue**: basic and **black** non-polar. **B)** Alanine scan for peptide 6. Every amino acid can be replaced by alanine without impairing the interaction.

Ynl086w peptides incubated with Grd19p GST fusion protein



Spot	Sequence
1	ELRESQALLILMLRK
2	ESQALLILMLRKVRD
3	ALLILMLRKVRDKLR
4	KPTMDKLADLSARVR
5	LADLSARVRILSQRY

Figure 36: Grd19p mapping on Ynl086w peptides

Peptide array of Ynl086w probed with Grd19p-GST fusion protein. Peptides were analyzed as in Figure 33. Sequences of interacting peptides are shown in the table below. Amino acids were coloured according to their properties, **red**: acidic, **green**: uncharged polar, **blue**: basic and **black** non-polar.

Yip1p HIITKTKM motif analysis on Ypt35-PX interaction

When mapping the interacting region of Yip1 family members with peptide arrays the most promising result was obtained with Yip1p. We could clearly depict a motif on the cytosolic N-terminus of Yip1p which could be restricted to the amino acids ITK due to the 3 residue shift on the peptide arrays (Fig. 23 A). As mentioned before, the alanine scan diverged from the result obtained with the peptide arrays (Box in Fig. 23 A). To be able to specify wether these amino acids are truly essential for the interaction between Yip1p and Ypt35-PX, two strategies were chosen.

1) Mutational analysis:

3 amino acids of B3 Yip1p peptide, when replaced with alanine, abolished the interaction with Grd19p (KTK). These were also replaced by 3 alanines in the full length Yip1p in an *in vitro* translated protein. Subsequently, GST pulldowns with Ypt35-PX protein and this mutant were performed.

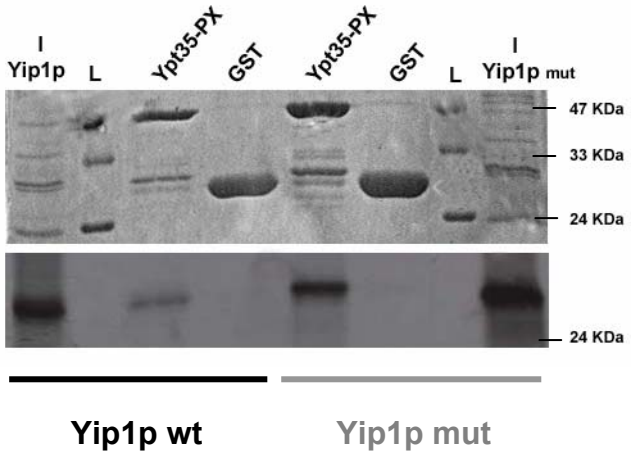


Figure 37: Replacement of Yip1p N-terminal KTK with AAA does not abolish its interaction with Ypt35-PX

Pictures on top of the panel shows Coomassie Blue-stained GST fusion proteins while bottom row shows autoradiography of bound Yip1p proteins (see Experimental Procedures for details).
I: Input, **L:** Ladder (molecular mass marker), **Yip1p mut**: Yip1p full-length protein with K87, T88 and K89 replaced by alanine.

I was not able to confirm by means of this method the data obtained from the alanine peptide scan for Grd19p (Fig. 23). I still obtained an *in vitro* interaction of mutated Yip1p (KTK → AAA) with the PX domain of Ypt35p (Fig. 37).

2) Competition assay:

An 8-mere peptide composed of the amino acids HIITKTKM was synthesized (NMI Peptides, Reutlingen, Germany) and added to the GST pulldowns of Ypt35-PX with *in vitro* translated Yip1p protein at different concentrations. This peptide contains the main binding sequence of Yip1p to Ypt35-PX as obtained from the arrays results (Figures 22, 23). I therefore expected a strong competition between the peptide and the *in vitro* translated full-length protein for the binding to Ypt35-PX.

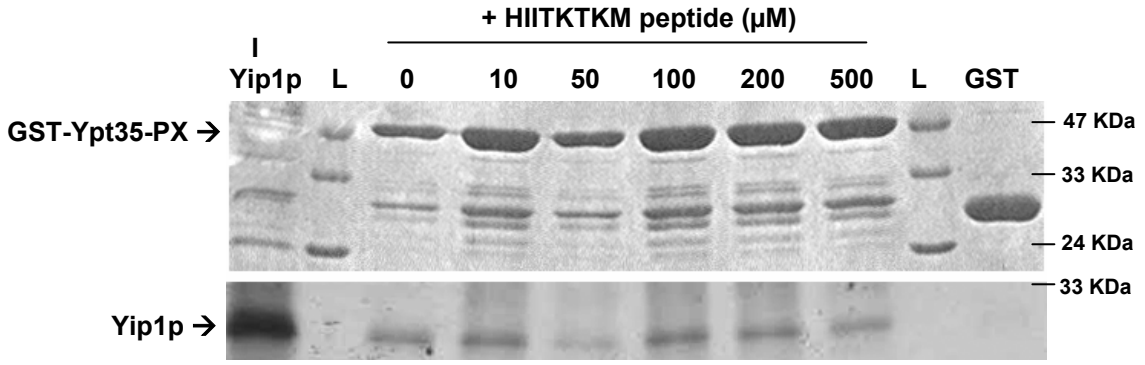


Figure 38: HIITKTKM peptide does not block Yip1p - Ypt35-PX interaction

GST fused to Ypt35-PX was incubated with *in vitro* translated Yip1p protein and synthetic peptide (HIIKTKM) at different concentrations. Subsequently GST pulldowns were performed (see experimental procedures). Picture on top of the panel shows Coomassie Blue-stained GST fusion proteins while bottom row shows autoradiography of bound Yip1p protein.
I: Input, **L:** Ladder (molecular mass marker), **0-500:** Different concentrations (in µM) of the synthetic peptide.

However, this competition was not observed in this experiment. The synthetic peptide was not able to block the interaction even at a concentration of 500µM (Fig. 38).

Temperature-sensitive mutant Yip1-2 (G175E) is able to interact in vitro but not in vivo with PX proteins and domains

When Yip1p was discovered (Yang et al. 1998) temperature sensitive mutants were obtained for functional analysis of this essential gene. The mutant Yip*1-2* showed a slightly disturbed maturation of CPY which was completely inhibited at 36°C. The ER form of CPY was accumulated in Yip*1-2* at 36°C as shown by electrophoretic mobility (Yang et al. 1998). These results suggested a transport defect at early stages in the biosynthetic pathway due to Yip1 loss of function. I decided to look for PX domain interactions of this temperature sensitive mutant and constructed a prey corresponding to Yip*1-2* for yeast two-hybrid assay (see experimental procedures). Figure 39 shows the interactions of Yip1p and Yip*1-2* with PX proteins.

The Yip*1-2* temperature-sensitive mutation prohibits the interactions with PX proteins and domains when performing yeast two-hybrid at 30°C (Fig. 39). An interaction may also be prevented by destabilization of the protein due to the mutation. However, this mutant protein is translated *in vitro* at 30°C and interacted with Ypt35-PX when performed GST-pull-downs at 4°C (Fig. 40). With this experimental setup I am not able to make any conclusions for it may be possible that protein stability is temperature dependent. In this case I will perform a yeast two-hybrid assay as well as GST pull-down assays at the permissive temperature of 25°C and observe if I obtain the same results.

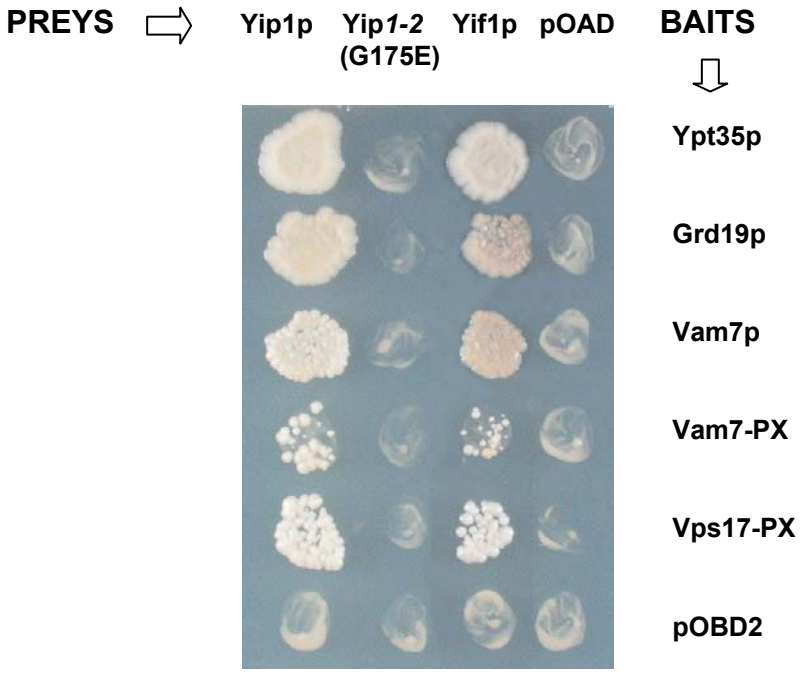


Figure 39: Yip*1-2* temperature sensitive mutant does not interact with PX proteins and domains via yeast two-hybrid at 30°C

Diploids on selective media (without histidine) showing growth of colonies where interaction takes place. **Yip*1-2***: (G175E) mutant; **pOAD:** empty prey vector; **pOBD2:** empty bait vector.

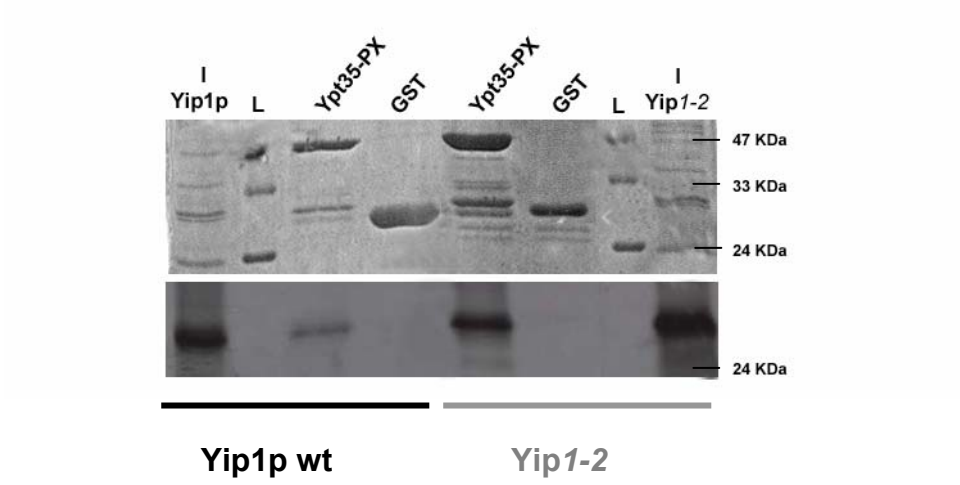


Figure 40: Yip*1-2* temperature sensitive mutant interacted with Ypt35p PX domain

Pictures on top of the panel shows Coomassie Blue-stained GST fusion proteins while bottom row shows autoradiography of bound Yip1p proteins (see Experimental Procedures for details).
I: Input, **L:** Ladder (molecular mass marker), **Yip*1-2***: (G175E) mutant.

DISCUSSION

PI binding domains as protein interaction domains

I describe here the first systematic study of PX domain interactions with other proteins. My study identifies for the first time the PX domain as a bona-fide protein-protein interaction module**.** This knowledge opens a new perspective on PX-phospholipid binding domains as being bifunctional, i.e. participating in both protein and phospholipid recognition.

The nature of PX protein interactors

Although PX proteins in the yeast genome are diverse in function, most of the 15 yeast PX domain-containing proteins are involved in vesicular transport. The over representation of proteins involved in vesicular transport among the interacting partners (36%, Fig. 16) was therefore expected. Unexpected was the large amount of transmembrane and membrane associated proteins detected with this method (53%).

False negatives

Detection of membrane proteins has been considered as being difficult with the yeast two-hybrid system. This is due to the hydrophobic transmembrane domains which may prevent the proteins from reaching the nucleus. Also unsuitable for analysis by the yeast two-hybrid method are transcriptional activators which may activate transcription without any interaction. Other unproductive baits may require modification by cytoplasmic or membrane associated enzymes in order to interact with binding partners. To overcome these limitations a considerable number of alternative systems have been developed (Vollert and Uetz in press).

Localization of PX interactors

The localization of the obtained interactions also validated the selected method for detecting of protein-interaction partners. 50% of the preys were localized to the ER, Golgi, endosomes or the vacuole where most of the PX proteins are situated (genome: 8,8%).

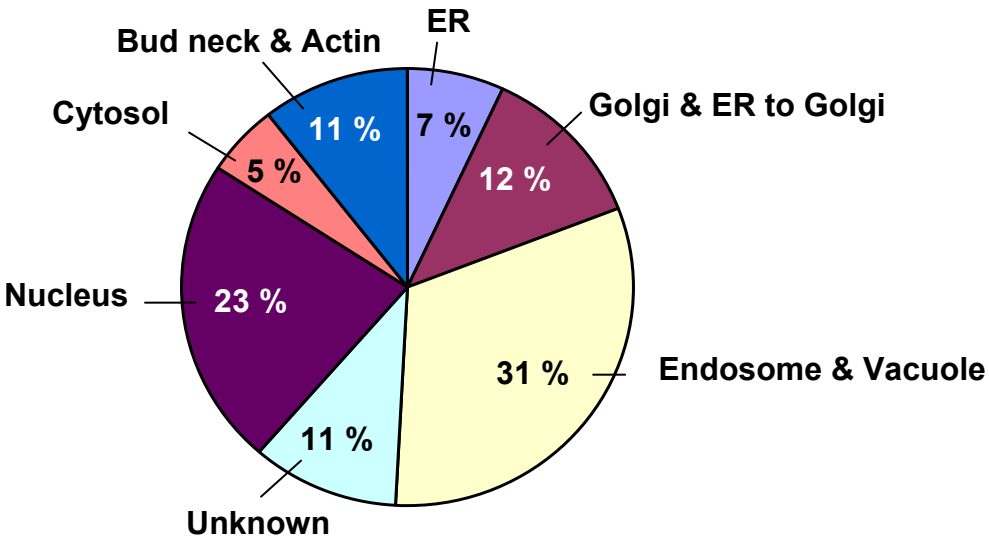


Figure 41: Localization of PX interacting partners

Nuclear PX interactions

I was surprised to find that several PX domain-containing proteins showed preferential interactions with nuclear proteins. Although this was initially unexpected, my findings can be rationalized by the known relationships between inositol phosphates and nuclear functions (reviewed in Jones and Divecha 2004).

Mvp1p, a protein required for sorting proteins to the vacuole, interacted weakly with **Std1p**, a suppressor of a TBP deletion. Std1p together with Mth1p is required for Rgt1p-mediated repression of transcription in the absence of glucose through interaction with Rgt1p (Lakshmanan et al. 2003).
Mvp1p also interacted with **Yra2p** (Yeast RNA annealing), a protein that is involved in mRNA export from the nucleus and possesses a RNA recognition motif (RRM), (Letunic et al. 2004):

Yra2p

Interestingly Yra2p has been isolated by systematic mass spectrometry with 34 members named the Hap2p-associated complex. A member of this complex is Apg17p, a protein required for activation of Apg1 protein kinase, involved in autophagy and interacting partner of further two PX proteins: Cvt20p/Snx42p and Cvt13p/Snx4p. Although the significance of both interactions is unclear, it is notable that both of these Mvp1p interactors have been found both in the nucleus as well as in the cytoplasm (Habeler et al. 2002; Huh et al. 2003). Std1p has also been localized to the plasma membrane (Schmidt et al. 1999).

Vps5p and ***Snx42p*** also have interactions with nuclear proteins of which Snx42p's interaction with **Rrn10p** is remarkable because it appears to be specific for the PX domain of Snx42p. Rrn10 (Regulation of RNA polymerase I) is a component of the upstream activation factor (UAF) complex involved in activation of RNA polymerase (Siddiqi et al. 2001). The UAF motif has been recognized and described by the Pfam database (Bateman et al. 2004):

Rrn10p

Additional support for an Snx42p-Rrn10p interaction comes from the observation that Rrn10 null mutants are hypersensitive to wortmannin, an inhibitor of phosphoinositides (PI) synthesis (Stt4p: PI 4-kinase; Mss4p: PI(4)P 5-Kinase) and PI turnover (PLCp: Phospholipase C) (Zewail et al. 2003).

The most remarkable example of nuclear protein interactions was seen with ***Snx4p***, which has 7 interactions with nuclear proteins. Two of these were highly reproducible, namely those with Ecm11p and Sds3p.

Ecm11p is an unusual protein in that its mutants exhibit phenotypes related to extracellular matrix functions such as zymolyase hypersensitivity. Nevertheless, the protein has been localized to the nucleus (Huh et al. 2003). Its molecular function remains obscure.

The other nuclear Snx4p interactor, **Sds3p**, is a suppressor of defective silencing, i.e. it is involved in silencing the HMR mating cassette locus. Its precise biochemical activity is also unknown but, interestingly, it binds phosphoinositol 4-phosphate *in vitro* (Zhu et al. 2001). Rrn10 and Sds3 are two of 38 genes whose deletion results in hypersensitivity to wortmannin but the mode of action remains to be determined (Zewail et al. 2003).

PX domain interaction map: a pool of new information

The obtained interaction map confirmed 17 published interactions (Table 6) belonging mainly to scores 2 and 3 which confirms their validity and my experimental setup.

False negatives

I also obtained several false negative interactions (see appendix: Table III), i.e. expected positive interactions not detected in my screens. Inefficient diploid formation, the use of different strains, plasmids or reporter genes and missfolding of proteins due to the GAL4 fusion may account for these results.

New high confidence interactions

Vam7p/Vam7 PX - Hsv2p

Most important, the interaction map describes a pool of new and interesting interactions. For example, Vam7p interacted reproducibly (score 3) with **Ygr223c** now named **Hsv2p** (Homologous with Svp1p). This protein was detected as a Svp1p like protein belonging to a new family of phosphoinositide effectors (Dove et al. 2004). The Svp1 itself is a specific PtdIns(3,5)P_2-binding protein that participates in the recycling of membrane proteins from the vacuole to the late endosome. Hsv2p is likely to be PtdIns(3,5)P_2 effector, but its loss causes none of the known FAB1/PtdIns(3,5)P_2-dependent phenotypes. It is therefore likely that undiscovered PtdIns(3,5)P_2-dependent cellular processes remain to be found (Dove et al. 2004). Interaction of Hsv2p with ***Vam7p*** a SNARE (protein which is required for vacuolar homotypic membrane fusion) may elucidate these undiscovered additional PtdIns(3,5)P_2-dependent cellular process. Hsv2p possesses two WD40 repeats which can act as a site for protein-protein interaction. Proteins containing WD40 repeats are known to serve as platforms for the assembly of protein complexes or mediators of a transient interplay among other proteins.

Vam7p/Vam7 PX – Bsp1p

A further novel reliable interesting interactor of ***Vam7p*** and its isolated PX domain is **Bsp1p**. Bsp1p (binding protein of synaptojanin PPIP domains) is proposed to function as an adapter that links the synaptojanins (inositol polyphosphate 5-phosphatases) to the cortical actin cytoskeleton. An interaction with Vam7p (vacuolar SNARE) can be biologically meaningful as actin is required to produce fusion-competent docked vacuoles and its reassembly is needed for yeast vacuole fusion (Eitzen et al. 2002). The interaction of Vam7p and its PX domain with Bsp1p was therefore particularly interesting. Vam7-PX domain GST fusion protein was hardly expressed or insoluble in *E.coli* cells. I decided therefore to use another strategy to characterize this interaction in cooperation with Birgit Singer-Krüger (University of Stuttgart, Germany). I TAP-tagged genomic VAM7 (Rigaut et al. 1999) and tested by means of the lucifer yellow assay (Singer and Riezman 1990) if the TAP tag influences the proteins function. Tap-Tagged yeast cells had normal vacuolar morphology like the wild type control. Birgit then crossed this haploid strain with a genomic HA- tagged BSP1 and selected double-tagged diploid cells. Currently I am

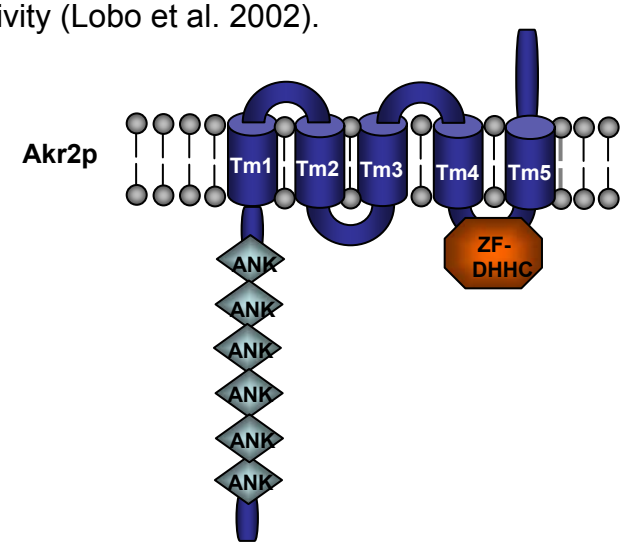
performing immunoprecipitations from yeast extracts of these diploids to verify this interaction. A positive interaction would link homotypic vacuolar fusion to the actin cytoskeleton.

Rbd2p

The predicted transmembrane protein of unknown function **Rbd2p** is also interesting as it interacted reproducibly (score 3) with the PX only proteins Grd19p and Ypt35p. These interactions may give clues about the function of this protein. Rbd2p has been recently described as a member of the Rhomboid protein family for it shows relation to the rhomboid protein of Drosophila (RBD2 = RhomBoiD) (McQuibban et al. 2003). Analysis suggests that Rhomboid-1 is a novel intramembrane serine protease that directly cleaves the membrane-anchored TGF-α–like growth factor Spitz, allowing it to activate the Drosophila EGF receptor (Bier et al. 1990; Urban et al. 2001). Proteins of this family contain three strongly conserved histidines in the putative transmembrane regions that may be involved in the peptidase function.
Yeast Rbd1p is localized in the inner mitochondrial membrane and mutant cells have disrupted mitochondria. Two substrates of this protein have been identified, c peroxidase and a dynamin-like GTPase (Mgm1p) which is involved in mitochondrial membrane fusion. Rbd2p has been predicted to be involved in cell wall synthesis, or protein-vacuolar targeting as suggested by network-based statistical analysis (Samanta and Liang 2003). Computational analysis additionally has predicted that this protein may be involved in vesicular transport (Brun et al. 2003). It would be interesting to analyse different GTPases and interactors of this protein amongst those the PX proteins as putative substrates.

Akr2p

A further interesting candidate is **Akr2p**, an ankyrin repeat protein involved in constitutive endocytosis of yeast pheromone receptors (Givan and Sprague 1997). Akr2p also interacted with the PX-only proteins Ypt35p (score 3) and Grd19p (score 1). Ankyrin repeats have been implicated in mediating protein-protein interactions although no common theme among the known ankyrin domain protein targets has been identified. The PX domain could be a possible target for this protein. An additional zf-DHHC motif has been found in Akr2p. This domain is predicted to be a zinc binding domain. The function of this domain is unknown, but it has been predicted to be involved in protein-protein or protein-DNA interactions (Putilina et al. 1999), and palmitoyltransferase activity (Lobo et al. 2002).



I hope all the new information obtained from the PX interaction map will encourage further studies on these proteins which may be able to eventually reveal their biological function.

Yip1 family members

The most striking result of the PX domain two-hybrid screens was the repeated appearance of Yip1-family proteins (Table 6 and 7). Yif1p, Yip1p, and Yip5p were consistently found with the PX domain-only proteins Grd19p and Ypt35p as baits, with Vam7p (PX domain alone and full-length protein), and with the PX domain from Vps17p. Yip4p was found with Ypt35p. The verification of PX domain interaction with Yip1p family members *in vitro* was successful (Figure 17, Table 8) although I obtained some notable differences compared to the two-hybrid data. Some expected interactions from the two-hybrid assay were not obtained *in vitro* and vice versa. These differences may rely on the methods which are based on different principles. On the one hand, yeast-two hybrid assays involve the construction of a fusion protein which may inhibit or impair interaction. Associated yeast proteins may also mediate an indirect interaction described as bridging effect. On the other hand, *in vitro* analysis may be more sensitive due to a higher concentration of the interacting partner but improper prey folding in a non-yeast based system may be limiting for interaction detection in some cases.

Yip1 family member's localization and function

Yif1p and Yip1p have been reported to be localized to the Golgi (Yang et al. 1998; Matern et al. 2000), and the two proteins have been postulated to be part of a complex (Matern et al. 2000). Recently, several studies showed that Yip1p/Yif1p proteins are necessary for COPII vesicle budding from the ER membrane (Heidtman et al. 2003) and for membrane fusion with the Golgi (Barrowman et al. 2003). Yip1p is thought to cycle between the ER and Golgi compartments (Heidtman et al. 2003), (Fig. 42)

Yip1 family member's interactors localization

It was therefore surprising that the strongest and most consistent PX domain-containing interactors of Yif1p/Yip1p were proteins that have been localized to the endocytic pathway. For example, Ypt35p is mainly found associated with endosomes (Huh et al. 2003), and Grd19p is localized to both the vacuolar membrane (Huh et al. 2003) and the cytosol (Voos and Stevens 1998), although it becomes associated with the prevacuolar compartment in *Δvps27* null mutant cells (Voos and Stevens 1998). Vps17p also localizes to the endosome, as reported by a C-terminally tagged GFP fusion protein (Huh et al. 2003) as well as to the prevacuolar compartment (Burda et al. 2002). So do Snx42p (Cvt13p) and Snx4p (Cvt20p) which have additionally been localized to the post-Golgi endosomes and the pre-autophagosomes (Hettema et al. 2003). Snx42p is also found at a perivacuolar structure (Nice et al. 2002). And finally, Vam7p is usually seen in association with vacuolar membranes (Sato et al. 1998; Boeddinghaus et al. 2002), (Fig. 42).

There are three possible explanations for this finding that a group of ER/Golgi proteins (the Yip1 family) interact with endosomal/vacuolar proteins (the PX domain-containing proteins).

First, a significant fraction of at least some PX proteins can be found in the cytoplasm, and could be recruited in small amounts to the ER membrane and act there during COPII vesicle formation. Although there is no direct evidence for this, while 5% of Vam7p associates with vacuolar membranes, the rest is soluble (Sato et al. 1998). Soluble and therefore diffusely stained cytoplasmic fractions of PX proteins could easily escape detection if there is at least one other membrane-related localization. In fact, sites of COPII vesicle formation are difficult to spot by light microscopy and neither are the proteins involved in such highly dynamic membrane spots.

Second, it remains likely that Yif1/Yip1 proteins have other, yet unnoticed functions (independent of COPII vesicle formation) in post-Golgi sorting or endocytosis. This idea is supported by the requirement for the Yip1/Yif1p-binding Rab GTPases in docking and fusion of transport vesicles, but not for budding (Heidtman et al. 2003). Furthermore, Sivars et al. 2003 (Sivars et al. 2003) showed that Yip3, a mammalian Yip1 family member, can act as a GDI-displacement factor for Rab GTPases, particularly Rab 9, which is localized in the endocytic pathway.

Third and last, I cannot completely rule out the possibility that at least some of the interactions are experimental artefacts with no physiological relevance. However, the specificity, apparent strengths of the interactions and confirmation by independent methods argue against this possibility.

Interestingly, Yif1p has been shown to localize to the vacuole under certain circumstances, for example in a BTN2 null mutant. The BTN2 gene encodes for a novel nonessential coiled coil protein (Chattopadhyay et al. 2003). Btn2 (Batten disease) was originally identified by gene expression profiling as one of only two proteins up-regulated in yeast strain lacking Btn1p (Pearce et al. 1999). BTN1 encodes for a non-essential gene that is 39% identical and 59% similar to human CLN3-protein (Pearce and Sherman 1997). Mutation of the human CLN3 gene results in the neurodegenerative disorder, Batten disease (1995). Btn2p has a role in trafficking Rsg1p to the cell periphery were it acts to down regulate arginine uptake through the Can1p permease (Chattopadhyay and Pearce 2002). Deletion of BTN2 results in altered intracellular pH maintenance (Chattopadhyay and Pearce 2002). Both findings indicate that Btn2p may be involved in maintaining the homeostatic environment within the cell. The accumulation of Yif1p in the vacuole in the absence of Btn2p may indicate a connection between intracellular homeostasis and protein trafficking (Chattopadhyay et al. 2003).

In addition, Yif1p was localized to the cytoplasm and may be free to diffuse to locations other than the ER (Kumar et al. 2002).

Even if Yif1p and Yip1p do not have a role in endocytosis or post-Golgi sorting, such a function may have been taken over by Yip4p and Yip5p as their localization remains unclear. Yip5p localizes to the cytoplasm, as determined for a C-terminally tagged GFP fusion protein (Huh et al. 2003) while Yip4p could not be localized so far.

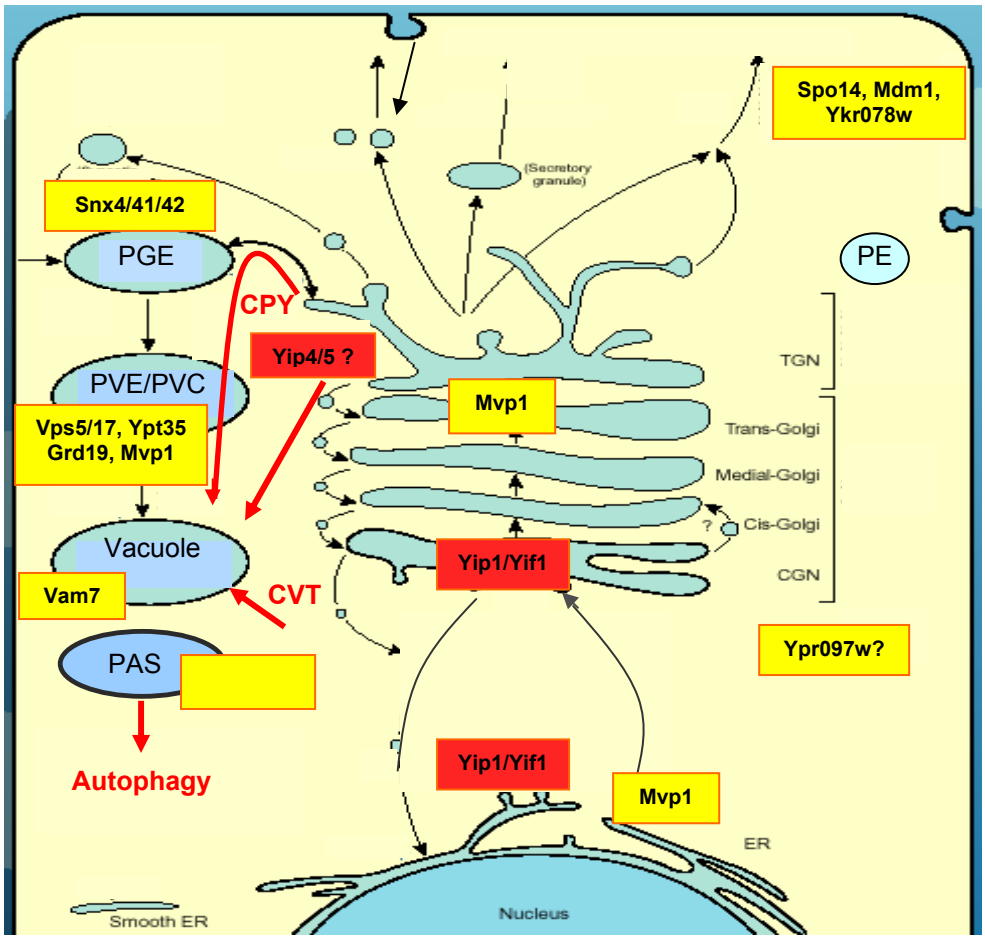


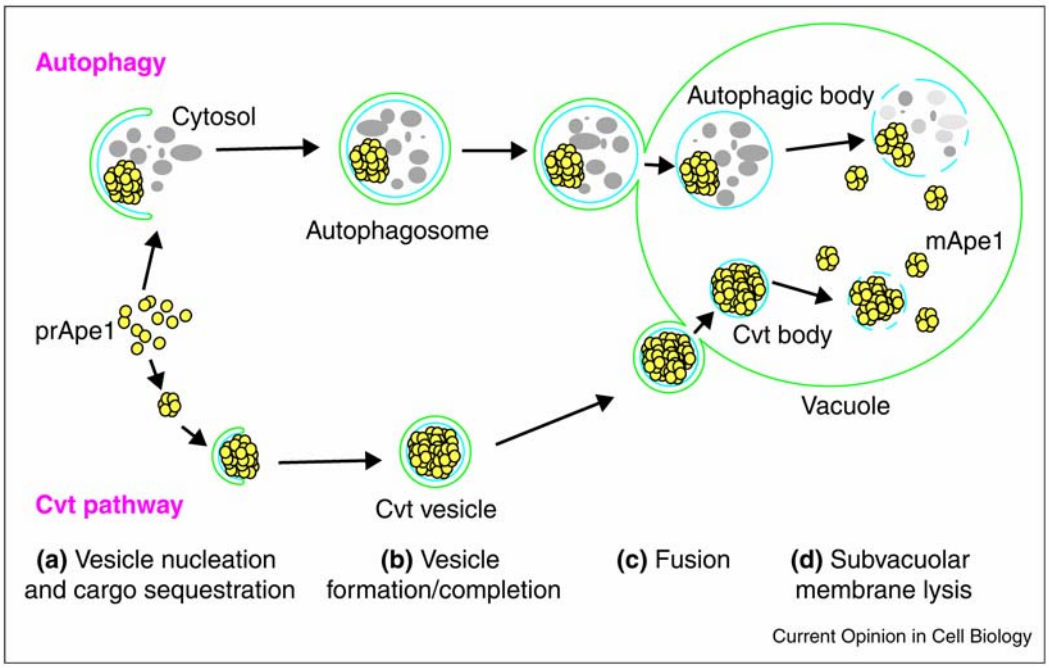
Figure 42: Primary localization of PX proteins and Yip1 family members in the cell

= PX proteins, = Yip proteins

PGE: post- Golgi endosome, PVE/PVC: pre-vacuolar endosome/compartment, PAS: pre-autophagosomal structure, PE: Peroxisome, CVT: Cytosol to valcuole targeting, ALP: Alkaline Phosphatase Pathway, CPY: Carboxy peptidase Y pathway

PX domain proteins in the Cvt pathway

Phosphoinositides play an important role in autophagy and the Cvt (cytoplasm to vacuole targeting) pathway, two pathways for the degradation and recycling of proteins and other components (Figures 43, 44). The Vps34 PI3 kinase complex (which also contains Vps15p, Vps30p/Apg6p and alternatively Vps38p or Apg14p) synthesizes PtdIns(3)P and is required for both autophagy and the Cvt pathway. Vps34p creates the PtdIns(3)P binding sites for two PX proteins that are required for the transport of the vacuolar hydrolase aminopeptidase I via the Cvt pathway, Cvt13p (= Snx4p) and Cvt20p (= Snx42p). Both proteins interact with Apg17p which in turn recruits the key regulator Apg1 kinase to the forming autophagic vesicle (Hettema et al. 2003) (Fig. 43). Snx4p and Snx41p both have a PX domain and predicted coiled-coil domains (see Table 5). Their PX domains do not appear to interact with Apg17p or any of their other interactors. I therefore suggest that the PX domain of each of these proteins serves to anchor the protein in the membrane while the C-terminal part recruits the Apg17p/Apg1p complex and other binding partners (Fig. 44).



Khalfan and Klionsky 2002

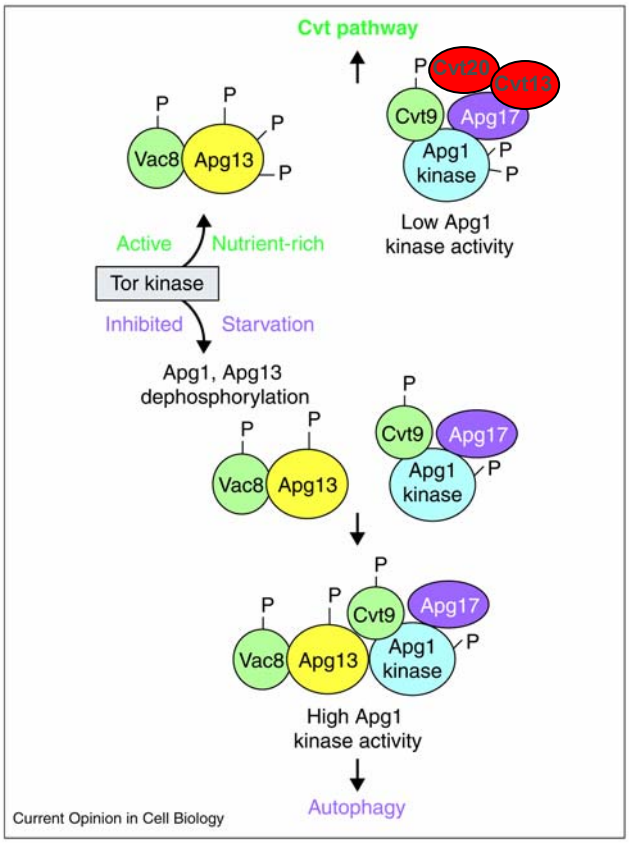
Figure 43: Morphological events marking autophagy and Cvt trafficking.

Autophagy is induced under starvation conditions. (a) A nucleation step initiates the formation of a sequestering vesicle. In autophagy, bulk cytoplasm is nonspecifically sequestered. The highly oligomerized precursor form of the resident vacuolar hydrolase aminopeptidase I (prApe1) can be selectively packaged into this isolation membrane. (b) This structure forms a closed double-membrane transport vesicle called the autophagosome. (c) The outer layer of the autophagosome membrane then fuses with the unit membrane of the vacuole. (d) The fusion event releases a subvacuolar vesicle, the autophagic body, which is degraded. During nutrient-rich conditions, the Cvt pathway transports prApe1 and the resident hydrolase α-mannosidase (not shown). The Cvt pathway is morphologically similar to autophagy, except that prApe1 is selectively targeted to double-membrane Cvt vesicles that exclude cytosol. Like autophagosomes, Cvt vesicles fuse with the vacuole and are subsequently degraded. Once exposed within the vacuole, the precursor form of Ape1 is proteolytically processed to yield the mature active enzyme (mApe1).

Recent work of the group of P.J.Cullen demonstrated the presence of a BAR domain in Snx1 (previously described as coiled coil region) which is able to form dimers and to sense membrane curvature (Carlton et al. 2004). It seems that several sorting nexins (SNX´s) belong to the BAR-domain family (Habermann 2004) including the yeast SNX proteins (P.J.Cullen, personal communication). Data on Arfaptin2 and adaptor proteins APPL1 and 2 suggest that another function of the BAR domain is to bind to small GTPases. It would be interesting to determine the role of putative BAR domains in yeast SNX proteins.

It is interesting that neither Snx4p nor Snx42p interacted with the Yip1 family in our two-hybrid tests, clearly separating them functionally from other PX domain containing proteins.

Particular interesting was the interaction of Snx4p with Snx41p because it could indicate that Snx41p may also be involved in the Cvt pathway. Deletions of SNX41 showed normal API maturation but the homologous gene in *P.pastoris* has deficient peroxisome degradation at a late stage when mutated (Michael Thumm personal communication). This could indicate a role of Snx41p in one of both pathways (autophagy or Cvt).



Adapted from Khalfan and Klionsky 2002

Figure 44: Proteins involved in autophagy and Cvt pathway

Autophagy is induced through formation of an Apg1p kinase complex. During nutrient-rich conditions, Tor kinase is active, and Apg13p and Apg1p are part of two separate protein complexes that function in the Cvt pathway. Apg13p is a phosphorylated protein that physically interacts with Vac8p, a protein that is relatively specific for the Cvt pathway. Apg1p kinase is also a phosphoprotein that physically associates with Cvt9p and Apg17p, proteins that are required primarily for the Cvt pathway and autophagy, respectively. The PX proteins Cvt20p (Snx42p) and Cvt13p (Snx4p) physically interact with Apg17p and are exclusively essential for the Cvt pathway. Under these conditions, Apg1p kinase activity is low. During starvation, Tor kinase activity is inhibited, causing partial dephosphorylation of Apg13p. Dephosphorylated Apg13p shows enhanced binding affinity for Apg1p, driving formation of an Apgp1–Apg13p complex. Consequently, the kinase activity of Apg1p increases and autophagy is induced. The level of phosphorylated Apg1 decreases under starvation conditions, and this might be an important feature that also helps to promote autophagy. The specificity factors Vac8p, Cvt9p and Apg17p, might be able to modulate the activity of Apg1p and Apg13p in order to favour one of the two pathways.

Mapping the interacting region on Yip1 family members

Based on their predicted topology (Calero et al. 2002) I was able to narrow down the interacting region of Yip1p and Yif1p (with Ypt35-PX) to their cytosolic N-terminus (Fig. 19). For Yip1p, I was also able to detect a weak binding of Ypt35-PX to the Yip1p C-terminus. I therefore suggest that cytoplasmic loops contribute to binding. It is possible that the PX-Yip1 interaction involves a discontinuous binding site, i.e. key residues contributing to the binding affinity are normally separated in the primary structure, but closer in the conformation on the surface of a folded protein to form a conjunct binding epitope (Fig. 11), Additional observations obtained from peptide arrays and competition assays confirm this hypothesis (see below).

The PI-binding site is different from the protein-protein interaction site

If this dual binding to lipids and proteins is a general feature of PX domains several important questions can be raised. Can an individual PX domain bind both protein and lipid ligands simultaneously? Or do they use the same site for lipid binding and protein interaction? Linkage between protein interaction and phospholipid binding sites of PX domains could give rise to interesting forms of regulation. For example, PX domains could require coordinated presentation of both phospholipids and proteins for proper cooperative binding and localization. Alternatively, the domain could function as a switch excluding the simultaneous binding of the two ligand types. To be able to solve some of these questions I introduced a point mutation into the PX domain of Ypt35p which should impair PtdIns(3)P binding (Sato et al. 1998; Cheever et al. 2001; Xu et al. 2001; Nice et al. 2002), (Fig. 21 A). For Ypt35-PX this mutation reduced lipid binding about 3 fold (Fig. 21 B). Importantly, the Y123A mutation did not prevent *in vitro* binding of Ypt35-PX to Yip1p, Yi1p and Yip4p (Fig. 21 C). This result suggests that interacting proteins use different surfaces than lipids or at least do not require the critical tyrosine 123 that is involved in binding the phosphate head group of PtdIns(3)P (Fig. 21 A).

Structural evidence for possible simultaneous binding of lipids and proteins

The obtained data would argue for a simultaneous binding of both ligands. Is there any structural evidence for this? The solved structures of the Grd19p PX domain in a ligand-free and ligand bound (diC_4PtdIns(3)P bound) state showed great similarity (Fig. 45). The position of the diC_4PtdIns(3)P in the recognition site of Grd19p is very similar to its position in the complex with the $p40^{phox}$ PX domain.

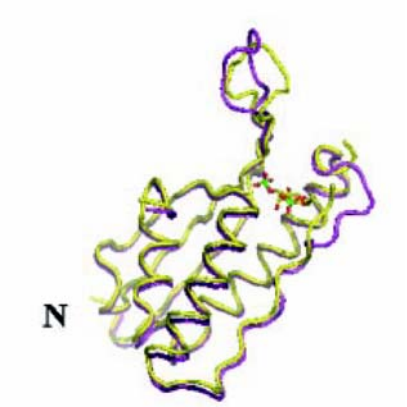


Figure 45: Global superposition of the free (*pink*) and ligand-bound (*green*) structures of Grd19p (Zhou et al. 2003)

N: N-Terminus. diC_4PtdIns(3)P bound to Grd19p is represented as “ball-stick” model

The Grd19p inositol-binding site undergoes small but significant structural changes upon ligand binding (Fig. 45). This are restricted to two sites: the first region, situated close to the diC_4PtdIns(3)P-binding site, is involved in dimer formation (at least in the crystal) and the second region provides one rim of the ligand-binding site (Zhou et al. 2003). The authors of this study believe that dimerisation in the presence of the ligand is probably induced by the crystallization conditions as gel filtration studies showed that the protein elutes as a monomer in the absence of ligand. It seems that the conformational changes of the structure are restricted to the lipid binding region, suggesting no activating conformational change for protein binding at putative protein interaction site/sites. This leaves open the possibilities of a simultaneous ligand binding or individual binding of each ligand independent from each other.

Simultaneous lipid and protein binding and phospholipid distribution in the cell

As shown in Figure 4, phospholipids are not distributed evenly throughout the cell but concentrate at distinct sub-cellular compartments.

PX domain containing proteins such as Vam7p, Snx4p and Snx42p, bind phospholipids (via their PX domain), and at the same time recruit proteins via an additional contact site to their proper cellular localization (Cheever et al. 2001; Boeddinghaus et al. 2002; Hettema et al. 2003). Many PX proteins do contain additional coil-coiled regions that possibly drive protein-protein interactions too. In the case of the PX-only proteins Grd19p and Ypt35p I assume that the recruitment of other proteins has to be achieved by the PX domain itself. As expected, I was able to prove that both proteins are involved in protein-protein interactions. This is additionally supported by several genetic observations in mutant PX proteins such as yeast strains with a deleted *GRD19* gene which missort the TGN membrane proteins A-ALP, Pep12p and Kex2p to the vacuole (Voos and Stevens 1998; Hettema et al. 2003). Assuming that phospholipid binding happens simultaneously to protein-protein interaction, is PtdIns(3)P (the yeast PX domain´s main lipid target) (Yu and Lemmon 2001), also present at the location of its interactors? As discussed before, the localization of Yip4p has not been studied, but Yip5p seems to localize to the cytoplasm although it is predicted to be a transmembrane protein. However, PX proteins may also cycle between membrane bound and cytosolic forms. Yip1p and Yif1p have been localized to the ER and Golgi (Yang et al. 1998; Matern et al. 2000), although Yif1p has been shown to localize to the vacuole under certain circumstances (Chattopadhyay et al. 2003). Assuming that the interaction of the PX proteins with Yip1p and Yif1p take place at the ER and Golgi, is PtdIns(3)P present at these sites?

Localization of phosphoinositides has been measured mainly by fusing lipid-binding protein modules to a green fluorescent protein to visualize these lipids and their changes within a single cell. Using this technique PtdIns(3)P could not be detected at the ER or Golgi. However, this technique has its limitations. It is quite possible that each of these lipid binding modules only recognizes the lipid in a very specific molecular context and, therefore, does not image all of the target inositides in every cellular compartment. It is not yet clear what fraction of the inositide pools are visualized by even the well-documented constructs. This aspect of inositide recognition by the domains is still being studied. Further, the amount of lipid that is produced and is available for binding by a fluorescent probe is limited. Therefore, in a cell that expresses a high concentration of the fusion protein, the membrane-bound fraction may not be distinguishable from the high cytosolic intensity of unlocalized fusion protein (Balla and Varnai 2002). I conclude that with the actual phospholipid localization detection methods, a failure to detect PtdIns(3)P in the Golgi and ER, does not mean that this lipid is absent in these membranes. The lipid concentration may be too low and the pools too short-lived to be detected by means of GFP-coupled lipid binding modules.

A possible experimental setup to distinguish between simultaneous or exclusive lipid/protein binding would be the use of PtdIns(3)P-loaded liposomes. These could be incubated with the PX domains of Grd19p or Ypt35p and the soluble N-terminal fraction of Yip1p. The liposomes are sedimented, washed and analysed for the presence of Yip1-N-terminus and PX domains. PtdIns(3)P binding specificity and strength of the PX domain of Ypt35p is enough to detect attachment of the domain to

the liposomes (Massimilano Mazza´s, personal communication). As comparison, the same experiment could be performed with PX domains containing a mutation in the PX lipid binding pocket.

Peptide arrays

Analysing different membranes

To solve the interacting region of Yip1 family members to the single amino acid resolution peptide arrays have been performed (Figures 22-29, 33-37 and Table 9). To test the reproducibility and reliability of this method in our lab we synthesized the arrays several times. We also used two different membranes for comparison: namely β-alanine membranes which are cellulose membranes coupled with β-alanine OH as a spacer, and alternatively the commercially available membranes from Intavis Bioanalytical Instruments which are also cellulose based. In my experience the commercially available membrane had clearly less background and was more sensitive. This can be clearly observed for the interactions of Grd19p and Ypt35-PX with Yip1p (Fig. 22). With the commercially available membranes I was able to obtain up to 5 spots in a row at positions B1-5 which was not the case for the β-alanine membranes (only 3 spots in row). Different supports and spacers might influence the folding of attached peptides and thus influence the recognition process.

Distribution of Yip1 family member binding peptides

The majority of the interacting peptides in Yip1 localized to the cytosolic N-terminus of the protein (Fig. 23). This was expected from the fragmentation experiments (Fig. 19). Additional binding to the luminal loop and C-terminus was also detected and expected from the pull-down experiments (Fig. 19). However, weak interacting signals were also detected in this region with GST alone. In the case of the other Yip1 family members interacting peptides were distributed quite evenly throughout the protein. This might indicate that not all proteins are suited for this analysis or point at a discontinuous interacting epitope (Fig. 11). Mapping this type of epitopes with overlapping peptide scans may be difficult as each of the single binding regions may contribute only with low binding affinity. Although the SPOT technology can immobilize peptides at a high density of up to 50 nmol/cm^2 and therefore increase the screening sensitivity it may also result in a non-specific background that hide all signals which arise form weak but specific interactions.

PX domain interacting motif on Yip1 family members

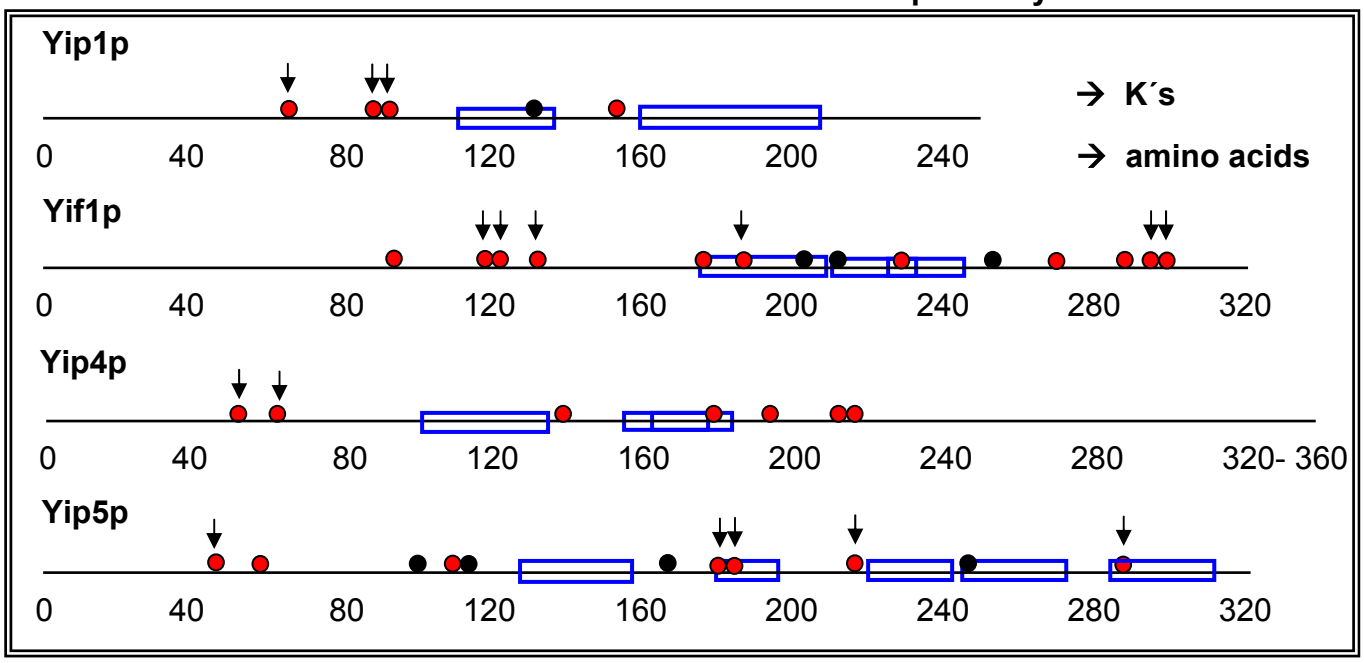
From all Yip1 family member interacting peptides (Table 9) I decided to select a core of peptides which either interacted with several PX domains and/or gave clear results with the alanine scans. I then searched those sequences for a common motif (Fig. 31). Indeed, I clearly observed a tendency for PX-binding peptides to have a concentration of basic residues at their C-terminus compared to non-Yip1 binding peptides which have a higher portion of acidic residues (Fig. 31). It should be kept in mind that non-Yip1 peptide arrays were only reproduced once. Based on the alanine scan results lysine seems to be essential for interaction with PX domains. I therefore aligned the essential lysines (Fig. 32) and defined according to this peptide set the following context in which these lysines are embedded:

β: Any basic residue
ε: Any hydrophobic residue
γ: Any uncharged polar residue

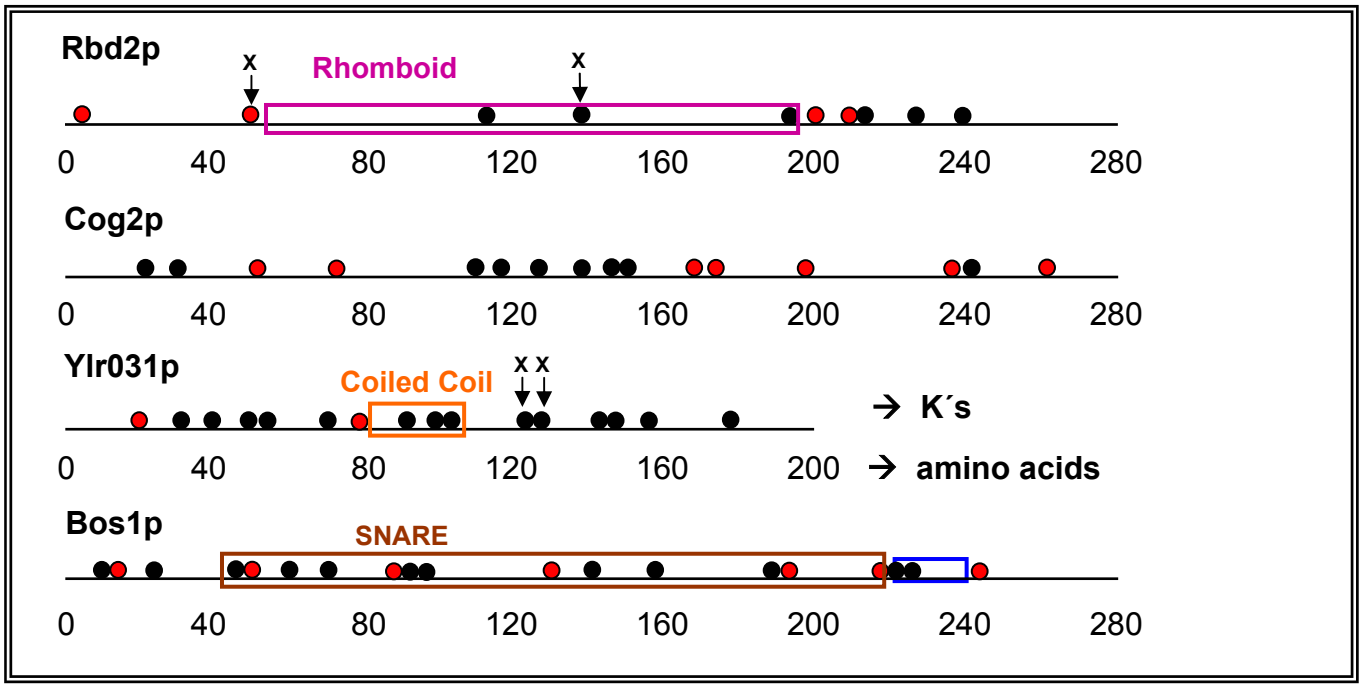
$$\begin{pmatrix} \beta & & \beta \\ \varepsilon\,\gamma\,\varepsilon & \mathbf{K} & \gamma \\ \gamma\,\varepsilon\,\gamma & & \varepsilon \end{pmatrix}$$

Only a subset of the possible variations was observed. For example no peptide composed of 3 hydrophobic residues N-terminal of the lysines was detected. With these criteria I decided to look whether lysines in this context are generally over-represented in all proteins or enriched only in Yip1 proteins or PX interacting partner? I selected the four Yip1 family members, four PX interacting partners with scores 3 or 2 and four random proteins (domain containing proteins and approximately the same size as Yip1 family proteins) for this study. Figure 46 shows various Yip proteins with the different K positions represented as circles. Red circles represent lysines which are surrounded by the selected motif, black circles those lysines with a different enviroment. Arrows show essential lysines as elucidated in my alanine scans. If these results did not show an abolishment of the interaction by replacing the lysine by alanine they were represented with an “x”.

Yip1 family members



Non-Yip PX interactors



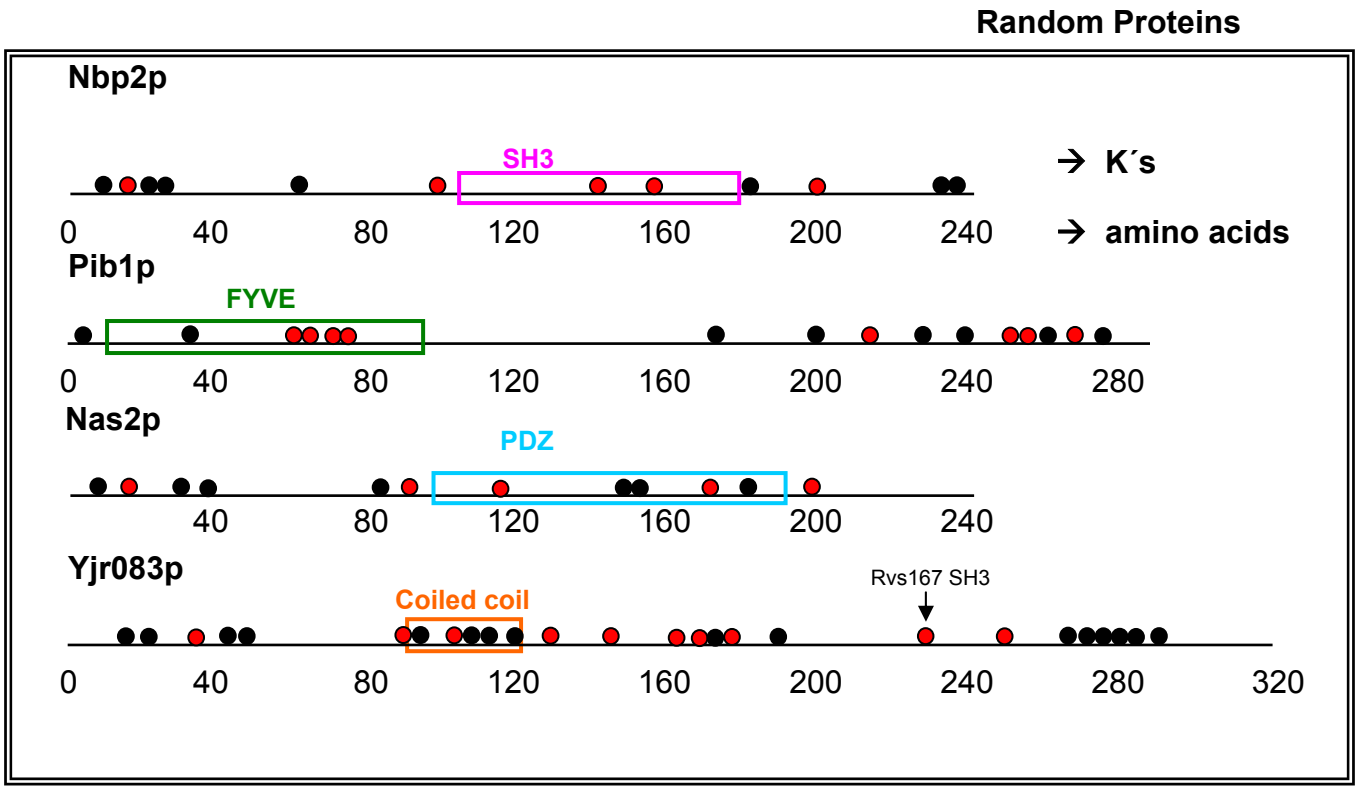


Figure 46: Lysine distribution throughout different protein groups

On the x axes the amino acid position is shown. Lysines (circles) which are surrounded by the motif: (εγ)(βγε)(εγ)**K**(βγε) are coloured red (●), the remaining ones are in black (●). ▭: Transmembrane regions. Domains and motifs are represented as coloured rectangles and labelled respectively. **Rvs167 SH3**: Binding of the SH3 domain of Rvs167p to this peptide according to (Landgraf et al. 2004)

The results are preliminar so interpretation should be taken with caution. I do observe that Yip1 family members contain few lysines and those which are present are surrounded mostly by the selected motif (65-100%). Non Yip PX interacting proteins do vary in their number of lysines. In this group the lysine motif occurs in 12,5 - 47% of the cases. Non related proteins show between 38-50% presence of lysines with the selected motif. When analysing the whole yeast proteome for the average of lysines per 100 amino acids I find the distribution shown in Figure 47**.** With these values we can clearly classify Yip1 family members as proteins with a low content of lysines namely 2 to 4,5 K /100 amino acids compared to the average protein with 5-8 K /100 amino acids. The analysis of further family members of other organisms could help to improve the elucidation of a possible interacting motif.

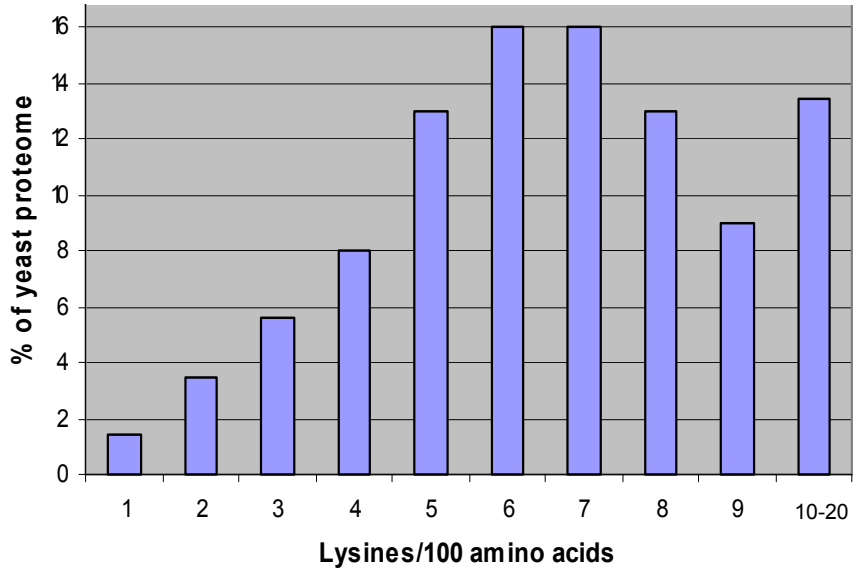


Figure 47: Average number of lysines per 100 amino acids in the yeast proteome

Analysis of the HITKTKM Yip1 motif on Ypt35-PX interaction

Using the peptide arrays I obtained the clearest binding motif (ITKTK) for the interactions of Ypt35-PX and Grd19p with Yip1p (Figures 22, 23, Table 9). The lysines of ITKTK are conserved in Yip1p and Yif1p (Fig. 30) and are essential for PX interaction (Figures 23, 25). I therefore chose this motif for further studies using mutational analysis and a competition assay (Figures 37, 38).

Mutational analysis

I decided to concentrate on the PX-domain of Ypt35p because I was able to detect clearly and reproducibly the interaction with Yip1p by means of GST pulldowns (Figures 17, 19). These experiments were repeated with a mutated version of the Yip1p protein. The mutation comprised the three amino acids which seemed to be essential for interaction according to the alanine scan results: K87, T88 and K89 (Fig. 23). Each one was replaced by alanine (i.e. KTK was replaced by AAA). Suprisingly, the interaction still took place *in vitro*. *In vivo* (i.e. analysing this mutant by means of two-hybrid) the constructed preys seem to have a mating defect (Fig. 48).

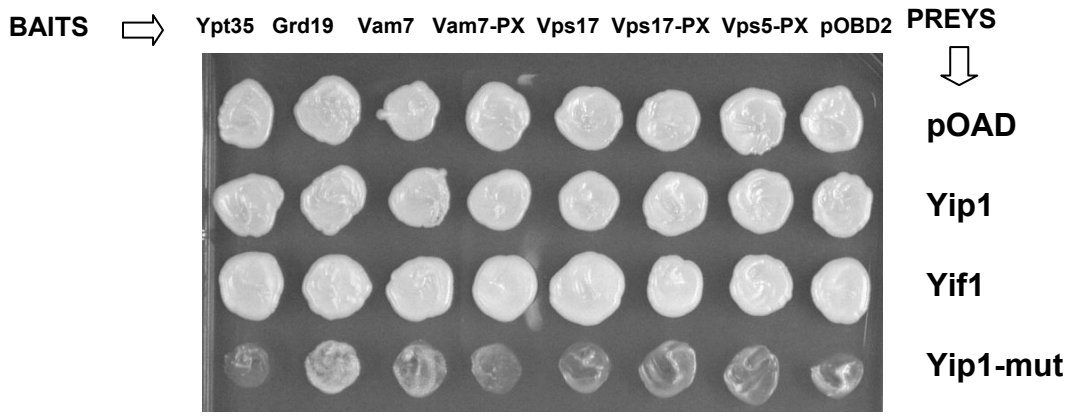


Figure 48: Yeast two-hybrid diploids on selective media. Yip1-mut is not able to mate

Selective media (lacking leucine and tryptophane) for diploids growth. Yip1-mut (K87A, T88A and K89A) was not able to mate properly as no diploids were formed (no growth on selective media)

The reason for this unexpected outcome is jet not understood. Further biochemical analyses have to be performed before a complete picture of this interaction will emerge

Competition assay

It is also possible that the amino acids surrounding the KTK motif (Fig. 32 B) play a role in the interaction. To test this possibility, GST pull-downs were performed with wild type Yip1p in the presence of different increasing concentrations of a synthetic peptide composed of HIKTKM (comprises the binding motif, Fig. 38). I expected that this peptide would compete for the interacting site and therefore (at least at an increased concentration) block the interaction between the full-length proteins. This was not observed. The peptide was used at a three fold higher concentration than the GST fusion protein. This concentration might not be sufficient to displace the interacting protein. This peptide may also have not been rightly folded or as indicated from several results (fragmentation, peptide arrays and mutational analysis) it may only be one of several interacting regions of a conformational discontinuous epitope (Fig. 11). In this case additional amino acid substitutions of putative additional binding sites could be performed. It would also be interesting to make systematic substitutions or deletions to see if only the interactions with particular proteins are lost. This would indicate that distinct interaction sites for different proteins are used.

PX domain´s interaction site

The alignment of the PX domains of Grd19p and Ypt35p (Fig.50) show few strictly conserved residues. Of particular interest are acidic residues which may represent putative sites for interaction with the basic motifs of Yip1 proteins.

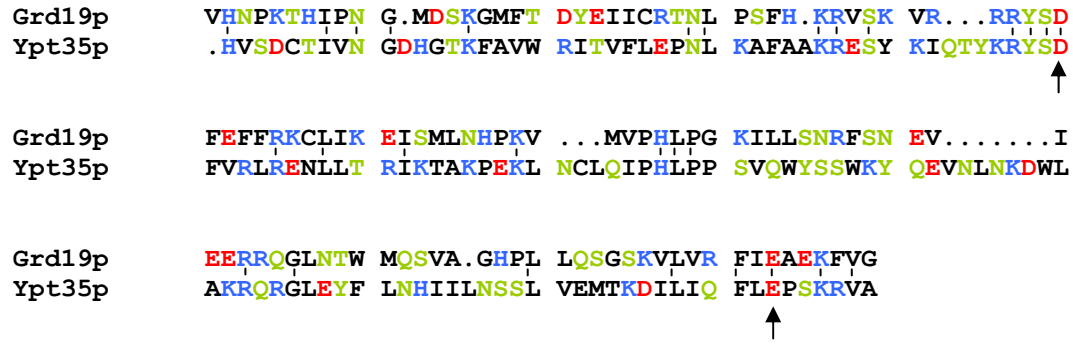


Figure 50 : Alignment of Ypt35p and Grd19p PX domains

The alignment was performed using the multiple alignment tool T-coffee (Notredame et al. 2000). Arrows: conserved acidic residues (D84 and E156 in the Grd19-PX domain). Amino acids were coloured according to their properties, **red**: acidic, **green**: uncharged polar, **blue**: basic and **black** non-polar.

When analysing the alignment, two acidic residues are strictly conserved: aspartic acid 84 (D84) and glutamic acid (E156). When represented in the crystal structure of Grd19-PX (Fig. 51) I can clearly observe that the D84 is too near to the lipid binding pocket to be a putative protein interaction site. On the other hand the glutamic acid at position 156 is nicely exposed and surrounded by basic residues from which K73 is also conserved in both domains. This region may be a putative protein interaction site and a good candidate for mutational studies to prove their role in the protein interaction.

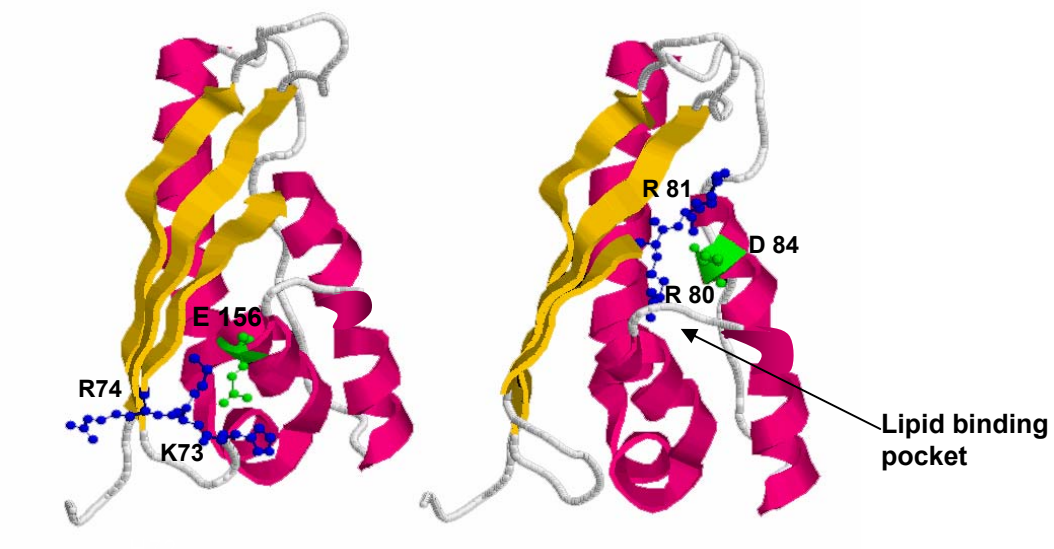
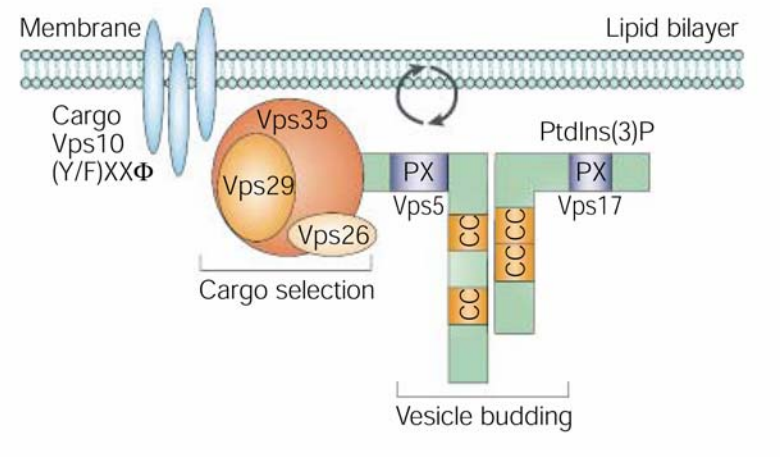


Figure 51: Grd19 PX domain structure and conserved charged residues

Crystal structure of the PX domain of Grd19p. Acidic and basic conserved residues are shown in ball and stick display (see text for details). Green: acidic residues; blue: basic residues. Cartoon generated with the program Rasmol (Sayle and Milner-White 1995).

The biological function of PX-Yip interactions

As described in detail in the introduction (*The PX-only proteins Grd19p and Ypt35p and their phenotypes*), the precise mechanistic role of Grd19p in vesicular trafficking remains unclear. Grd19p is required for the retrieval of Kex2p, A-ALP and Pep12p. The retrieval of these proteins also requires the retromer complex (Fig. 52) suggesting that Grd19p may associate with the retromer and improve the recruitment of some but not all cargo molecules.



(Worby and Dixon 2002)

Figure 52: Retromer complex in *Saccharomyces cerevisiae*

This complex functions in the endosome-to-Golgi retrieval of the vacuolar protein sorting receptor Vps10p, as well as the Golgi-resident membrane protein A-ALP and the endopeptidases Kex2p and dipeptidyl amino peptidase (DPAP). The protein subcomplexes that are involved in cargo selection and vesicle formation are bracketed. Vps35p acts as a 'receptor protein' for cargo proteins, and recruits them into retrograde vesicles by direct interaction with (Y/F)XXΦmotifs (where Φ is hydrophobic). PX: PX domain, cc: coiled coil motifs, arrows: cycling of retromer-complex proteins between cytoplasmic and membrane localization.

Pulse-Chase labelling of Kex2p

For *YPT35* deletion no mutant phenotype has been observed up to date. I therefore decided to look at the missorting of the TGN protease Kex2p to the vacuole in deletion strains of the *YPT35* gene. For this purpose I performed a pulse-chase labelling and immuno-precipitation of Kex2p (as described in Voos and Stevens 1998). The idea of this experiment is to mark all proteins radioactively with a short pulse and chase them at different time points. In this case I looked at the degradation of Kex2p through vacuolar proteases when the protein is misslocalized (to the vacuole). This is done by immuno-precipitating Kex2p from the radioactive labelled yeast extracts with a specific Kex2p antibody. The deletion of the *GRD19* gene is known to cause the misslocalization of Kex2p to the vacuole were it is degraded after 20 minutes (Voos and Stevens 1998). I took therefore this strain as a positive control. Unfortunately the antibody kindly provided by D.Gallwitz (polyclonal rabbit anti-Kex2p) was not specific enough. This resulted in the immuno-precipitation of several proteins from which it was difficult to clearly assign Kex2p (Fig. 53). I did not only perform this experiment for *YPT35* but decided to look at several non-essential Grd19p interacting partners (*RBD2*, *YIP4* and *YIP5*). Unfortunately the same unspecific results were obtained for these deletions (Fig. 53). The use of monoclonal antibodies may solve this problem. It is of particular interest to identify phenotypes of the selected interactors and therefore specify their role in endocytosis or post-Golgi sorting.

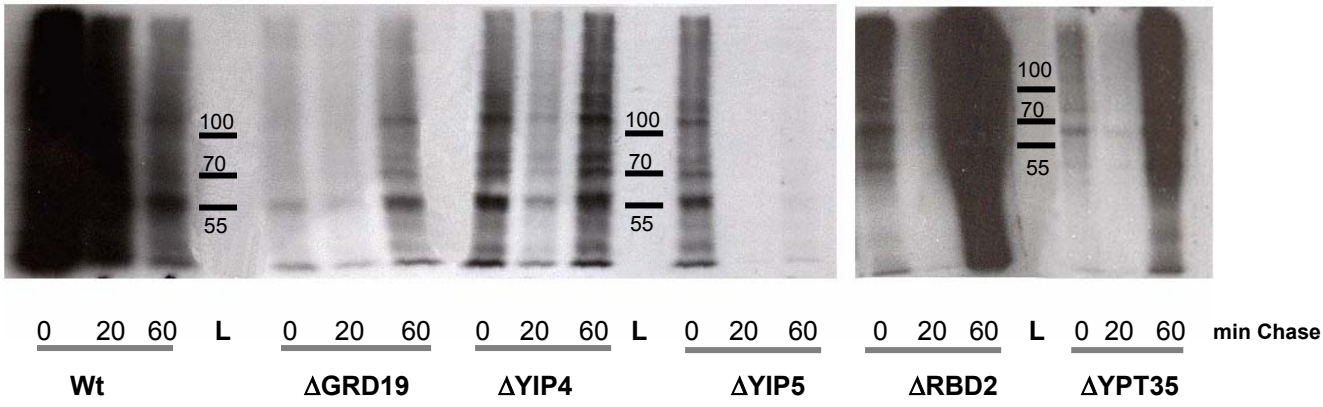


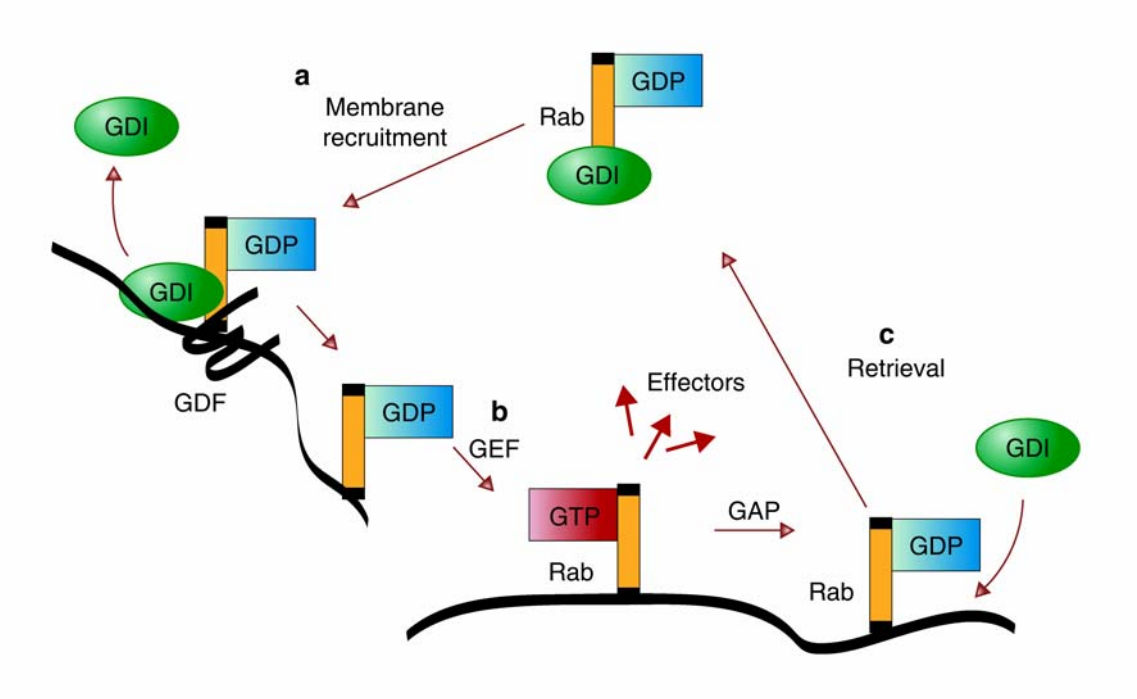
Figure 53: Pulse-chase labelling and immunoprecipitation with antibodies against Kex2p

Wild type (Wt) and deletions are BY4741 strains. Different time points were "chased". Kex2p was expected at 90 KDa but no band can be clearly assigned to Kex2p. L: ladder (molecular mass marker, mass in KDa).

Yip1p and Yif1p function in COPII vesicle formation as clearly assigned by Heidtman and co-workers. The authors were able to demonstrate in a cell-free transport assay that antibodies against the hydrophilic amino terminus of Yip1p (aa. 1-99) inhibited the budding of COPII vesicles at the ER membrane. The same observation has been made for a Yip*1-4* point mutant (E70K), where the mutation is situated in the cytoplasmic domain of Yip1p (Heidtman et al. 2003). Additionally, the amino terminus is also the region which interacts with the Golgi GTPase Ypt1 (Yang et al. 1998). The interaction with this GTPase is not required for COPII vesicle formation suggesting a multifunctional role for the proteins Yip1p and Yif1p.

Based on the recent evidence that the mammalian Yip3 acts as a GDI-displacement factor (GDF) for Rab GTPases (Fig. 54) in the endosomal pathway (Sivars et al. 2003), it may suggest a similar role of Yip1p at the ER.

Rab/Ypt GTPases are present in two states, inactive (GDP bound) and localized in the cytosol or active (GTP bound) and associated to the membrane. These states are regulated by accessory factors such as Guanine nucleotide Exchange Factors (GEFs) and GTPase activating proteins (GAPs). Rabs attach to membranes by their post-translational prenylation modification. The GTPase can be removed from the membrane through the action of Rab-GDI (GDP dissociation inhibitor). GDI is a soluble protein whose recognition site consists of both the GDP-bound Rab and its prenylation moiety (Fig. 54).



Barrowman and Novick 2003

Figure 54: Membrane recruitment and retrieval of Rab GTPases

(a) Prenylated GDP-bound Rabs bound to GDI are recruited to membranes, where a membrane-associated GDF catalyses release of GDI and insertion of the Rab into membranes. (b) After membrane recruitment, a GEF facilitates exchange of GDP for GTP. Activated Rabs can then interact with effector proteins. (c) Stimulation of the intrinsic GTP hydrolytic by a GAP converts the Rab into its GDP-bound form.GDI retrieves GDP-bound Rabs from membranes.

The membrane recruitment reaction of Rabs is highly specific, as each organelle of the secretory and endocytic pathways is found to associate with particular Rab proteins (Segev 2001). In yeast only one GDI has been described meaning that the specificity of Rab localization must be achieved by the factor that mediates the GDI release and membrane attachment, termed GDI displacement factor (GDF). No GDF function has been assigned to any yeast protein yet. Yip1 family members are therefore interesting candidates for this task. In this context it may be interesting to determine the effect of PX proteins on the recruitment of GDFs, their activity, or some downstream event such as the recruitment of GEFs. An indication in this direction is given by the interaction of Grd19p with the GTPase Ypt53p (score 2) involved in endocytosis and transport of proteins to the vacuole. Regulation of Ypt GTPases by

PX proteins in addition to other functions (multiple functions) appears to be possible as two recent studies demonstrated two distinct functions for each of the proteins Snx4p (Cvt13p) and Snx42p (Cvt20p) which depended on their localiztation in the cell (Nice et al. 2002; Hettema et al. 2003).
Against a role of Yip1p as a GDF for Ypt1p at the ER argues the fact that blocking of the Ypt1 GTPase function does not prevent COPII vesicle formation (Heidtman et al. 2003) and the localization of Ypt1p does not change when Yip1p is depleted (Barrowman et al. 2003).

Barrowman and co-workers assigned Yip1p/Yif1p a function in establishing fusion competence of ER derived vesicles. They were able to observe correct priming and tethering of ER derived vesicles to Golgi acceptor membranes but no fusion when Yip1p was depleted. This assay compared to the one of Heidtman and co-workers had a different outcome. Barrowman et al. 2003 were additionally able to prove an interaction of Yip1p with the ER to Golgi SNARES Bos1p and Sec22p. This is particularly interesting as Ypt35p interacted in my two-hybrid screens reproducibly (score 2) with Bos1p.

Yip1p temperature sensitive mutant interacts with Ypt35-PX *in vitro* but not *in vivo.*

The first functional studies of Yip1p (Yang et al. 1998) used the temperature sensitive mutant Yip*1-2* which showed a slightly disturbed maturation of CPY at 25°C which was completely inhibited at 36°C. I decided to study this functional mutant and look at its interactions with PX proteins and domains. The mutant could be translated *in vitro* at 30°C although the permissive temperature studied by Yang and co-workers was 25°C. The interaction took place when GST pull-downs were done although these were performed at 4°C (Fig. 40). I*n vivo* (Fig. 39) interactions when performing yeast two-hybrid at 30°C were abolished. At this temperature I cannot make any statement about the protein stability which is an important issue, for the interpretation of these results.
To rule out protein degradation I performed western blots of yeast extracts expressing the mutant in a prey vector (pOAD, see experimental procedures). I used the polyclonal GAL4 activation domain antibody (Sta. Cruz Biotechnology, sc-429) which revealed an extremely high background (Fig. 55). The fact that the pOAD vector is a low copy vector additionally complicated the detection.

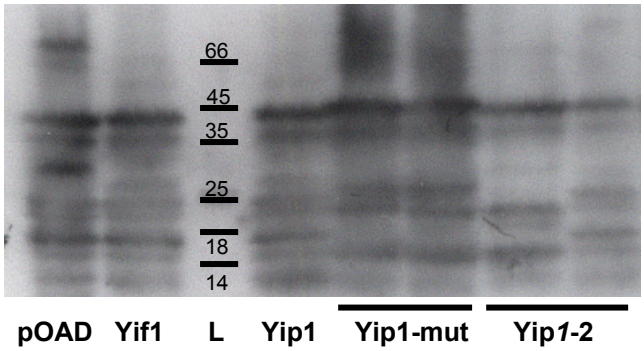


Figure 55: Western Blot of yeast extracts with antibodies against GAL4-AD

Yeast extracts of PJ-69 a strains carrying the low copy prey plasmid pOAD coding respective proteins (Yip1p: 27 KDa and Yif1p: 35 KDa) as GAL4-AD (18 KDa) fusions. **pOAD:** negative control, empty vector. **L:** ladder (molecular mass marker, mass in KDa). **Yip1-mut**: K87A, T88A and K89A, **Yip*1-2***: (G175E) mutant.

These contradictory results between both experiments can only be sorted out with further studies. A possibility would be to perform yeast two-hybrid assays as well as GST pull-down assays at the permissive temperature of 25°C and observe if I obtain the same results.

In this work I have shown that PX domains can interact with a number of different proteins in biological meaningful contexts. Most striking was their preference for membrane proteins of the Yip1 family which could be confirmed by independent techniques. Given the much greater diversity of both Yip and PX proteins in vertebrate genomes (Fig. 56, Fig. 57), I hope that my findings will encourage further studies on these proteins and so improve the understanding of both their diversity, specificity and, eventually, biological function.

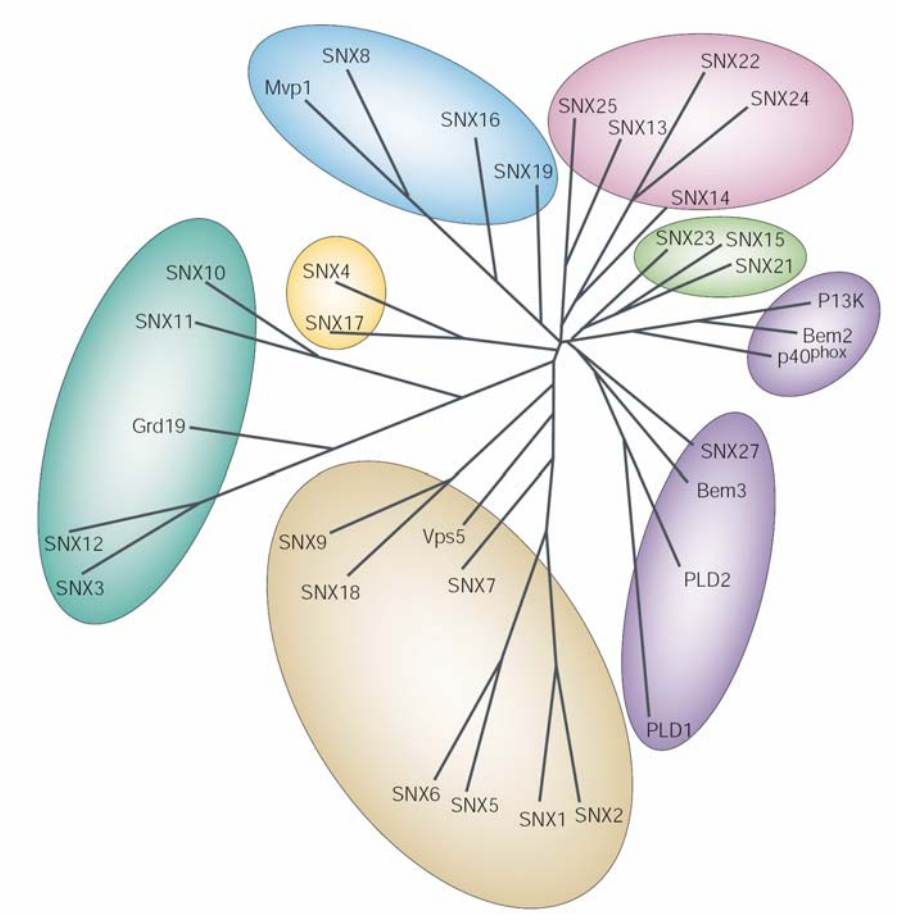


Figure 56: PX-domain phylogenetic tree

Phylogenetic clustering of PX domains in groups should give an overview of domain diversity. It is tempting to speculate that the SNXs that share conserved PX domains falling on the same branch of the phylogenetic tree might share common functions in the cell (Worby and Dixon 2002).

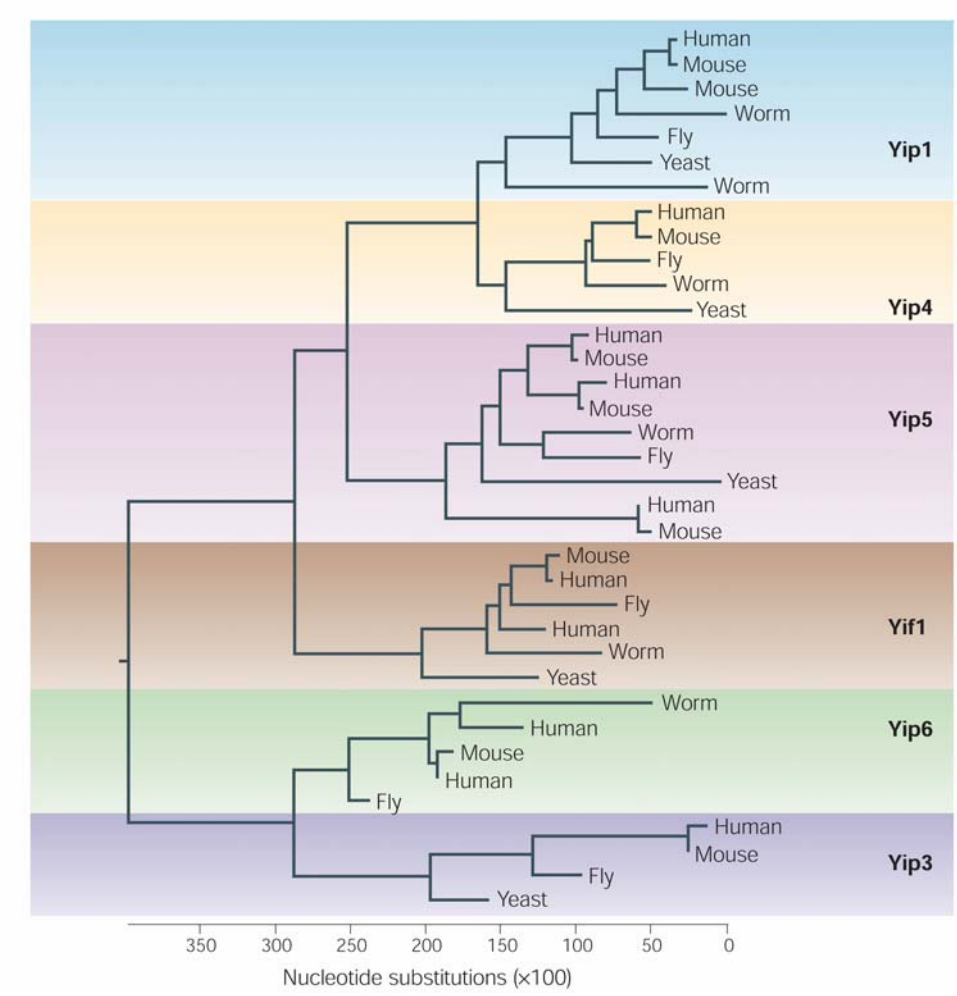


Figure 57: Phylogenetic-tree analysis of the Yip1 protein family

Of the five species shown (yeast, Saccharomyces cerevisiae; worm, Caenorhabditis elegans; fly, Drosophila melanogaster; mouse, Mus musculus; and human, Homo sapiens), each has at least one representative Yip protein in each of the main branches, except for worm, which has no apparent Yip3 orthologue, and yeast, which has no apparent Yip6 orthologue (Pfeffer and Aivazian 2004).

Open questions, strategies, and future perspectives

Results obtained during these 3 years opened new perspectives of the PX domain which entail several open questions:

How do PX domains and proteins contribute to Yip1 family function?

Yip1p/Yif1p

The role of PX proteins in COPII vesicle formation could be tested by a tethering (Barrowman et al. 2003) and COPII cell-free transport assay (Heidtman et al. 2003) to which I would add PX fusion proteins or domains and test if any change in COPII vesicle formation or fusion of the ER-derived vesicles to the Golgi-donor membranes occurs.

Yip4p/Yip5p

For these two Yip1 family members no mutant phenotype, function or localization in the cell is known. My first steps would be to look at some phenotypes which are observed in mutants/deletions their interacting partners, the PX only-proteins Grd19p and Ypt35p. As mentioned before (*Biological function of PX-Yip interactions)* I am planning to look at the missorting of the Golgi endopeptidase Kex2p in deletions of Yip4p and Yip5p by means of pulse and chase experiments (Voos and Stevens 1998). Also interesting would be the analysis of missorting of Pep12p by immunoblotting Pep12p in p13 and p100 fractions (13,000 g pellet, 100,000 g pellet). In *GRD19* and retromer mutants endogenous Pep12 shifts from p100 (endosomes) to p13 (vacuole) (Hettema et al. 2003). This experiment is semi-quantitative, unbiased, looks at an endogenous protein rather than an overexpressed mutant chimera and seems quite specific (Hugh Pelham, personal communication).
Localization of these proteins can be performed by immunofluorescence or GFP/YFP constructs. It would be interesting to see if they are localized to the same membranes and form a complex. An interesting observation was for PRA (prenylated Rab acceptor = Yip3) isoforms. These are targeted to distinct membrane compartments and this localization could be mapped to a particular signal localized at the C-terminus of both isoforms (Abdul-Ghani et al. 2001). It would be interesting to analyse Yip4p and Yip5p for this type of localization signal.

Can we find out more by about PX proteins looking at their genetic interactions?

A future plan is to establish in our lab the Systematic Genetic Array (SGA), for we possess the knock-out library of all yeast non-essential genes and the robotic equipment. SGA analysis is an approach that automates the isolation of yeast double mutants and enables large-scale mapping of genetic interactions. In a typical SGA screen, a mutation in a query gene of interest is crossed into an array of viable gene deletion mutants to generate an output array of double mutants, which can then be scored for specific phenotypes. Synthetic interactions, in which the combination of mutations in two genes causes cell death or reduced fitness, respectively, are of particular interest because they can identify genes whose products buffer one another and impinge on the same essential biological process (Tong et al. 2001). I have performed *GRD19* and *YPT35* as query genes and tested a subset of deletions according to the SGA protocols. I was not able to efficiently obtain double deletion haploids though. This was probably due to inefficient sporulation, different media and other factors which have to be optimized. Looking at genetic interactions systematically for PX proteins would be the first direct comparison of the same set of proteins used for genome wide two-hybrid screens.

Do PX domains bind simultaneously lipids and proteins?

As suggested already (*Possible simultaneous lipid and protein binding and phospholipid distribution in the cell)*, a liposome based assay may give insight into the possibilities of independent or simultaneous protein and lipid interaction of PX domains.

Which surfaces interact in Yip-PX interactions?

The best way to map the interacting region of Yip1 family members is to crystallize the complex of Yip proteins with PX domains. Efforts are being made in this direction in cooperation with Alexey Rak (Max-Planck Institute for Molecular Physiology, Dortmund, Germany). This would give us insight into the protein interaction site/sites on the PX domain surface as well as on the Yip proteins.

Further more, point mutations in the PX domain or putative interacting partners (Figures 50 and 51) or domain swap may allow us to map the protein interacting regions.

The domain swap project consists of the following steps: The first step would be to divide the PX domains of Snx4p and Ypt35p into two parts, each containing only the N-terminal β-sheets or the C-Terminal α-helices (Fig. 7 A). Both parts would be then exchanged (domain swap) and GST pull downs of these chimeras would be performed. Snx4-PX interacts only with Yip4p in contrast to Ypt35-PX which interacts with Yip1p, Yif1p and Yip4p. After the domain swap it would be interesting to observe if the interacting partners have also been exchanged. In this way I would be able to map the interacting region and determine aspects about the differences between these two PX domains.

I hope that future work and cooperation's will able to answer some of these exciting open questions and we can so improve the understanding of the functions, diversity and specificity of these proteins.

Appendix

Systematic complex purifications

Several large-scale projects have attempted the systematic isolation and identification of protein complexes in yeast (Gavin et al. 2002, Ho et al. 2002). Surprisingly few of them recovered any PX domain proteins and their interactions did not turn out to be very helpful for my discussion, so I did not discuss those results in detail.

Protein complexes containing PX proteins or their interactors based on Gavin et al. 2002 **(G)**, Ho et al. 2002 **(H)** and Hazbun et al. 2003 **(Ha)**; bait proteins are listed first.

Table I a: PX proteins in systematic complex purifications

BAIT	ORF	Complex	Ref.
Bem1p	YBR200W	Bem1 Boi2 Cdc24 Rsc2	**G**
Bem3p	YPL115C	Dop1(cs), Yil055c, Yta7	**H**
Grd19p	YOR357C	none	
Mdm1p	YML101C	none	
Mvp1p	YMR004W	none	
Spo14p	YKR031C	Mum2(tag) Mtc2 **Spo14**	**G**
Snx4p	YJL036W	none	
Snx41p	YDR425W	none	
Snx42p	YDL113C	none	
Vam7p	YGL212W	none	
Vps17p	YOR132W	none	
Vps5p	YOR069W	Sup35 Sup45 Clu1 Crn1 Ecm29 Kap123 Mec1 Mip6 Mis1 Mrpl23 Nab3 Nrd1 Rom2 Rpa135 Sen54 Sui3 Tao3 Uso1 **Vps5** Ybl004w Yjl017w Ylr386w Yml117w	**G**
Ypt35p	YHR105W	Vps13	**H**
Ykr078p	YKR078W	none	
Ypr097p	YPR097W	none	

Table I b: PX interactors in systematic complex purifications

PREY	ORF	Complex	Ref.
Adh1p	YOL086C	1. Aos1-Adh1 Aos1 Uba2 Yef3 2. Ecm1-Adh1 Ecm1 Gcn1 Hsc82 Kap123 Lys2 Pse1 Sxm1 3. Esa1-Act1 Adh1 Arp4 Eaf3 Epl1 Esa1 Tra1 Vid21 Yaf9 Yap1 4. Fth1-Adh1 Fet5 Fks1 Fth1 Gea2 Kap123 Rpn3 5. Isw2-Adh1 Isw2 Itc1 6. Leu1-Adh1 Leu1 7. Med7-Ada2 Adh1 Cse2 Eno2 Fab1 Fba1 Med1 Med11 Med4 Med6 Med7 Med8 Nut1 Pgk1 Rgr1 Sin4 Srb2 Srb4 Srb5 Srb6 Srb7 Srb8 Srb9 Srp1 Taf60 Tif1 YIL077C 8. Mot1-Adh1 Gcn1 Isw1 Mot1 Pdc1 Spt15 Vps1 Yef3 9. Myo4-Act1 Adh1 Gcn1 Hsc82 Kap123 Mlc1 Myo4 Nip1 She2 She3 YDR101C Yef3 10. Nmr1-Adh1 Nmr1 Spo72 11. Rad16-Adh1 Mir1 Pdc1 Pep4 Rad16 Rim1 Tdh3 Vma4 Vps1 Wrs1 YMR315W 12. Sec1-Adh1 Pgi1 Sec1 13. Sec7-Adh1 Cse1 Dhh1 Fks1 Gcn20 Gfa1 Idh2 Kap123 Ktr3 Mir1 Sam1 Sec7 YFR044C YHR020W 14. Sir2-Adh1 Bgl2 Net1 Sir2 Sir4 Tdh3 Yef3 15. Ubp14-Adh1 Ils1 Nip1 Ubp14 16. YAL027W-Adh1 Gin4 Hmo1 Kcc4 Pdc1 Pgi1 Rad1 Rad10 Yra1 [YAL027W] 17. YAL034C-Adh1 Kap123 Sin3 Taf145 Taf90 YAL034C 18. YNL127W-Adh1 Eno2 Far3 Fba1 Gpm1 Hfm1 Hsc82 Pdc1 Rom2 Sse2 Tpi1 YGL131C YNL127W YOR056C-Adh1 Enp1 YDL060W YNL207W YOR056C	**G**
Adh1p	YOL086C	YKL195W YER110C (KAP123 YMR203W (TOM40) YOL086C (ADH1)	**H**
Bsp1p	YPR171W	Sla1-Bzz1 Ecm25 Inp52 Las17 Sla1 Stm1 YCR030C YFR024C-A YPR171W Ysc84	**G**
Bub1p	YGR188C	ARO9, ENO1, KAR2, PDC1, PGI1, VMA2	**H**
Dam1p	YGR113W	Pse1-Dam1 Dbp9 Gfa1 Ino4 Kap95 Lys12 Mds3 Nud1 Pda1 Pdb1 Pre10 Pre5 Pre6 Pre8 Pse1 Rpt3 Rpt5 Scl1 Spa2 Srp1 Ulp1 YGR081C YMR310C YPL012W Yra1	**G**
Lcp5p	YER127W	YDR299W (BFR2), YDR365C, YER127W (LCP5), YGR145W, YHR196W (UTP9), YJL033W (HCA4), YOR310C (NOP58)	**H**
Rrn10p	YBL025W	PDA1	**H**
Rvs167p	YPR097W	ALD6, ARG1, ARO8, ARO9, CDC33, CIT1, COR1, CYS4, GAL1, GDH1, GLK1, GND1, HXK1, HXK2, INO1, KGD2, KRS1, LPD1, MET6, PDC1, PDI1, PGI1, PMI40, RSP5, RVB2, RVS161, SAH1, SES1, SSC1, TEF2, TIF2, TKL1, TRP5, TUB1, UGP1, VMA2	**H**

Protein complexes containing PX proteins or their interactors based on Gavin et al. 2002 **(G)**, Ho et al. 2002 **(H)** and Hazbun et al. 2003 **(Ha)**; bait proteins are listed first.

Table I b: PX interactors in systematic complex purifications

PREY	ORF	Complex	Ref.
Sds3p	YIL084C	1. Ecm16-Ecm16 Pho23 Sds3 Ume1 2. Rpd3-Cka1 Ckb2 Dep1 Eaf3 Nrd1 Pho23 Rpd3 Sap30 Sds3 Sin3 Sto1 Top1 Ume1 Vps1 YBR095C YMR075W YPL181W 3. Sin3-Abf2 Cka1 Cka2 Ckb1 Eaf3 Hta2 Ioc3 Isw1 Pho23 Rpd3 Rsc8 Sap30 Sds3 Sin3 Tup1 Ume1 YBR095C YDL076C YMR075W YPL181W 4. YDL076C-Dot6 Med4 Nhp10 Rpd3 Sap30 Sds3 Sin3 Ume1 Ume6 YDL076C YPL181W	**G**
Vma8p	YEL051W	1. Vma7-Vma1 Vma10 Vma13 Vma4 Vma6 Vma7 Vma8 Vph1 2. Vma8-Vma1 Vma13 Vma4 Vma5 Vma7 Vma8	**G**
Vps21p	YOR089C	ACO1 ARO9, BMH1, BMH2, ENO1, GDH1, GDI1, HSC82, MET6, MRS6, PDC1, PYC1, RHR2, RPT3, SAH1, SSB1, STE11, TDH1, TDH2, TPS1, TSA1, VAS1, YPT52, YPT53	**H**
Ylr424p	YLR424W	1. Prp43-Asc1 Clf1 Nop1 Prp43 Prp46 Snu114 Sro9 YGL128C YLR424W YNL224C 2. Prp8-Aar2 Cef1 Clf1 Lsm2 Lsm6 Prp19 Prp3 Prp31 Prp4 Prp43 Prp6 Prp9 Rse1 Smb1 Smd3 Snu114 Snu66 Syf1 YCR063W YDL209C YGL128C YGR278W YHR156C YLR424W [Prp8] 3. Smx2-Brr1 Clf1 Cus1 Dib1 Hsh155 Lea1 Lsm4 Luc7 Mud1 Prp11 Prp19 Prp21 Prp3 Prp31 Prp4 Prp40 Prp46 Prp6 Prp9 Rse1 Smb1 Smd1 Smd2 Smd3 Snp1 Snu114 Snu56 Snu66 Snu71 Sto1 Yhc1 YJR084W YLR424W [Smx2] 4. Smx3-Cus1 Dib1 Ecm2 Hsh155 Lea1 Luc7 Msl1 Prp19 Prp31 Prp39 Prp4 Prp40 Prp42 Prp43 Prp46 Prp6 Prp9 Rse1 Smb1 Smd3 Snt309 Snu114 Snu71 Sto1 YDL209C YJR084W YLR424W [Smx3] 5. Yju2-Cdc40 Clf1 Lea1 Prp19 Prp43 Prp45 Prp46 Snt309 Snu114 Sto1 Syf1 YDL209C YGR278W Yju2 YLR424	**G**
		1. YAL032C (PRP45) YBR065C (ECM2) *YDL209C (CWC2) YDR364C (CDC40) YDR416W (SYF1) YER029C (SMB1) YER172C (BRR2) YGL120C (PRP43) YGL128C (CWC23) YGR074W (SMD1), YGR129W (SYF2), YHR165C (PRP8), YJR050W (ISY1), YKL095W (YJU2), YKL173W (SNU114), YKR022C, YLL036C (PRP19), YLR117C (CLF1), YLR147C (SMD3), *YLR424W, YMR213W (CEF1), YPL151C (PRP46), YPL213W (LEA1), YPR101W (SNT309) 2. YAL032C (PRP45), YBR065C (ECM2), *YDL209C (CWC2), YDR364C (CDC40), YDR416W (SYF1), YER029C (SMB1), YER172C (BRR2), YGL120C (PRP43), YGL128C (CWC23), YHR165C (PRP8), YKL173W (SNU114), YKR022C, YLL036C (PRP19), YLR117C (CLF1), YPL151C (PRP46), YPR101W (SNT309), YPR182W (SMX3)	**Ha**

PREY	ORF	Complex	Ref.
Ypt53p	YNL093W	BCY1, CDC33, MRS6, PAB1, POR1, THI21, TPK1, TPK3, VPS21, YNL227C, YPT52	**H**
Yra2p	YKL214C	Brr1 Bur2 Cbc2 Hta1 Kap95 Luc7 Msh4 Mud1 Nab3 Nrd1 Prp40 Prp42 Rlr1 Rse1 Scp160 Sen1 Sgv1 Smd1 Smd2 Smd3 Snp1 Snu71 Sro9 Srp1 Sto1 Tif4631 Tif4632 YDL175C YKL214C	**G**

Table I b: PX interactors in systematic complex purifications

Protein complexes containing PX proteins or their interactors based on Gavin et al. 2002 **(G)**, Ho et al. 2002 **(H)** and Hazbun et al. 2003 **(Ha)**; bait proteins are listed first.

References

Gavin, A.C., Bosche, M., Krause, R., Grandi, P., Marzioch, M., Bauer, A., Schultz, J., Rick, J.M., Michon, A.M., Cruciat, C.M. et al. 2002. Functional organization of the yeast proteome by systematic analysis of protein complexes. *Nature* **415:** 141-147.

Hazbun, T.R., Malmstrom, L., Anderson, S., Graczyk, B.J., Fox, B., Riffle, M., Sundin, B.A., Aranda, J.D., McDonald, W.H., Chiu, C.H. et al. 2003. Assigning function to yeast proteins by integration of technologies. *Mol Cell* **12:** 1353-1365.

Ho, Y., Gruhler, A., Heilbut, A., Bader, G.D., Moore, L., Adams, S.L., Millar, A., Taylor, P., Bennett, K., Boutilier, K. et al. 2002. Systematic identification of protein complexes in Saccharomyces cerevisiae by mass spectrometry. *Nature* **415:** 180-183.

Table II: PX protein interactions not found in this work

A number of interactions have been reported for PX proteins that we failed to identify. It is difficult to explain this discrepancy but it has been shown that differences in strains, plasmids, or reporter genes may account for these results.

Bait	Prey	Method	Refs.
Bem1p	**Act1p**		(1)
	Boi1p	2-hybrid, affinity column	(2)
	Boi2p	2-hybrid, affinity column, coippt	(3)(2)(4)
	Cdc42p	affinity column, coippt, 2-hybrid, in vitro binding	(5)(4)(6)
	Cla4p	coippt, in vitro binding	(6)
	Far1p	2-hybrid	(7)(8)(4)(9)
	Rsr1p	affinity column	(10)
	Sec15p	2-hybrid	(4)
	Ste20p	2-hybrid, coippt, in vitro binding	(1)(11)(4)(12)
	Ste5p	2-hybrid, coippt, copurification	(7)(1)
	Swe1p	2-hybrid	(4)
Bem3p	**Bem4p**	2-hybrid	(4)
	Cdc42p	2-hybrid	(13)
	Cla4p	2-hybrid	(4)
	Lsm2p	2-hybrid	(14)
	Zds1p	2-hybrid	(4)
	Zds2p	2-hybrid	(4)
Grd19p	**Ste13p**	in vitro binding	(15)
Mdm1p	**Mdm1p**	in vitro binding	(16)(17)(18)
	Ypr099c	2-hybrid	(18)
Mvp1p	**Hxt10p**	2-hybrid	(18)
	Ybr077c	2-hybrid	(19)
Snx4p	**Atg17p**	2-hybrid	(18)
	Atg20p	coippt, 2-hybrid	(19)(20)
	Snc1p	crosslinking	(20)
	Snx41p	coippt, 2-hybrid	(18)(20)
Snx41p	**Snx4p**	2-hybrid	(18)
Snx41p	**Yip5p**	2-hybrid	(18)
	Yip4p	2-hybrid	(18)
	Yip1p	2-hybrid	(18)

Bait	Prey	Method	Refs.
Snx41p	**Yjr110w**	2-hybrid	(18)
	Ypl280w	2-hybrid	(18)
	Ypt52p	2-hybrid	(18)
Snx42p	**Nip100p**	2-hybrid	(18)
	Rrn10p	2-hybrid	(19)
	Snx4p	coippt, 2-hybrid	(19)(20)
	Yhr022p	(2-hybrid	(21)
	Ylr424p	2-hybrid	(18)
Spo14p	**Dcp2p**	2-hybrid	(14)
Vam7p	**Apg17p**	2-hybrid	(19)
	Ktr3p	2-hybrid	(19)
	Nyv1p	in vitro binding, coippt, copurification	(22)(23)
	Vam3p	in vitro binding, coippt, copurification, 2-hybrid, affinity column	(22)(23)(19)(24)
	Vtip	in vitro binding, coippt, copurification, affinity column	(22)(23)(24)
	Ykt6p	coippt, copurification	(23)
	Rbd2p	2-hybrid	(19)
	Sec10p	2-hybrid	(19)
	Sna3p	2-hybrid	(19)
	Spc72p	2-hybrid	(19)
	Stb2p	2-hybrid	(19)
	Tvp18p	2-hybrid	(19)
	Vps68p	2-hybrid	(19)
	Vps73p	2-hybrid	(19)
	Yif1p	2-hybrid	(19)
	Yip5p	2-hybrid	(19)
	Yor292c	2-hybrid	(19)
	Ypt7p	2-hybrid	(19)
Vps5p	**Smf3p**	2-hybrid	(18)
	Vps29p	crosslinking	(25)
	Vps35p	crosslinking	(25)
	Vps17p	hybrid	(19)
Vps17p	**Vps29p**	crosslinking	(25)
	Vps35p	crosslinking	(25)

Table II: PX protein interactions not found in this work

References

(1) Leeuw, T., Fourest-Lieuvin, A., Wu, C., Chenevert, J., Clark, K., Whiteway, M., Thomas, D. Y., and Leberer, E. Pheromone response in yeast: association of Bem1p with proteins of the MAP kinase cascade and actin. Science 270, 1210-3 (1995).

(2) Bender, L., Lo, H. S., Lee, H., Kokojan, V., Peterson, V., and Bender, A. Associations among PH and SH3 domain-containing proteins and Rho-type GTPases in Yeast. J Cell Biol 133, 879-94 (1996).

(3) Matsui, Y., Matsui, R., Akada, R., and Toh-e, A. Yeast src homology region 3 domain-binding proteins involved in bud formation. J Cell Biol 133, 865-78 (1996).

(4) Drees, B. L., Sundin, B., Brazeau, E., Caviston, J. P., Chen, G. C., Guo, W., Kozminski, K. G., Lau, M. W., Moskow, J. J., Tong, A., Schenkman, L. R., McKenzie, A. 3rd, Brennwald, P., Longtine, M., Bi, E., Chan, C., Novick, P., Boone, C., Pringle, J. R., Davis, T. N., Fields, S., and Drubin, D. G. A protein interaction map for cell polarity development. J Cell Biol 154, 549-76. (2001).

(5) Gladfelter, A. S., Moskow, J. J., Zyla, T. R., and Lew, D. J. Isolation and characterization of effector-loop mutants of cdc42 in yeast. Mol Biol Cell 12, 1239-55. (2001).

(6) Bose, I., Irazoqui, J. E., Moskow, J. J., Bardes, E. S., Zyla, T. R., and Lew, D. J. Assembly of scaffold-mediated complexes containing Cdc42p, the exchange factor Cdc24p, and the effector Cla4p required for cell cycle-regulated phosphorylation of Cdc24p. J Biol Chem 276, 7176-86 (2001).

(7) Lyons, D. M., Mahanty, S. K., Choi, K. Y., Manandhar, M., and Elion, E. A. The SH3-domain protein Bem1 coordinates mitogen-activated protein kinase cascade activation with cell cycle control in Saccharomyces cerevisiae. Mol Cell Biol 16, 4095-106 (1996).

(8) Butty, A. C., Pryciak, P. M., Huang, L. S., Herskowitz, I., and Peter, M. The role of Far1p in linking the heterotrimeric G protein to polarity establishment proteins during yeast mating. Science 282, 1511-1516 (1998).

(9) Butty, A. C., Perrinjaquet, N., Petit, A., Jaquenoud, M., Segall, J. E., Hofmann, K., Zwahlen, C., and Peter, M. A positive feedback loop stabilizes the guanine-nucleotide exchange factor Cdc24 at sites of polarization. EMBO J 21, 1565-1576. (2002).

(10) Park, H. O., Bi, E., Pringle, J. R., and Herskowitz, I. Two active states of the Ras-related Bud1/Rsr1 protein bind to different effectors to determine yeast cell polarity. Proc Natl Acad Sci U S A 94, 4463-8 (1997).

(11) Ito, T., Matsui, Y., Ago, T., Ota, K., and Sumimoto, H. Novel modular domain PB1 recognizes PC motif to mediate functional protein-protein interactions. EMBO J 20, 3938-3946. (2001).

(12) Evangelista, C., Lockshon, D., and Fields, S. The yeast two-hybrid system: prospects for protein l inkage maps. Trends In Cell Biology 6, 196-199 (1996).

(13) Richman, T. J., and Johnson, D. I. Saccharomyces cerevisiae Cdc42p GTPase Is Involved in Preventing the Recurrence of Bud Emergence during the Cell Cycle. Mol Cell Biol 20, 8548-8559 (2000).

References

(14) Fromont-Racine, M., Mayes, A. E., Brunet-Simon, A., Rain, J. C., Colley, A., Dix, I., Decourty, L., Joly, N., Ricard, F., Beggs, J. D., and Legrain, P. Genome-wide protein interaction screens reveal functional networks involving Sm-like proteins. Yeast 17, 95-110 (2000).

(15) Voos, W., and Stevens, T. H. Retrieval of resident late-Golgi membrane proteins from the prevacuolar compartment of Saccharomyces cerevisiae is dependent on the function of Grd19p. J Cell Biol 140, 577-90 (1998).

(16) McConnell, S. J., and Yaffe, M. P. Intermediate filament formation by a yeast protein essential for organelle inheritance. Science 260, 687-9 (1993).

(17) Fisk, H. A., and Yaffe, M. P. Mutational analysis of Mdm1p function in nuclear and mitochondrial inheritance. J Cell Biol 138, 485-94 (1997).

(18) Ito, T., Chiba, T., Ozawa, R., Yoshida, M., Hattori, M., and Sakaki, Y. A comprehensive two-hybrid analysis to explore the yeast protein interactome. Proc Natl Acad Sci U S A 98, 4569-4574. (2001).

(19) Uetz, P., Giot, L., Cagney, G., Mansfield, T. A., Judson, R. S., Knight, J. R., Lockshon, D., Narayan, V., Srinivasan, M., Pochart, P., QureshiEmili, A., Li, Y., Godwin, B., Conover, D., Kalbfleisch, T., Vijayadamodar, G., Yang, M. J., Johnston, M., Fields, S., and Rothberg, J. M. A comprehensive analysis of protein-protein interactions in Saccharomyces cerevisiae. Nature 403, 623-627 (2000).

(20) Hettema, E. H., Lewis, M. J., Black, M. W., and Pelham, H. R. Retromer and the sorting nexins Snx4/41/42 mediate distinct retrieval pathways from yeast endosomes. EMBO J 22, 548-57. (2003).

(21) Schwikowski, B., Uetz, P., and Fields, S. A network of protein-protein interactions in yeast. Nat Biotechnol 18, 1257-1261 (2000).

(22) Fukuda, R., McNew, J. A., Weber, T., Parlati, F., Engel, T., Nickel, W., Rothman, J. E., and Sollner, T. H. Functional architecture of an intracellular membrane t-SNARE. Nature 407, 198-202. (2000).

(23) Ungermann, C., vonMollard, G. F., Jensen, O. N., Margolis, N., Stevens, T. H., and Wickner, W. Three v-SNAREs and two t-SNAREs, present in a pentameric cis-SNARE complex on isolated vacuoles, are essential for homotypic fusion. J Cell Biol 145, 1435-1442 (1999).

(24) Sato, T. K., Rehling, P., Peterson, M. R., and Emr, S. D. Class C Vps protein complex regulates vacuolar SNARE pairing and is required for vesicle docking/fusion. Mol Cell 6, 661-71. (2000).

(25) Seaman, M. N. J., McCaffery, J. M., and Emr, S. D. A membrane coat complex essential for endosome-to-Golgi retrograde transport in yeast. J Cell Biol 142, 665-681 (1998).

Abbreviations

AA:	Amino acid
A-ALP:	cytosolic domain of DPAP A fused to the transmembrane and lumenal domains of the alkaline phosphatase
Amp:	Ampicillin
bp:	Base pairs
BSA:	Albumin bovine serum
CPY:	Carboxypeptidase Y
DMSO:	Dimethyl sulfoxide
DNA:	Desoxy ribonucleic acid
DPAP A:	dipeptidyl aminopeptidase A
dNTP:	Desoxy nucleosid triphosphate
E.coli:	Escherichia coli
EDTA:	Ethylenediaminetetraacetic acid
GAL4:	Yeast transcription factor of the galactose pathway
GAL4-AD:	GAL4-Activation Domain
GAL4-DBD:	GAL4-DNA Binding Domain
HMM:	Hidden Markov Models
HRP:	Horse radish peroxidase
IPTG:	Isopropyl-β-D-thiogalactopyranoside
KDa:	Kilo Dalton
kb:	Kilo base
MAT:	Mating type
µl:	microliter
µM:	micromolar
M:	Molar
mM:	milimolar
ORF:	open reading frame
PCR:	Polymerase chain reaction
PI:	Phosphoinositides
Pr A:	Protein A
PtdIns:	Phosphatidylinositol
RT:	Room Temperature
SDS:	Sodium dodecyl sulfate
SNARE:	soluble N-ethyl-maleimide-sensitive fusion protein attachment protein receptor

SNX: Sorting Nexins

TGN: Trans-Golgi Network

YTH: Yeast two-hybrid

References

(1995). "Isolation of a novel gene underlying Batten disease, CLN3. The International Batten Disease Consortium." Cell **82**(6): 949-57.

Abdul-Ghani, M., et al. (2001). "PRA isoforms are targeted to distinct membrane compartments." J Biol Chem **276**(9): 6225-33.

Ago, T., et al. (2001). "The PX domain as a novel phosphoinositide- binding module." Biochem Biophys Res Commun **287**(3): 733-8.

Ashburner, M., et al. (2000). "Gene ontology: tool for the unification of biology. The Gene Ontology Consortium." Nat Genet **25**(1): 25-9.

Balla, T. and P. Varnai (2002). "Visualizing cellular phosphoinositide pools with GFP-fused protein-modules." Sci STKE **2002**(125): PL3.

Barrowman, J. and P. Novick (2003). "Three Yips for Rab recruitment." Nat Cell Biol **5**(11): 955-6.

Barrowman, J., et al. (2003). "The Yip1p.Yif1p complex is required for the fusion competence of endoplasmic reticulum-derived vesicles." J Biol Chem **278**(22): 19878-84.

Bateman, A., et al. (2004). "The Pfam protein families database." Nucleic Acids Res **32 Database issue**: D138-41.

Berridge, M. J. (1981). "Phosphatidylinositol hydrolysis: a multifunctional transducing mechanism." Mol Cell Endocrinol **24**(2): 115-40.

Bier, E., et al. (1990). "rhomboid, a gene required for dorsoventral axis establishment and peripheral nervous system development in Drosophila melanogaster." Genes Dev **4**(2): 190-203.

Bluthner, M., et al. (2000). "Identification of an alpha-helical epitope region on the PM/Scl-100 autoantigen with structural homology to a region on the heterochromatin p25beta autoantigen using immobilized overlapping synthetic peptides." J Mol Med **78**(1): 47-54.

Boeddinghaus, C., et al. (2002). "A cycle of Vam7p release from and PtdIns 3-P-dependent rebinding to the yeast vacuole is required for homotypic vacuole fusion." J Cell Biol **157**(1): 79-89.

Bonangelino, C. J., et al. (2002). "Genomic screen for vacuolar protein sorting genes in Saccharomyces cerevisiae." Mol Biol Cell **13**(7): 2486-501.

Bravo, J., et al. (2001). "The crystal structure of the PX domain from p40(phox) bound to phosphatidylinositol 3-phosphate." Mol Cell **8**(4): 829-39.

Brun, C., et al. (2003). "Functional classification of proteins for the prediction of cellular function from a protein-protein interaction network." Genome Biol **5**(1): R6.

Burda, P., et al. (2002). "Retromer function in endosome-to-Golgi retrograde transport is regulated by the yeast Vps34 PtdIns 3-kinase." J Cell Sci **115**(Pt 20): 3889-900.

Cagney, G., et al. (2000). "High-throughput screening for protein-protein interactions using two-hybrid assay." Methods Enzymol **328**: 3-14.

Calero, M., et al. (2002). "Identification of the novel proteins Yip4p and Yip5p as Rab GTPase interacting factors." FEBS Lett **515**(1-3): 89-98.

Carlton, J., et al. (2004). "Sorting nexin-1 mediates tubular endosome-to-TGN transport through coincidence sensing of high- curvature membranes and 3-phosphoinositides." Curr Biol **14**(20): 1791-800.

Chattopadhyay, S. and D. A. Pearce (2002). "Interaction with Btn2p is required for localization of Rsglp: Btn2p-mediated changes in arginine uptake in Saccharomyces cerevisiae." Eukaryot Cell **1**(4): 606-12.

Chattopadhyay, S., et al. (2003). "The yeast model for Batten disease: a role for Btn2p in the trafficking of the Golgi-associated vesicular targeting protein, Yif1p." Biochem Biophys Res Commun **302**(3): 534-8.

Cheever, M. L., et al. (2001). "Phox domain interaction with PtdIns(3)P targets the Vam7 t-SNARE to vacuole membranes." Nat Cell Biol **3**(7): 613-8.

Cozier, G. E., et al. (2004). "Membrane targeting by pleckstrin homology domains." Curr Top Microbiol Immunol **282**: 49-88.

De Matteis, M., et al. (2002). "Phosphoinositides and the golgi complex." Curr Opin Cell Biol **14**(4): 434-47.

DiNitto, J. P., et al. (2003). "Membrane recognition and targeting by lipid-binding domains." Sci STKE **2003**(213): re16.

Dove, S. K., et al. (2004). "Svp1p defines a family of phosphatidylinositol 3,5-bisphosphate effectors." Embo J **23**(9): 1922-33.

Dwight, S. S., et al. (2004). "Saccharomyces genome database: underlying principles and organisation." Brief Bioinform **5**(1): 9-22.

Eitzen, G., et al. (2002). "Remodeling of organelle-bound actin is required for yeast vacuole fusion." J Cell Biol **158**(4): 669-79.

Ekena, K. and T. H. Stevens (1995). "The Saccharomyces cerevisiae MVP1 gene interacts with VPS1 and is required for vacuolar protein sorting." Mol Cell Biol **15**(3): 1671-8.

Fields, S. and O. Song (1989). "A novel genetic system to detect protein-protein interactions." Nature **340**(6230): 245-6.

Frank, R. (1992). "SPOT synthesis: an easy technique for the positionally addressble, parallel chemical synthesis on a membrane support." Tetrahedron **48**(42): 9217-9232.

Gavin, A. C., et al. (2002). "Functional organization of the yeast proteome by systematic analysis of protein complexes." Nature **415**(6868): 141-7.

Geysen, H. M., et al. (1984). "Use of peptide synthesis to probe viral antigens for epitopes to a resolution of a single amino acid." Proc Natl Acad Sci U S A **81**(13): 3998-4002.

Ghaemmaghami, S., et al. (2003). "Global analysis of protein expression in yeast." Nature **425**(6959): 737-41.

Giaever, G., et al. (2002). "Functional profiling of the Saccharomyces cerevisiae genome." Nature **418**(6896): 387-91.

Givan, S. A. and G. F. Sprague, Jr. (1997). "The ankyrin repeat-containing protein Akr1p is required for the endocytosis of yeast pheromone receptors." Mol Biol Cell **8**(7): 1317-27.

Godi, A., et al. (2004). "FAPPs control Golgi-to-cell-surface membrane traffic by binding to ARF and PtdIns(4)P." Nat Cell Biol **6**(5): 393-404.

Goffeau, A., et al. (1996). "Life with 6000 genes." Science **274**(5287): 546, 563-7.

Grigoriev, A. (2004). "Understanding the yeast proteome: a bioinformatics perspective." Expert Review of Proteomics **1**(2): 193-205.

Gruenberg, J. (2003). "Lipids in endocytic membrane transport and sorting." Curr Opin Cell Biol **15**(4): 382-8.

Habeler, G., et al. (2002). "YPL.db: the Yeast Protein Localization database." Nucleic Acids Res **30**(1): 80-3.

Habermann, B. (2004). "The BAR-domain family of proteins: a case of bending and binding?" EMBO Rep **5**(3): 250-5.

Hazbun, T. R., et al. (2003). "Assigning function to yeast proteins by integration of technologies." Mol Cell **12**(6): 1353-65.

Heidtman, M., et al. (2003). "A role for Yip1p in COPII vesicle biogenesis." J Cell Biol **163**(1): 57-69.
Hettema, E. H., et al. (2003). "Retromer and the sorting nexins Snx4/41/42 mediate distinct retrieval pathways from yeast endosomes." Embo J **22**(3): 548-57.
Hiroaki, H., et al. (2001). "Solution structure of the PX domain, a target of the SH3 domain." Nat Struct Biol **8**(6): 526-30.
Hishigaki, H., et al. (2001). "Assessment of prediction accuracy of protein function from protein--protein interaction data." Yeast **18**(6): 523-31.
Ho, Y., et al. (2002). "Systematic identification of protein complexes in Saccharomyces cerevisiae by mass spectrometry." Nature **415**(6868): 180-3.
Hudson, J. R., Jr., et al. (1997). "The complete set of predicted genes from Saccharomyces cerevisiae in a readily usable form." Genome Res **7**(12): 1169-73.
Hughes, T. R., et al. (2000). "Functional discovery via a compendium of expression profiles." Cell **102**(1): 109-26.
Huh, W. K., et al. (2003). "Global analysis of protein localization in budding yeast." Nature **425**(6959): 686-91.
Hurley, J. H. and T. Meyer (2001). "Subcellular targeting by membrane lipids." Curr Opin Cell Biol **13**(2): 146-52.
Ito, H., et al. (1983). "Transformation of intact yeast cells treated with alkali cations." J Bacteriol **153**(1): 163-8.
Ito, T., et al. (2001). "A comprehensive two-hybrid analysis to explore the yeast protein interactome." Proc Natl Acad Sci U S A **98**(8): 4569-74.
Itoh, T. and T. Takenawa (2002). "Phosphoinositide-binding domains: Functional units for temporal and spatial regulation of intracellular signalling." Cell Signal **14**(9): 733-43.
James, P., et al. (1996). "Genomic libraries and a host strain designed for highly efficient two-hybrid selection in yeast." Genetics **144**(4): 1425-36.
Jones, D. E. and N. Divecha (2004). "Linking lipids to chromatin." Curr Opin Gen Dev **14**: 1-7.
Jones, S. and J. M. Thornton (1996). "Principles of protein-protein interactions." Proc Natl Acad Sci U S A **93**(1): 13-20.
Kanai, F., et al. (2001). "The PX domains of p47phox and p40phox bind to lipid products of PI(3)K." Nat Cell Biol **3**(7): 675-8.
Kanemaki, M., et al. (2003). "Functional proteomic identification of DNA replication proteins by induced proteolysis in vivo." Nature **423**(6941): 720-4.
Karathanassis, D., et al. (2002). "Binding of the PX domain of p47(phox) to phosphatidylinositol 3,4-bisphosphate and phosphatidic acid is masked by an intramolecular interaction." Embo J **21**(19): 5057-68.
Khalfan, W. A. and D. J. Klionsky (2002). "Molecular machinery required for autophagy and the cytoplasm to vacuole targeting (Cvt) pathway in S. cerevisiae." Curr Opin Cell Biol **14**(4): 468-75.
Kumar, A., et al. (2002). "Subcellular localization of the yeast proteome." Genes Dev **16**(6): 707-19.
Lakshmanan, J., et al. (2003). "Repression of transcription by Rgt1 in the absence of glucose requires Std1 and Mth1." Curr Genet **44**(1): 19-25.
Landgraf, C., et al. (2004). "Protein interaction networks by proteome Peptide scanning." PLoS Biol **2**(1): E14.

Lawe, D. C., et al. (2000). "The FYVE domain of early endosome antigen 1 is required for both phosphatidylinositol 3-phosphate and Rab5 binding. Critical role of this dual interaction for endosomal localization." J Biol Chem **275**(5): 3699-705.

Lemmon, M. A. (2003). "Phosphoinositide recognition domains." Traffic **4**(4): 201-13.

Leto, T. L., et al. (1994). "Assembly of the phagocyte NADPH oxidase: binding of Src homology 3 domains to proline-rich targets." Proc Natl Acad Sci U S A **91**(22): 10650-4.

Letunic, I., et al. (2004). "SMART 4.0: towards genomic data integration." Nucleic Acids Res **32 Database issue**: D142-4.

Levine, T. P. and S. Munro (1998). "The pleckstrin homology domain of oxysterol-binding protein recognises a determinant specific to Golgi membranes." Curr Biol **8**(13): 729-39.

Levine, T. P. and S. Munro (2001). "Dual targeting of Osh1p, a yeast homologue of oxysterol-binding protein, to both the Golgi and the nucleus-vacuole junction." Mol Biol Cell **12**(6): 1633-44.

Levine, T. P. and S. Munro (2002). "Targeting of Golgi-specific pleckstrin homology domains involves both PtdIns 4-kinase-dependent and -independent components." Curr Biol **12**(9): 695-704.

Liu, Z., et al. (1999). "Fine mapping of the antigen-antibody interaction of scFv215, a recombinant antibody inhibiting RNA polymerase II from Drosophila melanogaster." J Mol Recognit **12**(2): 103-11.

Lobo, S., et al. (2002). "Identification of a Ras palmitoyltransferase in Saccharomyces cerevisiae." J Biol Chem **277**(43): 41268-73.

Lu, J., et al. (2002). "Solution structure of the Vam7p PX domain." Biochemistry **41**(19): 5956-62.

Ma, H., et al. (1987). "Plasmid construction by homologous recombination in yeast." Gene **58**(2-3): 201-16.

Matern, H., et al. (2000). "A novel Golgi membrane protein is part of a GTPase-binding protein complex involved in vesicle targeting." Embo J **19**(17): 4485-92.

McConnell, S. J. and M. P. Yaffe (1992). "Nuclear and mitochondrial inheritance in yeast depends on novel cytoplasmic structures defined by the MDM1 protein." J Cell Biol **118**(2): 385-95.

McQuibban, G. A., et al. (2003). "Mitochondrial membrane remodelling regulated by a conserved rhomboid protease." Nature **423**(6939): 537-41.

Merrifield, R. B. (1963). J. Am. Chem Soc **85**: 2149-2154.

Nice, D. C., et al. (2002). "Cooperative binding of the cytoplasm to vacuole targeting pathway proteins, Cvt13 and Cvt20, to phosphatidylinositol 3-phosphate at the pre-autophagosomal structure is required for selective autophagy." J Biol Chem **277**(33): 30198-207.

Nothwehr, S. F., et al. (1999). "Distinct domains within Vps35p mediate the retrieval of two different cargo proteins from the yeast prevacuolar/endosomal compartment." Mol Biol Cell **10**(4): 875-90.

Nothwehr, S. F., et al. (1996). "The newly identified yeast GRD genes are required for retention of late-Golgi membrane proteins." Mol Cell Biol **16**(6): 2700-7.

Nothwehr, S. F., et al. (2000). "Sorting of yeast membrane proteins into an endosome-to-Golgi pathway involves direct interaction of their cytosolic domains with Vps35p." J Cell Biol **151**(2): 297-310.

Notredame, C., et al. (2000). "T-Coffee: A novel method for fast and accurate multiple sequence alignment." J Mol Biol **302**(1): 205-17.

Odorizzi, G., et al. (2000). "Phosphoinositide signaling and the regulation of membrane trafficking in yeast." Trends Biochem Sci **25**(5): 229-35.

Oliver, S. (1996). "A network approach to the systematic analysis of yeast gene function." Trends Genet **12**(7): 241-2.

Otte, L., et al. (2003). "WW domain sequence activity relationships identified using ligand recognition propensities of 42 WW domains." Protein Sci **12**(3): 491-500.

Pawson, T. and P. Nash (2003). "Assembly of cell regulatory systems through protein interaction domains." Science **300**(5618): 445-52.

Pearce, D. A., et al. (1999). "Action of BTN1, the yeast orthologue of the gene mutated in Batten disease." Nat Genet **22**(1): 55-8.

Pearce, D. A. and F. Sherman (1997). "BTN1, a yeast gene corresponding to the human gene responsible for Batten's disease, is not essential for viability, mitochondrial function, or degradation of mitochondrial ATP synthase." Yeast **13**(8): 691-7.

Pfeffer, S. and D. Aivazian (2004). "Targeting Rab GTPases to distinct membrane compartments." Nat Rev Mol Cell Biol **5**(11): 886-96.

Ponting, C. P. (1996). "Novel domains in NADPH oxidase subunits, sorting nexins, and PtdIns 3-kinases: binding partners of SH3 domains?" Protein Sci **5**(11): 2353-7.

Putilina, T., et al. (1999). "The DHHC domain: a new highly conserved cysteine-rich motif." Mol Cell Biochem **195**(1-2): 219-26.

Rameh, L. E., et al. (1997). "A new pathway for synthesis of phosphatidylinositol-4,5-bisphosphate." Nature **390**(6656): 192-6.

Reddy, J. V. and M. N. Seaman (2001). "Vps26p, a component of retromer, directs the interactions of Vps35p in endosome-to-Golgi retrieval." Mol Biol Cell **12**(10): 3242-56.

Reineke, U., et al. (1999). "Antigen sequence- and library-based mapping of linear and discontinuous protein-protein-interaction sites by spot synthesis." Curr Top Microbiol Immunol **243**: 23-36.

Rigaut, G., et al. (1999). "A generic protein purification method for protein complex characterization and proteome exploration." Nat Biotechnol **17**(10): 1030-2.

Ron, D. and H. Dressler (1992). "pGSTag--a versatile bacterial expression plasmid for enzymatic labeling of recombinant proteins." Biotechniques **13**(6): 866-9.

Ross-Macdonald, P., et al. (1999). "Large-scale analysis of the yeast genome by transposon tagging and gene disruption." Nature **402**(6760): 413-8.

Rubin, G. M., et al. (2000). "Comparative genomics of the eukaryotes." Science **287**(5461): 2204-15.

Samanta, M. P. and S. Liang (2003). "Predicting protein functions from redundancies in large-scale protein interaction networks." Proc Natl Acad Sci U S A **100**(22): 12579-83.

Sanchez-Diaz, A., et al. (2004). "Rapid depletion of budding yeast proteins by fusion to a heat-inducible degron." Sci STKE **2004**(223): PL8.

Sato, T. K., et al. (1998). "Vam7p, a SNAP-25-like molecule, and Vam3p, a syntaxin homolog, function together in yeast vacuolar protein trafficking." Mol Cell Biol **18**(9): 5308-19.

Sayle, R. A. and E. J. Milner-White (1995). "RASMOL: biomolecular graphics for all." Trends Biochem Sci **20**(9): 374.

Schmidt, M. C., et al. (1999). "Std1 and Mth1 proteins interact with the glucose sensors to control glucose-regulated gene expression in Saccharomyces cerevisiae." Mol Cell Biol **19**: 4561-4571.

Sciorra, V. A., et al. (1999). "Identification of a phosphoinositide binding motif that mediates activation of mammalian and yeast phospholipase D isoenzymes." Embo J **18**(21): 5911-21.

Seaman, M. N., et al. (1998). "A membrane coat complex essential for endosome-to-Golgi retrograde transport in yeast." J Cell Biol **142**(3): 665-81.

Seaman, M. N. and H. P. Williams (2002). "Identification of the functional domains of yeast sorting nexins Vps5p and Vps17p." Mol Biol Cell **13**(8): 2826-40.

Segev, N. (2001). "Ypt and Rab GTPases: insight into functions through novel interactions." Curr Opin Cell Biol **13**(4): 500-11.

Siddiqi, I. N., et al. (2001). "Transcription of chromosomal rRNA genes by both RNA polymerase I and II in yeast uaf30 mutants lacking the 30 kDa subunit of transcription factor UAF." Embo J **20**(16): 4512-21.

Simonsen, A., et al. (2001). "The role of phosphoinositides in membrane transport." Curr Opin Cell Biol **13**(4): 485-92.

Singer, B. and H. Riezman (1990). "Detection of an intermediate compartment involved in transport of alpha-factor from the plasma membrane to the vacuole in yeast." J Cell Biol **110**(6): 1911-22.

Sivars, U., et al. (2003). "Yip3 catalyses the dissociation of endosomal Rab-GDI complexes." Nature **425**(6960): 856-9.

Song, X., et al. (2001). "Phox homology domains specifically bind phosphatidylinositol phosphates." Biochemistry **40**(30): 8940-4.

Sprong, H., et al. (2001). "How proteins move lipids and lipids move proteins." Nat Rev Mol Cell Biol **2**(7): 504-13.

Sumimoto, H., et al. (1994). "Role of Src homology 3 domains in assembly and activation of the phagocyte NADPH oxidase." Proc Natl Acad Sci U S A **91**(12): 5345-9.

Toker, A. (2002). "Phosphoinositides and signal transduction." Cell Mol Life Sci **59**(5): 761-79.

Tong, A. H., et al. (2001). "Systematic genetic analysis with ordered arrays of yeast deletion mutants." Science **294**(5550): 2364-8.

Tong, A. T., et al. (2004). "Transesophageal echocardiography improves risk assessment of thrombolysis of prosthetic valve thrombosis: results of the international PRO-TEE registry." J Am Coll Cardiol **43**(1): 77-84.

Uetz, P., et al. (2000). "A comprehensive analysis of protein-protein interactions in Saccharomyces cerevisiae." Nature **403**(6770): 623-7.

Urban, S., et al. (2001). "Drosophila rhomboid-1 defines a family of putative intramembrane serine proteases." Cell **107**(2): 173-82.

Vanhaesebroeck, B., et al. (2001). "Synthesis and function of 3-phosphorylated inositol lipids." Annu Rev Biochem **70**: 535-602.

Vollert, C. S. and P. Uetz (2004). "The Phox Homology (PX) Domain Protein Interaction Network in Yeast." Mol Cell Proteomics **3**(11): 1053-64.

Vollert, C. S. and P. Uetz (in press). "The Two-Hybrid System." Encyclopedic Reference of Genomics and Proteomics in Molecular Medicine, Springer Verlag.

Voos, W. and T. H. Stevens (1998). "Retrieval of resident late-Golgi membrane proteins from the prevacuolar compartment of Saccharomyces cerevisiae is dependent on the function of Grd19p." J Cell Biol **140**(3): 577-90.

Wach, A., et al. (1994). "New heterologous modules for classical or PCR-based gene disruptions in Saccharomyces cerevisiae." Yeast **10**(13): 1793-808.

Winzeler, E. A., et al. (1999). "Functional characterization of the S. cerevisiae genome by gene deletion and parallel analysis." Science **285**(5429): 901-6.

Wishart, M. J., et al. (2001). "Phoxy lipids: revealing PX domains as phosphoinositide binding modules." Cell **105**(7): 817-20.

Worby, C. A. and J. E. Dixon (2002). "Sorting out the cellular functions of sorting nexins." Nat Rev Mol Cell Biol **3**(12): 919-31.

Xing, Y., et al. (2004). "Structural basis of membrane targeting by the Phox homology domain of cytokine-independent survival kinase (CISK-PX)." J Biol Chem **279**(29): 30662-9.

Xu, Y., et al. (2001). "SNX3 regulates endosomal function through its PX-domain-mediated interaction with PtdIns(3)P." Nat Cell Biol **3**(7): 658-66.

Xu, Y., et al. (2001). "The Phox homology (PX) domain, a new player in phosphoinositide signalling." Biochem J **360**(Pt 3): 513-30.

Yang, X., et al. (1998). "Specific binding to a novel and essential Golgi membrane protein (Yip1p) functionally links the transport GTPases Ypt1p and Ypt31p." Embo J **17**(17): 4954-63.

Yu, J. W. and M. A. Lemmon (2001). "All phox homology (PX) domains from Saccharomyces cerevisiae specifically recognize phosphatidylinositol 3-phosphate." J Biol Chem **276**(47): 44179-84.

Zewail, A., et al. (2003). "Novel functions of the phosphatidylinositol metabolic pathway discovered by a chemical genomics screen with wortmannin." Proc Natl Acad Sci U S A **100**(6): 3345-50.

Zhou, C. Z., et al. (2003). "Crystal structure of the yeast Phox homology (PX) domain protein Grd19p complexed to phosphatidylinositol-3-phosphate." J Biol Chem **278**(50): 50371-6.

Zhu, H., et al. (2001). "Global analysis of protein activities using proteome chips." Science **293**(5537): 2101-5.

Summary

The phox homology (PX) domain is a phosphoinositide-binding domain that is conserved from yeast to man. In this work I show for the first time by yeast genome-wide two-hybrid screens and *in vitro* binding assays that, the PX domain is a bona fide protein interaction domain and that it forms a complex network of interactions in yeast.

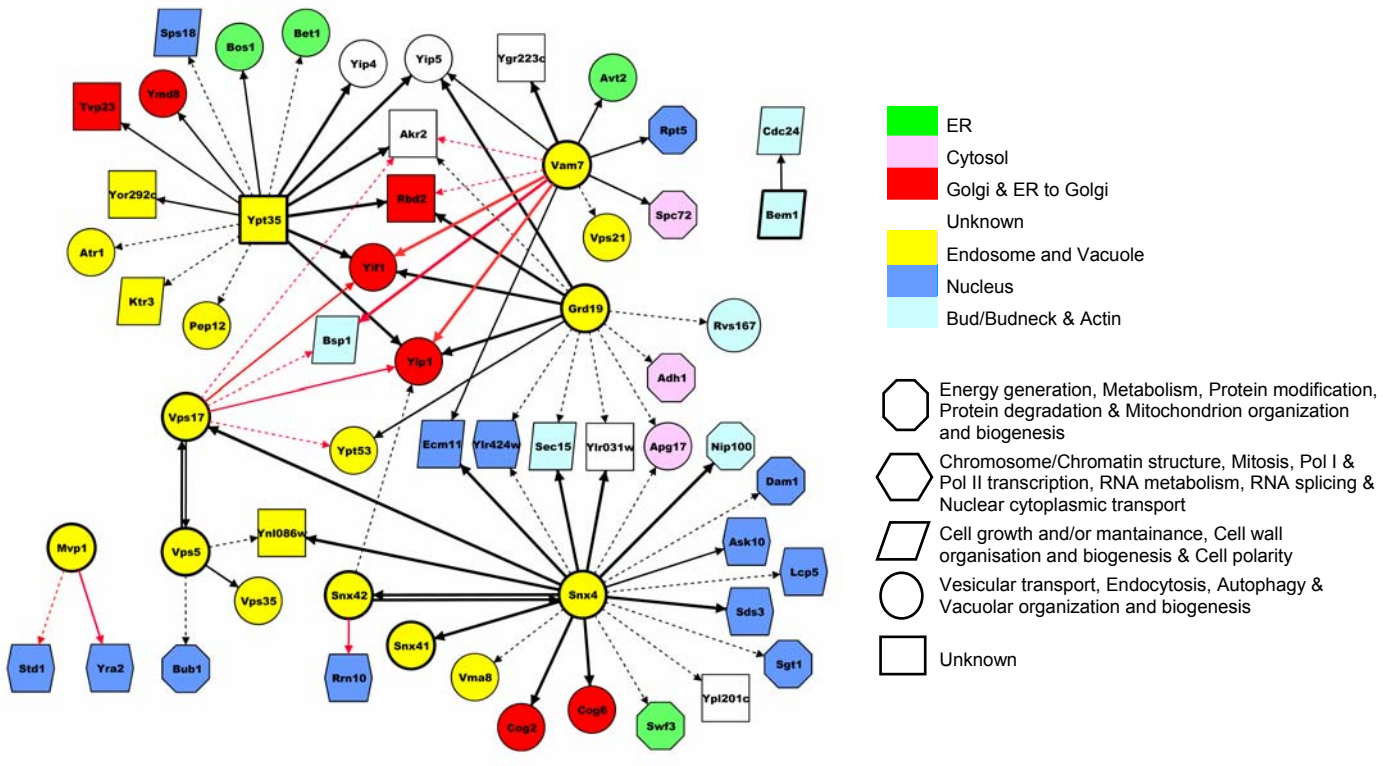
The yeast PX domain-only proteins Grd19p (YOR357C) and Ypt35p (YHR105W), as well as the isolated PX domains from Mvp1p (YMR004W), Snx42p/Cvt20p/Atg20p (YDL113C), Vam7p (YGL212W), and Vps17p (YOR132W) yielded a total of 40 reproducible two-hybrid interactions. 35 interactions were found for the full-length proteins of Bem1p (YBR200W), Snx42p, Snx4p/Cvt13p (YJL036W), Vam7p, Vps5p (YOR069W), and Vps17p, but these appear not to require the PX domain, since these interactions could not be reproduced with their isolated PX domains.

Interactions of Grd19p, Vam7p, Vps5p, Vps17p, and Ypt35p with members of the Yip1p family of proteins were detected consistently and were verified by *in vitro* binding assays. The N-terminal cytoplasmic domain of Yip1p and Yif1p mediates these interactions with PX domains although C-terminal cytosolic loops seem to contribute to the interactions.

Peptide array results demonstrated that PX-domains bind to Yip1 family peptides which contain basic residues at their C-terminus. It was not the case for peptides interacting with PX domains of non-Yip1 family interactors.

Alanine scans further identified lysines which are essential for Yip-PX protein interactions. Compared to the yeast proteome, Yip1 family proteins contain relatively few lysines of which most are embedded in a candidate consensus motif.

A mutation in the lipid-binding pocket of Ypt35-PX that reduces lipid-binding markedly does not affect these PX domain protein interactions, arguing that lipid binding uses a different interaction surface than protein binding.



The PX domain interaction network in yeast

PX-domain containing proteins (*thick border*) and isolated PX-domains (red arrows) in yeast.
Thickness of the arrows represents the reproducibility of the interactions (i.e. scores 1-3 in Table 6)

Zusammenfassung

Die PX Domäne ist eine Phosphatidylinositol-bindende Domäne, die von der Hefe bis zum Menschen konserviert wurde. In dieser Arbeit zeige ich mittels systematischen two-hybrid Screens, die das gesamte Hefegenom umfassen, und *in vitro* Bindungsanalysen, dass die PX-Domäne eine neuartige Protein-Interaktionsdomäne ist und ein komplexes Interaktions-Netzwerk in Hefe bildet.

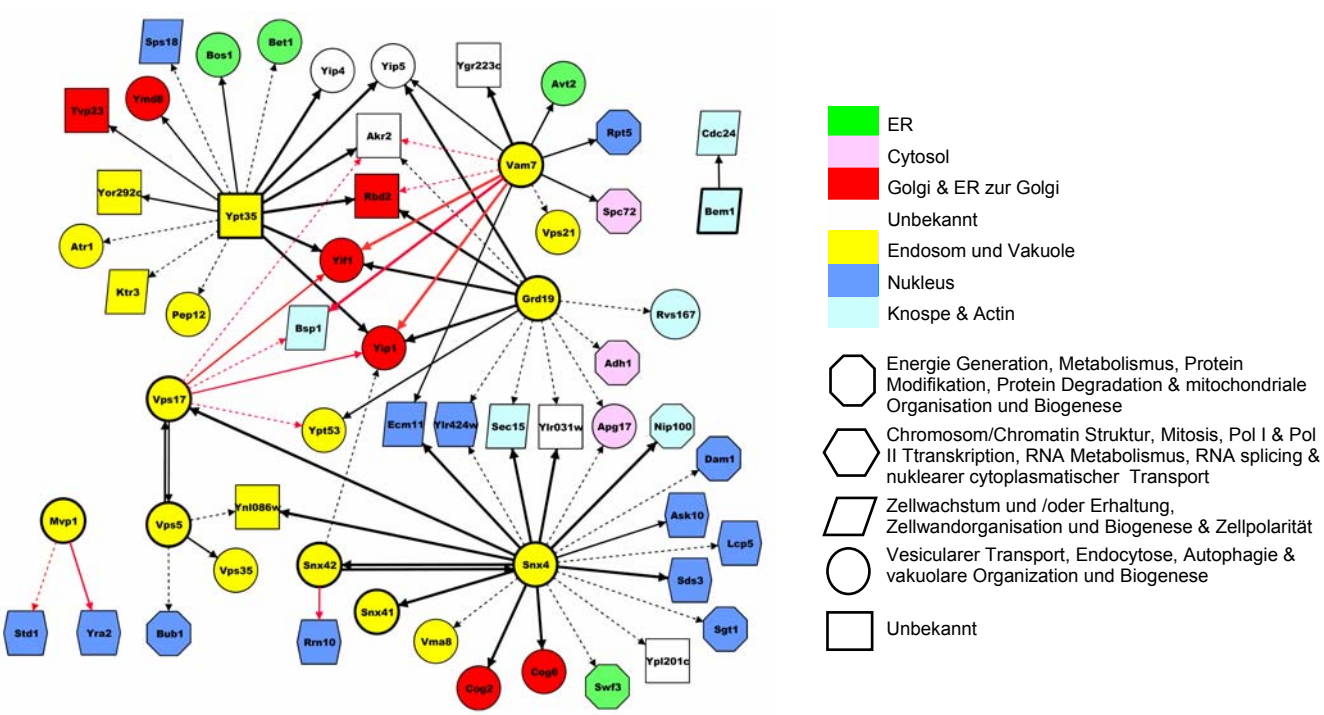
Die hauptsächlich aus der PX-Domäne bestehenden Proteine Grd19p (YOR357C) und Ypt35p (YHR105W), und die isolierten PX Domänen von Mvp1p (YMR004W), Snx42p/Cvt20p/Atg20p (YDL113C), Vam7p (YGL212W), und Vps17p (YOR132W) ergaben insgesamt 40 reproduzierbare two-hybrid-Interaktionen. 35 Interaktionen wurden für die Gesamtproteine von Bem1p (YBR200W), Snx42p, Snx4p/Cvt13p (YJL036W), Vam7p, Vps5p (YOR069W), und Vps17p gefunden, aber diese scheinen die PX-Domäne nicht zu benötigen, da diese mit den entsprechenden PX-Domänen, nicht reproduziert werden konnten.

Interaktionen zwischen Grd19p, Vam7p, Vps5p, Vps17p, and Ypt35p und Mitgliedern der Yip1 Proteinfamilie wurden mehrfach detektiert und über *in vitro* Bindeversuche verifiziert. Die N-terminale cytoplasmatische Domäne von Yip1p und Yif1p vermittelt diese Interaktionen mit der PX Domäne, obwohl C-terminale, cytosolische Schleifen zu den Interaktionen beizutragen scheinen.

Peptid-array-Ergebnisse zeigten bei Yip1-Proteinen, die eine Affinität zur PX-Domäne aufwiesen, dass diese Affinität mit basischen Aminosäuren am C-Terminus einherging. Bei entsprechenden Versuchen mit Proteinen, die nicht zur Yip1-Familie gehören, konnte eine solche Korrelation nicht gezeigt werden.

Alanin-Scans identifizierten Lysine, die unabdingbar für Yip-PX-Protein-Interkationen sind. Verglichen zum Hefe-Proteom, enthalten Yip-Proteine einen relativ niedrigen Gehalt an Lysinen, von denen die meisten in dem von mir identifiziertem Motiv auftreten.

Eine Mutation in der Lipid-bindenden-Tasche von Ypt35-PX, die die Lipid-Bindefähigkeit signifikant reduziert, beeinflusst diese Interaktion mit der PX-Domäne nicht. Dies legt nahe, dass die Lipid-Bindung eine andere Interaktionsstelle benötigt als die Proteinbindung.



Das PX-Domänen Interaktions-Netzwerk in der Hefe

PX-Domäne-haltige Proteine (*dick umranden*) und isolierte PX-Domäne (rote Pfeile) in Hefe. Die Dicke der Pfeile stellt die Reproduzierbarkeit der Interaktionen dar (d.h. scores 1-3 in Tabelle 6).

Acknowledgements

I would like to thank to:

Peter Uetz for his supervision, constant support, scientific freedom and good ideas, although some times too many!!!

Nils Johnsson for useful scientific discussions and taking over the role of evaluator.

Adam Bertl for useful comments on my work and also taking over the evaluation.

Tanja Kuhn especially for her technical assistance throughout my 3 years, for her constant support and pleasant philosophic talks in the morning.

Claudia Ester for her calm personality which encouraged me to continue also through hard times. I also thank her for establishing the peptide array method which I could exhaustly use.

Raja Gopala for his nice smile, his always good mood, and for taking stuff in/out on weekends.

Bjorn Titz for being a hard motivated worker that was particularly helpful at the end of my work when the batteries are low. He was also great help for the bioinformatical stuff.

Sindhu Thomas came “recently” to our lab and I thank her for making some media for my studies.

Wolf Thies for making phylogenetical studies on PX domains (that unfortunately did not work out) and for all type of informatics help (converting yeast genome data systematically from swissprot numbers to the official nomenclature, and so on). Thanks to his helpful kind and calm way, it was always a pleasure to work with him.

During my years some students, diploma-candidates and technicians performed sub-projects of my work, thanks to them: **Sebastian Heucke**, **Marco Wendel**, **René Günther** and **Thomas Dürr**.

Xian Wang who helped me with yeast-specific assays.

Christopher Melcher for helping me with the confocal microscope.

Massimilano Mazza and **Michael Knop** at the EMBL that allowed me to perform there my Lipid overlay assays.

Jong W.Yu who provided me with his pGSTag PX domain vectors and **Elizabeth Conibear** provided me with some bait constructs.

Birgit Singer-Krüger for her cooperation with the Vam7p-Bsp1p interaction.

Xiaoping Yang who came at the right time to talk to our institute and her tips rescued my work.

Last but not least I would like to thank **my parents** for supporting me throughout my whole carrier and **Alex** for being my cable to earth.

Curriculum Vitae

CAROLINA S. MÜLLER

PERSONAL DATA

Name	Carolina S. Müller geb. Vollert
Date of birth	26.12.1974, Buenos Aires, Argentine
Nationality	German/Argentine
Address	Bongartstraße 26 71131 Jettingen e-Mail: carolina.mueller@c-grit.de

SCHOOL

1988 - 1992	St. Andrew´s Scots School, Buenos Aires, Argentina

UNIVERSITY

1994 - 1996	Studies in Biology at the University of Buenos Aires, Argentina
1996 - 1998	Biology “Vordiplom” (bachelors degree equivalent) at the Johannes-Gutenberg University, Mainz, Germany
1998 - 2001	Biology “Diplom” (masters degree equivalent) at the University of Hohenheim, Stuttgart, Germany
	Diploma Thesis: at the Institute for Plant Physiology and Biotechnology, University of Hohenheim, Germany
	Supervisor: Prof. Dr. D. Hess
	Subject: Analysis of inducible promoters in sunflower and tobacco

PHD STUDIES

2002 - 2005	PhD at the Institute for Genetics and Toxicology, Forschungszentrum Karlsruhe, Germany
	Supervisor: Dr. P. Uetz
	Subject: The PX domain protein interaction network in yeast