

The identification of novel interactions of the vesicle tethering complex TRAPP

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A Thesis

in

The Department

of

Biology

Presented in Partial Fulfillment of the Requirements

for the Degree of Master of Science (Biology) at

Concordia University

Montreal, Quebec, Canada

March 2010

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ISBN: 978-0-494-67113-9
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ISBN: 978-0-494-67113-9

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Abstract

The identification of novel interactions of the vesicle tethering complex TRAPP

Audrey Morin

Membrane trafficking is an essential cellular process where tethering factors offer the first level of specificity for the proper localization of vesicles to target membranes. The localization of the tethers themselves may depend on their associations with a unique subset of lipids or activated GTPases. The TRAPP vesicle tethering complex localizes to the Golgi in an unknown manner but its localization may be mediated by the Bet3 protein. To gain insight into the mechanism of localization of TRAPP a synthetic lethal yeast screen with the *bet3-4* mutant was employed. The screen is based on a plasmid-dependent assay which exploits the adenine biosynthesis pathway and red/white colony sectoring. A *Saccharomyces cerevisiae bet3-4 ade2 ade3* mutant strain with a counter-selectable plasmid harbouring the *BET3* and *ADE3* genes was randomly mutated with ethyl methanesulfonate (EMS). Double mutants, identified as uniformly red colonies, were obtained and initially screened for the presence of a mutation in either *BET3* or another gene encoding a TRAPP subunit. Mutants that were judged to be in non-TRAPP genes were then screened with a yeast library to try to identify the mutated gene. One synthetic lethal mutant was suppressed by *VPS16*, a gene encoding a component of another vesicle tethering complex. Since *VPS16* does not suppress *bet3-4* and since sequencing failed to reveal any mutations in *VPS16*, our results suggest that the defective gene in this particular mutant can be suppressed by *VPS16*. The identity of the mutated gene remains unknown at present.

Acknowledgments

I would like to show my gratitude to my supervisor, Dr. Michael Sacher, whose guidance and continued encouragement throughout the years enabled me to develop my understanding of molecular biology. It is an honor for me to have worked with him and to be one of his first graduates. Thank you!

To my committee members, Dr. P. Joyce and Dr. V. Titorenko, I am grateful for your time, help and encouragement. You have guided this project from beginning to end and for this I thank you both.

Lastly, I offer my regards to my colleagues and lab members who supported me in any respect. The camaraderie I found while working with you has been a blessing.

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List of acronyms and abbreviations

5-FOA: 5-fluoroortic acid

AIR: 5-aminoimidazole ribonucleotide

Amp: ampicillin

CIP: Calf intestinal alkaline phosphatase

COG: conserved oligomeric Golgi

COPI: coat protein complex I

COPII: coat protein complex II

CORVET: class C core vacuole/endosomes tethering

C-terminal: carboxy terminal

dH₂O: distilled water

DNA: deoxyribonucleic acid

EB: elution buffer

EDTA: ethylenediaminetetra acetic acid

EMS: ethyl methanesulfonate

ER: endoplasmic reticulum

GAP: GTPase-activating protein

GARP: Golgi-associated retrograde protein

GDP: guanosine diphosphate

GEF: guanosine exchange factor

GTP: guanosine triphosphate

GTPase: guanosine-5'-triphosphatase

HA: hemmagglutinin

HOPS: homotypic fusion and vacuole protein sorting

LB: Luria-Bertani

N-terminal : amino-terminal

OD: optical density

ODCase: orotidine 5-phosphate decarboxylase

ORF: open reading frame

PCR: polymerase chain reaction

PEG: polyethylene glycol

S. cerevisiae: *Saccharomyces cerevisiae*

SDS: sodium dodecyl sulfate

SED1: spondyloepiphyseal dysplasia tarda

SNARE: soluble N-ethylmaleimide-sensitive factor attachment protein receptors

SPO: sporulation

TE buffer: Tris EDTA buffer

TRAPPI/II: transport protein particle I/II

Tris: Tris (hydroxymethyl) aminomethane

t-SNARE: target soluble N-ethylmaleimide-sensitive factor attachment protein receptors

v-SNARE: vesicle soluble N-ethylmaleimide-sensitive factor attachment protein
receptors

wt: wild type

YPD: yeast peptone dextrose

1. Introduction

1.1 Intracellular traffic in the early secretory pathway

In eukaryotic cells, proteins are transported from a donor organelle to a specific target organelle by transport vesicles in a process referred to as membrane traffic. Membrane trafficking is essential for cell viability and organelle functionality. The secretory pathway, a branch of membrane traffic that ultimately leads to the release of proteins from the cell, begins in the endoplasmic reticulum (ER) where newly synthesized proteins are packaged into coat protein complex II (COPII)-coated vesicles which bud off the ER (Schekman and Orci, 1996). COPII is composed of the small guanosine-5'-triphosphatase (GTPase) Sar1p (Nakano and Muramatsu, 1989) and two heterodimers, Sec23p/24p and Sec13p/31p (Barlowe *et al.*, 1994). Studies have shown that these components are the minimal requirements for the formation of vesicles from liposomes *in vitro* (Higashio *et al.*, 2008).

Activation of GTPases is an essential step in membrane trafficking. GTPases are present in one of two nucleotide-bound forms: guanosine diphosphate (GDP-) or guanosine triphosphate (GTP-) bound. The GDP-bound form is considered the “inactive” form while the GTP-bound form is considered the “active” form (Bourne *et al.*, 1990). Only the active GTP-bound form can transduce a signal. GTPase-activating proteins (GAPs) interact with GTP-bound GTPases and facilitate the hydrolysis of the GTP into GDP thus inactivating the GTPase. GDP-bound GTPases require accessory factors called

guanine nucleotide exchange factors (GEFs) to facilitate the release of GDP and allow GTP to bind (Bourne *et al.*, 1990).

Budding is triggered by the activation of Sar1p by its GEF Sec12p. Once in its active form, Sar1p-GTP recruits Sec23/24p and Sec13/31p which mediates membrane curvature and protein sorting (Behnia and Munro, 2005). Vesicle cargo selection is determined in part by the Sar1p-Sec23p/24p prebudding complexes (Aridor *et al.*, 1998; Kuehn *et al.*, 1998). Different sites on Sec24p recognize different protein motifs and allow for the packaging of specific cargo inside vesicles (Higashio *et al.*, 2008). Finally the bud is pinched off the ER and forms a COPII-coated transport vesicle (Barlowe *et al.*, 1994). Vesicle uncoating is mediated by the inactivation of Sar1p by its GAP Sec23p (Sato and Nakano, 2004; Yoshihisa *et al.*, 1993). The coats are recycled back to the ER while the vesicles are tethered, docked and fused with the target membrane, the Golgi (Bonifacino and Glick, 2004). Resident ER proteins that escaped are trafficked back to the ER by virtue of several characterized sequences at or near their carboxy-terminus (e.g. KKXX and KDEL) (Nilsson *et al.*, 1989; Pelham, 1989; Teasdale and Jackson, 1996). These proteins are transported back to the ER in association with COPI (coat protein complex I) coated vesicles (Letourneur *et al.*, 1994).

There are three levels of specificity which allow binding of vesicles with their target membrane(s). First, vesicle tethering factors and coiled-coil proteins tether vesicles to acceptor membranes. Second, small Rab GTPases and effector proteins regulate vesicle tethering and docking through their cycling between GDP- and GTP-

bound forms (Pfeffer, 2001). Thirdly, the binding of SNAREs (soluble N-ethylmaleimide-sensitive factor attachment protein receptors) on both the vesicle and target membranes mediate membrane fusion (Ungar and Hughson, 2003). Each of these levels of specificity will be discussed in the following sections.

1.2 The Golgi

The Golgi is a major site for protein sorting and processing (e.g. glycosylation) (Dean, 1999; Lowe and Marth, 2003; Ungar and Hughson, 2003). Processing enzymes are segregated based on their requirements for function and are concentrated in specific regions (*cis*, medial or *trans*) of the Golgi. The mechanism of transport of material through the various compartments of the Golgi is unclear and three models have been proposed: the forward-trafficking model, the cisternal-maturation model, and the rapid partitioning model (Jackson, 2009).

In the forward-trafficking model, cargo proteins are transported from the *cis*-Golgi to the *trans*-Golgi in COPI vesicles and undergo modifications in each compartment (Palade, 1975). The Golgi compartments are said to be static in this model because the resident proteins do not change. This model explains the forward transport of cargo from the ER to the Golgi in COPII vesicles but fails to explain experimental observations of the retrograde traffic of COPI vesicles (Lanoix *et al.*, 1999; Martinez-Menarguez *et al.*, 2001). In an attempt to better represent traffic of enzymes through the

Golgi the cisternal-maturation model was proposed. In this model, it is the processing enzymes themselves that are transported from the late to the early Golgi resulting in the maturation of early compartments into late compartments while the cargo remains static (Bonifacino and Glick, 2004; Elsner *et al.*, 2003; Morre D.J. and Mollenhauer, 2007). A more recent model, the rapid partitioning model, suggests that the trafficking of lipids is coupled to cargo and enzyme traffic (Patterson *et al.*, 2008). There are regions of the Golgi enriched in certain lipids creating a gradient of lipid composition through the Golgi and among the cisternae themselves. Cargo proteins are rapidly exposed to the different Golgi cisternae until they associate with their preferred lipid environments. It is noteworthy that, in this model, cargo proteins and processing enzymes can move bi-directionally (*cis-to-trans* and *trans-to-cis*) among the Golgi stacks (Pelham and Rothman, 2000; Patterson *et al.*, 2008). In all three models, once the cargo proteins have undergone the proper post-translational modifications they are transported to other intracellular locations such as the plasma membrane, secretory vesicles, or organelles of the endocytic pathway (Bard and Malhotra, 2006; McNiven and Thompson, 2006; Pelham and Rothman, 2000).

1.3 SNAREs

SNARE proteins have been shown to co-localize and directly interact with specific tethering complexes (Fridmann-Sirkis *et al.*, 2006; Price *et al.*, 2000; Suvorova *et al.*, 2002). Tethers function in bringing vesicles in close proximity to target membranes

establishing a loose interaction with the target membrane. Once tethered, vesicles establish a more stable interaction through the binding of compatible SNAREs in a process referred to as docking (Waters and Pfeffer, 1999). SNAREs allow for vesicle fusion with acceptor membranes and provide an additional layer of specificity (Jahn, 2008; Shorter *et al.*, 2002).

Fusion is mediated by the formation of a functional four-helix bundle, referred to as SNAREpins, composed of one α -helix from a vesicle-SNARE (v-SNARE) to three α -helices of a cognate target-SNARE (t-SNARE) on the target membrane (Fasshauer *et al.*, 1998; Sutton *et al.*, 1998; Antonin *et al.*, 2002). The four-helix bundle links the donor and acceptor membranes (Lupashin and Sztul, 2005) and provides the necessary energy to pull the donor and acceptor membranes together resulting in membrane fusion since no additional energy input is required (Li *et al.*, 2007). SNAREs are further classified into four subfamilies (Qa-, Qb-, Qc- and R-SNAREs) according to highly conserved glutamine or arginine residues at the center of the four-helix bundle (Fasshauer *et al.*, 1998). Q-SNAREs are usually found on target membranes while R-SNAREs are usually found on vesicles.

1.4 Vesicle tethering complexes

Tethering is a complex process involving multiple protein interactions which occur at various steps along the secretory pathway. The localization of tethers is thought

to depend on their associations with a unique subset of lipids or activated small GTPases present on target membranes. Tethering factors are believed to impart the initial recognition of specific vesicles with their target membranes which is then amplified by the engagement of cognate SNAREs (Cai *et al.*, 2007a; Lupashin and Sztul, 2005; Waters and Pfeffer, 1999). There are two types of tethering factors: coiled-coil tethers and multisubunit tethering complexes (Lupashin and Sztul, 2005). The focus of the remainder of this section will be on the multisubunit tethering complexes.

The multisubunit tethering complexes are evolutionarily conserved peripheral membrane protein complexes that act prior to SNAREpin formation (Oka and Krieger, 2005; Swennen and Beckerich, 2007). There are four known multisubunit Golgi tethering complexes: TRAPP (transport protein particle) I, TRAPP II, COG (conserved oligomeric Golgi) and GARP (Golgi-associated retrograde protein) (Cai *et al.*, 2007a; Lupashin and Sztul, 2005). In addition, there are four other known multisubunit tethering complexes on other subcellular compartments: homotypic fusion and vacuole protein sorting (Stroupe *et al.*, 2006), class C core vacuole/endosomes tethering (CORVET), the exocyst and Dsl1 (Cai *et al.*, 2007a). These multisubunit tethering complexes interact with different factors to facilitate tethering.

TRAPP I/II (see below) and COG mediate ER to Golgi and intra-Golgi transport. The COG complex is found in the *cis*/medial Golgi (Suvorova *et al.*, 2001; Ungar *et al.*, 2002) and functions in the retrograde trafficking of proteins from endosomes to the early-Golgi (Bruinsma *et al.*, 2004; Suvorova *et al.*, 2002). It is composed of eight subunits

(Cog1-8p) (Ram *et al.*, 2002; Suvorova *et al.*, 2002; Whyte and Munro, 2001). The COG complex subunits interact with proteins involved in Golgi trafficking indicating a role for COG in membrane traffic (Suvorova *et al.*, 2002; VanRheenen *et al.*, 1998; VanRheenen *et al.*, 1999). Importantly, the COG complex interacts with activated GTP-bound Rab Ypt1p, Golgi associated SNAREs and the COPI coat (Suvorova *et al.*, 2002). In HeLa cells, it was observed that knock-down of one of the components of COG did not block anterograde transport but did, however, lead to a block in retrograde transport (Zolov and Lupashin, 2005). This further confirmed its role in the retrograde transport of COPI vesicles to the early-Golgi. Database searches using the amino (N)-terminal domains of COG components revealed structural similarity with other tethering complexes (the exocyst and GARP) indicating a possible common ancestor which diverged to specialize at different steps of the secretory pathway (Whyte and Munro, 2001).

The exocyst is found at the plasma membrane (Guo *et al.*, 1999) and helps target vesicles to bud tips in post-Golgi secretion (Finger *et al.*, 1998; Hsu *et al.*, 2004; TerBush *et al.*, 1996). It is composed of eight subunits (Sec3p, Sec5p, Sec6p, Sec8p, Sec10p, Sec15p, Exo70 and Exo84p) (Guo *et al.*, 1999; TerBush *et al.*, 1996) which arrange in a tree-like configuration with subunits branching off from a central point (Hsu *et al.*, 1998). The exocyst complex, like the COG complex, does not possess GEF activity. However, the exocyst does interact with the activated form of the Rab protein Sec4p (Guo *et al.*, 1999). Most exocyst subunits are recruited to post-Golgi vesicles by the interaction of the Sec15p subunit with active GTP-bound Sec4p (Guo *et al.*, 1999). Two exocyst subunits, Sec3p and Exo70p, localize to the plasma membrane (Boyd *et al.*, 2004; Finger *et al.*,

1998). Post-Golgi vesicles are tethered to the plasma membrane and the exocyst complex can then assemble (Boyd *et al.*, 2004). The assembled exocyst complex can then promote SNARE-mediated membrane fusion by binding Sec1p which binds to a SNARE complex and promotes fusion with the plasma membrane (Carr *et al.*, 1999; Wiederkehr *et al.*, 2004).

The GARP complex is found associated with the late-Golgi and mediates the transport of endosome derived vesicles to the late-Golgi (Conibear and Stevens, 2000; Conibear *et al.*, 2003; Reggiori *et al.*, 2003). The GARP complex is composed of four subunits: Vps51p, Vps52p, Vps53p and Vps54p. Mutations in *VPS52*, *VPS53* or *VPS54* lead to the mislocalization of proteins in the late-Golgi while protein sorting in the early-Golgi remains unaffected (Conibear and Stevens, 2000). These results indicate that the complex has a role in protein sorting in the late-Golgi. The GARP subunit Vps51p associates with the t-SNARE Tlg1p found in the late-Golgi and thus may provide a link between vesicle tethering and fusion (Conibear *et al.*, 2003).

The HOPS and CORVET complexes are very similar to each other. Each is composed of six subunits, four of which are common to both complexes: Vps11, Vps16, Vps18, and Vps33 (Peplowska *et al.*, 2007). These homologous complexes can interconvert, forming intermediate complexes by exchanging a few subunits. However each is localized to a different intracellular region (HOPS at the vacuole and CORVET at endosomes) and have distinct functions (Peplowska *et al.*, 2007). The HOPS complex is involved in vacuole-vacuole and vesicle-vacuole fusion (Haas *et al.*, 1995; Mayer and

Wickner, 1997; Price *et al.*, 2000; Wurmser *et al.*, 2000), while the CORVET complex mediates endosome-lysosome transport (Peplowska *et al.*, 2007). The interaction of HOPS and Ypt7p is required for vacuole-vacuole and vesicle-vacuole fusion (Price *et al.*, 2000; Seals *et al.*, 2000; Wurmser *et al.*, 2000). Activated Ypt7p and its interaction with Vps33p promotes the binding of HOPS to the SNARE Vam3p (Laage and Ungermann, 2001; Seals *et al.*, 2000; Wang *et al.*, 2001). In addition, the binding of HOPS to the SNARE Vam7p may facilitate membrane fusion (Stroupe *et al.*, 2006). HOPS contains Vps41p and Vps39p, while CORVET contains their homologues Vps8p and Vps3p respectively (Peplowska *et al.*, 2007). Vps39p and Vps3p act as GEFs for Ypt7p and Vps21p respectively (Peplowska *et al.*, 2007; Wurmser *et al.*, 2000). Vps41p and Vps8p act as effector proteins recognizing active GTP-bound forms of these GTPases (Ypt7p and Vps21p respectively). Exchanging subunits would change the GEF activity of the complex from the small GTPase Ypt7p (Stroupe *et al.*, 2006) to Vps21p (COVET) and vice/versa (Peplowska *et al.*, 2007).

The Dsl1 complex is involved in the tethering of retrograde COPI Golgi derived vesicles with the ER (Andag *et al.*, 2001; Reilly *et al.*, 2001). It is composed of only three subunits: Dsl1p, Tip20p and Sec39p, which all localize to the ER (Kraynack *et al.*, 2005; Reilly *et al.*, 2001; VanRheenen *et al.*, 2001). The subunits are linked together like a chain: Sec39p-Dsl1p-Tip20p (Tripathi *et al.*, 2009). Interactions with both the COPI coat and SNAREs elucidates a mechanism for the tethering of vesicles by the Dsl1 complex. At the centre of the complex, Dsl1p has overlapping binding sites for two COPI vesicle coat proteins (Andag *et al.*, 2001; Andag and Schmitt, 2003). As well, at each end of the

complex, there are t-SNARE binding sites. Sec39p and Tip20p bind to the ER t-SNAREs Ufe1p and Sec20p, respectively (Kraynack *et al.*, 2005; Sweet and Pelham, 1993; Tripathi *et al.*, 2009). Interaction with these t-SNAREs as well as the t-SNARE Ufe1p and the v-SNARE Sec22p are proposed to form the SNARE complex which allows fusion of the vesicle with the ER (Tripathi *et al.*, 2009).

1.4.1 TRAPPI and TRAPPII complexes

As mentioned above, TRAPP is a family of protein complexes with two members, TRAPPI and TRAPPII. In yeast, both TRAPPI and TRAPPII are composed of the same core of seven subunits (Bet5p, Bet3p, Trs20p, Trs23p, Trs31p, Trs33p and Trs85p), while TRAPPII has three additional subunits (Trs65p, Trs120p and Trs130p). As well, a novel TRAPPII subunit called YEL048c/Tca17p in yeast (TRAPPC2L in mammals) has recently been identified (Scrivens *et al.*, 2009).

TRAPP has specific GEF activity for certain GTPases which are important vesicular transport regulators. The three key GTPases which TRAPP interacts with are Ypt1p, Ypt31p and Ypt32p. TRAPPI has GEF activity toward the specific Rab protein Ypt1p (Wang *et al.*, 2000) while TRAPPII has GEF activity toward YPT31/32 (Jones *et al.*, 2000; Morozova *et al.*, 2006). Bet3p, Bet5p, Trs31p and Trs23p represent the minimal TRAPP subunits required for GEF activity for Ypt1p (Kim *et al.*, 2006; Cai *et al.*, 2008). Consistent with its role as a GEF, mutations in some TRAPP subunits lead to a

loss in GEF activity and a defect in the secretory pathway (Sacher *et al.*, 2001). For instance, the Bet3p mutant (*bet3-1*) was created which has a temperature-sensitive (ts) phenotype and results in a defect in the tethering of vesicles to the Golgi (Jiang *et al.*, 1998; Rossi *et al.*, 1995). TRAPP containing the mutant bet3-1p has a decreased ability to stimulate the release of ³[H]GDP from Ypt1p at 30°C and almost a complete loss in GEF activity is observed at 37°C (Wang *et al.*, 2000). This is consistent with the finding that Bet3p is necessary for Ypt1p-directed GEF activity (Kim *et al.*, 2006; Cai *et al.*, 2008).

Co-expressed yeast TRAPPI subunits are able to assemble into a functional recombinant complex (Kim *et al.*, 2006). However, the co-expressed vertebrate subunits were only able to form smaller subcomplexes and not fully assembled recombinant TRAPP. This indicates that either there is an as yet unknown vertebrate subunit which links the two subcomplexes or post-translational modifications are required for complex assembly (Kim *et al.*, 2006).

While both TRAPP I and TRAPP II are found on the Golgi, they act at different steps in the secretory pathway. TRAPPI is associated with the *cis*-Golgi (Sacher *et al.*, 1998) and functions in ER-to-Golgi transport while TRAPPII is associated with the *trans*-Golgi/ early endosome (Cai *et al.*, 2005; Morozova *et al.*, 2006) and is thought to mediate endosome-to-*trans*-Golgi transport (Cai *et al.*, 2005; Sacher *et al.*, 2001). The detailed mechanism of vesicle tethering by the TRAPP complexes remains obscure but may involve interactions with GTPases and the recognition of vesicle coat proteins.

The ways in which the different subunits interact are likely key to the functions of the complexes. For example, as only TRAPPI and not TRAPP II recognizes uncoated ER-derived (COPII) vesicles (Sacher *et al.*, 2001), the two unique and essential subunits of TRAPP II may in some way be blocking this recognition from occurring. As well, the subunits unique to TRAPP II may be blocking GEF activity towards Ypt1p and allowing GEF activity for Ypt31/32p (Morozova *et al.*, 2006). Since the TRAPP complexes activate Ypt1p and Ypt31/32p in ER-to-Golgi and trans-Golgi traffic, respectively, it has been proposed that they mediate coordinated entry and exit of material at the two ends of the Golgi (Morozova *et al.*, 2006).

Also, the specific composition of the COPII protein coat and possibly the vesicle content itself may have a role in association with specific tethers and thus target membranes. Indeed, one component of the COPII coat, Sec23p, has been shown to bind to TRAPPI and this interaction was reported to be mediated by the Bet3 protein (Cai *et al.*, 2007b). Vesicle tethering was successfully reconstituted *in vitro* using TRAPPI and COPII vesicles and no other tethering factors were required for association with the complex (Sacher *et al.*, 2001). This indicates that TRAPPI is sufficient to tether COPII vesicles and that recruitment of other tethering factors occurs either in parallel to strengthen the tether *in vivo* or after this event (Sacher *et al.*, 2001). Such additional factors may include Uso1p in yeast, and p115 and GM130 in mammals.

Membrane trafficking is essential to cell survival and mutations in the tethering factors can have adverse effects on the function of a cell. Spondyloepiphyseal dysplasia

tarda (SED1) is an X-linked recessive disease caused by mutations in the sedlin gene (*S. cerevisiae* Trs20 protein) a component of the TRAPP complex (Suvorova *et al.*, 2001). This disease causes skeletal abnormalities and early-onset osteoarthritis. Although the sedlin message is found in all cells examined, its specific effect may indicate a tissue specific role for TRAPP (Barrowman *et al.*, 2000; Gedeon *et al.*, 1999; Sacher, 2003).

1.4.2 BET3

Multisubunit tethering complexes are involved in the initial stages of vesicle recognition. Therefore, their correct localization to the appropriate subcellular compartments is essential. While none of the TRAPP subunits have a membrane spanning domain (Kim *et al.*, 2005b), biochemical studies of yeast and mammalian TRAPP suggest that it is anchored to the Golgi (Sacher *et al.*, 2000) through electrostatic interactions or post-translational modifications (such as lipidation) (Kim *et al.*, 2005b; Turnbull *et al.*, 2005). The mechanism by which this is achieved is unclear, however special interest has been placed on the Bet3p subunit due to several unique structural features.

1.4.2.1 The crystal structure of BET3 and BET3 mutants

The crystal structure of full length mouse Bet3p was solved to a resolution of 1.9Å. Its structure consists of four α -helices and five β -strands. One hairpin structure (β 2 and β 3) is juxtaposed to a second hairpin structure (β 4 and β 5) forming an anti-parallel β -sheet. One face of this β -sheet forms hydrophobic interactions with α 3 and α 4 while the other face is exposed to the solvent (Kim *et al.*, 2005b).

Complete chain tracing identified a central hydrophobic channel. This channel is lined by well conserved apolar side chains contributed by α 2, α 3 and α 4 and also by Leu18 on α 1 (Kim *et al.*, 2005b). Curiously, the channel enclosed a long alkyl chain that could be attributed to a myristate group in the electron density map. A conserved cysteine at position 68 (C68) near the entrance of the channel was clearly observed in crystals of truncated Bet3p (residues 8-172) (Kim *et al.*, 2005b). A Bet3 mutant was generated in yeast with a C68S substitution (inhibiting acylation) and a carboxy (C)-terminal hemmagglutinin (HA) tag. This mutant had no temperature-sensitive phenotype nor was there any observable change in the localization or subcellular fractionation of the mutant versus the wild type. These results indicate that acylation is not a requirement for membrane-anchoring of Bet3p (Kim *et al.*, 2005b).

It has been speculated that the hydrophobic channel may be needed to bind specifically to the Golgi membrane through the insertion of a hydrophobic anchor protein. Therefore, to study the function of the hydrophobic channel, a Bet3p A94L

mutant was generated where a conserved alanine residue in the middle of the channel was changed to a leucine residue. This substitution has no predicted effect on the structure of the channel surrounding it but would effectively block access to most of the channel (Kim *et al.*, 2005b). This mutant, referred to as *bet3-4*, is conditionally lethal at 37°C. Immunofluorescence visualization of the HA tagged mutant showed localization to many intracellular compartments suggesting that localization of Bet3p to the Golgi is lost when the hydrophobic channel is blocked (Kim *et al.*, 2005b).

The hydrophobic channel on Bet3p is located adjacent to a wide flat surface with an overall positive charge due to seven exposed basic residues. Five of these residues are well conserved (>84%): Lys13, Arg62, Arg67, Lys80 and Lys84. Although Bet3p in the channel blocked mutant did not localize correctly to the Golgi, it was able to bind less specifically to other membranes. The conserved positive patch of amino acids on Bet3p may account for the interactions with the negatively charged lipids on these membranes (Kim *et al.*, 2005b). To examine the role of the positive surface on Bet3p, a mutant called *bet3-3* was generated containing both K13E and K84E substitutions. These changes generated a conditionally-lethal phenotype and inhibited TRAPP from binding to the Golgi membrane (Kim *et al.*, 2005b) suggesting that this positive patch on Bet3p is necessary for the proper association with this membrane.

Bet3p and Trs33p are structurally similar even though their amino acid sequences are very different (Kim *et al.*, 2005a). The heterodimerization of these two proteins allows for the co-precipitation of Bet5p with Bet3p (Kim *et al.*, 2005a). This indicates

that Trs33p facilitates the interaction of Bet3p with other proteins and thus may also have a role in the assembly of TRAPP (Kim *et al.*, 2005a) and its localization to the Golgi membrane.

1.4.2.2 Genetic interactions

BET3 was first identified as a genetic interactor with the SNARE mutant *bet1-1* in a synthetic lethal screen (Rossi *et al.*, 1995). Immunopurified c-myc tagged Bet3p was shown to be part of TRAPP. *BET5*, *TRS20*, *TRS23* and *TRS33* are high copy suppressors of the temperature-sensitive mutant *bet3-1* (Sacher *et al.*, 1998), as could be expected from proteins in the same complex. Overexpression of genes that encode SNAREs (*BOS1*, *SEC22*, *SED5*, *BET1*) and genes whose products are involved in membrane trafficking (*YPT1*, *SLY1*, *SEC17*, *SEC18*) are also able to suppress this mutant (Sacher *et al.*, 1998). Co-localization and subcellular co-fractionation with the t-SNARE Sed5p indicate that Bet3p resides on the *cis*-Golgi membrane (Sacher *et al.*, 1998; Banfield *et al.*, 1994). Bet3p is the most highly conserved TRAPP subunit, it is essential for cell viability, it localizes to the Golgi, and it genetically interacts with SNAREs and other membrane trafficking factors (Sacher *et al.*, 1998). For these reasons, Bet3p is thought to be a determining factor in the ability of TRAPP to attach specifically to the Golgi membrane.

1.5 Synthetic lethal yeast screen

Synthetic lethality is a phenomenon by which the combination of two otherwise non-lethal mutations results in lethality. Such a genetic interaction oftentimes suggests that the corresponding gene products act in the same or in a parallel pathway (Guarente, 1993). In the present study a synthetic lethal screen will be employed using the channel-blocked *bet3-4* mutation. By using the channel blocked mutant *bet3-4* in this screen, we hope to identify a gene whose protein product may be responsible for TRAPP's ability to attach specifically to the Golgi membrane.

1.5.1 Overview of project

The variation of the screen chosen for the present study is based on a plasmid-dependent assay which exploits red/white colony sectoring (Bender and Pringle, 1991). In the adenine biosynthetic pathway, the product of the *ADE3* gene acts upstream of the product of the *ADE2* gene. An *ade2* mutation blocks the 5-aminoimidazole ribonucleotide carboxylase step (Patterson *et al.*, 2008) and leads to the accumulation of the oxidized and polymerized intermediate AIR (Smirnov *et al.*, 1967) in the vacuoles (Weisman *et al.*, 1987). However, a single *ade3* mutation and a double *ade2 ade3* mutation result in colonies that are white in appearance since the pathway is blocked and the red intermediate is not produced (Appling, 1999). Yeast genetics can be used to control the red/white appearance by inserting a counter-selectable plasmid harbouring a

wild type *ADE3* gene into *ade2 ade3* cells. In this scenario the preferred counter-selectable gene is *URA3* which can be counter-selected on medium containing 5-fluoroortic acid (5-FOA) (Boeke *et al.*, 1984). The *URA3* gene codes for the enzyme orotidine 5-phosphate decarboxylase (ODCase) which is involved in the synthesis of pyrimidine ribonucleotides where it catalyses the decarboxylation of orotidine 5-phosphate into uridylic acid (Umezue *et al.*, 1971). Mutations in this gene, which lead to a defect in enzyme activity, result in lethality unless supplemented with uracil in the growth media. ODCase also catalyses the conversion of 5-FOA into a toxic compound (5-fluorouracil) (Boeke *et al.*, 1984). Therefore, only cells which have lost the *URA3*-containing plasmid will grow on 5-FOA media.

Treatment of yeast with ethyl methanesulfonate (EMS) results in random mutagenesis (mainly point mutations resulting in G to A base substitutions but also A to G substitutions and base insertions and deletions) (Sega, 1984). When performed in a strain with an *ade2 ade3* background that carries a mutation (e.g. *bet3-4*) as well as the counter-selectable plasmid mentioned above (containing *URA3* and *ADE3* with a wild type copy of the mutant gene (e.g. *BET3*)), cells with mutations that are not synthetically lethal with the mutation of interest will be capable of losing the *URA3/ADE3*-containing plasmid and will appear as white colonies or red colonies with white sectors. However, if a resulting mutation is synthetically lethal with the mutation of interest, then the colonies will not be capable of losing the plasmid and will remain uniformly red.

Once these mutants have been identified, a yeast plasmid library is used to screen for complementation. By complementing the EMS-induced mutation in the unknown

gene, red mutant colonies will be able to lose the *ADE3*-containing plasmid and will have a white or sectorized phenotype.

2. Materials and Methods

2.1 Strains, media and oligonucleotides

The *S. cerevisiae* strains used in the present study are listed in Table 2.1. Bacterial strains and plasmid information are listed in Table 2.2. Strains were grown in the appropriate media listed in Table 2.3. All oligonucleotides (Dean, 1999) used are listed in Table 2.4.

2.2 Construction of yeast strains for the synthetic lethal yeast screen

In order to generate the starting strain for the screen (*bet3-4 ade2 ade3*), strains MSY116 and MSY15a were patched one on top of the other to allow mating on a yeast peptone dextrose (YPD) plate and grown for 5 hours at 30°C. Diploids were then isolated after visual inspection using a micromanipulator and allowed to grow for 3 days on YPD at 30°C. These diploids were then patched onto a sporulation (SPO) plate and sporulated at 30°C for 7 days. Following dissection of the sporulated diploids, a red (*ade2*) colony was selected which was *leu*⁺ and temperature-sensitive at 38°C indicating it contained the *bet3-4* mutation. This strain was designated MSY188d.

This strain, MSY188d, was then crossed again to MSY116. Following dissection of the tetrads, a white (*ade2 ade3*) colony was selected which is also *leu*⁺ and

Table 2.1 *Saccharomyces cerevisiae* strains

Strain	Features	Source
MSY14c	MAT α his3 Δ 1 leu2 Δ 0 ura3 Δ 0 MET15 bet3 Δ ::KanMX Bet3p(K24/96E)::LEU2, ts at 38°C	Michael Sacher
MSY15a	MAT α his3 Δ 1 leu2 Δ 0 ura3 Δ 0 MET15 bet3 Δ ::KanMX Bet3p(A94L)::LEU2, lys-, ts at 38°C	Michael Sacher
MSY16	MAT α his1	Michael Sacher
MSY17	MAT α his1	Michael Sacher
MSY20	MAT α can100, leu2-3,112, his3-11 trp1 Δ , ura3-1, ade2-1	Martin Latterich
MSY21	MAT α can100 leu2-3,112 his3-11 trp1 Δ ura3-1 ade2-1 trs3 Δ ::HIS3	Michael Sacher
MSY22	MAT α can100 leu2-3,112 his3-11 trp1 Δ ura3-1 ade2-1 trs65 Δ ::HIS3	Michael Sacher
MSY116	MAT α ade2 ade3 ura3 leu2 lys2 his3	Brian Haarer
MSY146	MAT α lys2 ura3-1 his3-11,15 leu2-3,112 can1-100 ade2-1::ADE2 bet3-5 (Bet3p(G64E)), ts at 34°C	Kathy Ryan
MSY147	MAT α trp1-1 ura3-1 his3-11,15 leu2-3,112 can1-100 ade2-1::ADE2 bet3-5 (Bet3p(G64E)), ts at 34°C	Kathy Ryan
MSY165	MAT α ura3 Δ 0 leu2 Δ 0 his3 Δ 1 can1 Δ ::LEU2-Mfa1pr::HIS3 Trs20p(V92A, F133S)::URA3, ts at 38°C	Phil Hieter
MSY174	MAT α leu2-3,112 trs130::URA3 ura3 Δ 0 ade2-1 lys2 Δ 0, ts at 38°C	Michael Sacher
MSY179	MAT α leu2-3 112 his3-11 15 bet5::URA3 ura3 Δ 0, ts at 38°C	Michael Sacher
MSY188d	MAT α ade2 ura3 leu2 lys2 his3 bet3 Δ ::KanMX Bet3p(A94L)::LEU2, ts at 38°C	Present Study
MSY206	MAT α ade2 ade3 ura3 leu2 lys2 his3 bet3 Δ ::KanMX Bet3p(A94L)::LEU2, ts at 38°C	Present Study
MSY207	MAT α ade2 ade3 ura3 leu2 lys2 his3 bet3 Δ ::KanMX Bet3p(A94L)::leu2::LYS2, ts at 38°C	Present Study
MSY211a	MAT α ade2 ade3 ura3 leu2 lys2 his3 bet3 Δ ::KanMX Bet3p(A94L)::leu2::LYS2 pRS316-BET3 ADE3 URA3, ts at 38°C	Present Study
MSY214	MAT α can100, leu2-3,112, his3-11 trp1 Δ , ura3-1, ade2-1, trs85 Δ ::HIS3	Michael Sacher
MSY362	BY4742. MAT α his3 Δ 1 leu2 Δ 0 lys2 Δ 0 ura3 Δ 0 trs85 Δ ::KanMX	Vladimir Titorenko

Table 2.2 Bacterial strains and plasmid information

Strain	Plasmid Features	Source
MSB8	pRS315-BET3	Michael Sacher
MSB340	pHT4467 (pRS316)-ADE3 URA3	Ed Hurt
MSB365a	pRS316-BET3 ADE3 URA3	Present Study
MS7	<i>leu2::LYS2</i> Disruptor Converter	David Stillman
MSB297	pRS313-BET3	Michael Sacher
MSB299	pRS313-yBET5	Michael Sacher
MSB300	pRS313-yTRS20	Michael Sacher
MSB301	pRS313-yTRS31	Michael Sacher
MSB302	pRS313-yTRS23	Michael Sacher
MSB303	pRS313-yTRS85	Michael Sacher
MSB304	pRS313-yTRS65	Michael Sacher
MSB305	pRS313-yTRS120	Michael Sacher
MSB306	pRS313-yTRS33	Michael Sacher
MSB470	pRS315-YEL048c	Michael Sacher
MSB474	pRS425-TRS130	Michael Sacher
YE213 library	pBR322 derived high copy yeast plasmid library (LEU2)	Doreen Harcus

Table 2.3A Media

Media	Components
YPD	1% yeast extract, 2% peptone, 2% dextrose
drop-out	0.67% yeast nitrogen base, 0.08% amino acid mix, 2% dextrose
5-FOA	0.67% yeast nitrogen base, 0.08% complete amino acid mix, 2% dextrose, 0.1% 5'-FoA
SPO	1% potassium acetate, 0.1% yeast extract, 0.05% dextrose, 0.02% complete amino acid mix
LB+ amp	0.5% yeast extract, 1% tryptone, 1% sodium chloride, 34mg/ml Ampicillin
G418	1% yeast extract, 2% peptone, 2% dextrose, 200µg/ml G418

Note: For solid media 2% agar was added.

Table 2.3B Amino acid mix

Components (g/ 25L of media)
1g adenine
0.5g uracil
1g tryptophan
0.5g histidine
0.5g arginine
0.5g methionine
0.75g tyrosine
0.75g isoleucine
3.725g valine
0.75g lysine
1.25g phenylalanine
2.5g glutamic acid
5g threonine
1.5g leucine

Note: To make low adenine medium 0.15g of adenine is used in the mix instead of 1g which lowers the concentration of adenine from 40mg/L to 6mg/L.

Table 2.4 Oligonucleotides used in the present study

Oligo	Comments	Sequence
P336	to sequence yeast library	CATCGCCAGTCACTATG
P82	to sequence yeast library	CTGGGTTGAAGGCTCT
Vps16SacI-F	400bp from start	GTAGACGAGCTCCGTCGGTGAAATCATCGCAGCA
Vps16SacI-R	400bp past stop	GTAGACGAGCTCCATCTGTGACATCCTGGGGTAT
Vps16R-SacI-282bp	282bp past stop	ATGCGAGCTCACTTTGGTAGATTGGTAAT
Vps16-A	for sequencing	TGGAACGTACAATAAGGCACC
Vps16-B	for sequencing	ACCACTGGAGTTGCAATGATGAC
Vps16-C	for sequencing	CAGAAATGTTGTGACGGCTGC
Vps16-D	for sequencing	TGACTTGGCTCACGTATTACTGC
Trs23SacIF	400bp from start	GTAGACGAGCTCGGCGAGATGCAGTACTACACCAC
Trs23SacIR	400bp past stop	GTAGACGAGCTCGTTACACTTTGGTATCAGTCATC
Trs23seqF	for sequencing	AAGGAATCTGCCTTTGCATAAGTTC
Trs23seqR	for sequencing	AGACGGAGAGCGGCAACTTCTTC
Trs85SacIF	400bp from start	GTAGACGAGCTCCATCGCCTGAGTGATATGTTGTG
Trs85SacIR	400bp past stop	GTAGACGAGCTCGTAATGCAAGAATTGTCAATCC
Trs85seqA	for sequencing	TTCCCTCGGACAAAATTAGGCAA
Trs85seqB	for sequencing	TACAAGGCCATTAGGTGACTTGA
Trs85seqC	for sequencing	TCTTCCTTCAAGCTCCAACCTCA
Trs85seqD	for sequencing	TTACAGAGCCAGTGCCTTCGTA
Bet3BamHI-F	for sequencing	AGGGATCCATGGTTTCTACCACGCAATCGAGG
Bet3EcoRI-R	for sequencing	CGGAAATTCCTAATCTTCGCCGATCGGTAT

temperature-sensitive at 38°C and was designated MSY206. Strain MSY207 was obtained by swapping the *LEU2* gene in MSY206 for the *LYS2* gene using a standard marker swapping protocol (see below, section 2.8) (Voth *et al.*, 2003) and selecting a strain which is *lys*⁺ *leu*⁻ *ura*⁻ and temperature-sensitive at 38°C. The *leu2* marker was made available in this strain for the later use of a yeast plasmid library which is on a *LEU2* plasmid. MSY207 was then transformed with a balancing plasmid containing wild type *BET3* (plasmid from MSB365a) to obtain the strain MSY211a.

2.3 Plasmid construction

To construct the balancing plasmid used in the synthetic lethal screen, the wild type *BET3* open reading frame with 400 base pairs upstream and downstream was isolated from the plasmid from strain MSB8 (pRS315-*BET3*) by digesting with the restriction enzyme *XbaI* (New England Biolabs). The plasmid from strain MSB340 with the *ADE3* and *URA3* genes was also digested with *XbaI* for 1 hour at 37°C followed by a 30 minute incubation at 37°C with calf intestinal alkaline phosphatase (CIP) to dephosphorylate the 5' ends of the linearized deoxyribonucleic acid (DNA). The insert and linearized plasmid DNA were then fractionated on a 1% agarose gel and the bands were extracted using a gel extraction kit (Fermentas). The insert and plasmid were then ligated together with T4 DNA ligase (200 Units in a total volume of 10µl) at room temperature overnight. The following day, 5µl of the ligated DNA was transformed into 40µl of CaCl₂ competent DH5α cells and plated on solid Luria-Bertani (LB)+ampicillin

(LB+amp) overnight at 37°C. The following day a single colony was picked into 3ml of liquid LB+amp and again grown overnight. The following day the plasmid was isolated from 1.5ml of this culture using a kit (Fermentas). The presence of the insert was confirmed by its release from the plasmid following digestion with *XbaI*. The resulting plasmid is referred to as MSB365a.

2.4 Transformation of *Saccharomyces cerevisiae*

In order to transform cells with plasmids or DNA fragments a 5ml pre-culture was grown in the appropriate medium overnight at 30°C on a rotary shaker at 250rpm. The next day, the absorbance at 600nm of a 10 fold dilution of this culture was measured in a spectrophotometer. Commonly, a concentration of 1×10^6 cells/ml gives an optical density (OD)₆₀₀ of 0.1. Using this formula the approximate number of cells in culture at different points in this protocol could be calculated (Gietz and Woods, 2002).

Liquid media (50ml) was inoculated with 2.5×10^8 cells to give a starting concentration of 5×10^6 cells/ml equivalent to an OD₆₀₀ of 0.5 and grown at 30°C for 3-5 hours on a rotary shaker at 250rpm. When an OD₆₀₀ of 2 was reached ($\sim 2 \times 10^7$ cells/ml), cells were pelleted by centrifugation at 3000 x g for 5 minutes in a table-top centrifuge. The pellet was washed with 25mL of sterile distilled water (dH₂O) and resuspended in 1ml of sterile dH₂O. Cells were pelleted again at the same speed for 1 minute and resuspended in dH₂O to a final volume of 1ml. For each transformation, 100µl of this cell

suspension was transferred to a fresh 1.5ml microcentrifuge tube, pelleted at 13000rpm and the supernatant discarded. A transformation mix consisting of 33.3% polyethylene glycol (PEG) 3500, 100mM lithium acetate (LiAc) and 1.4µg/µl of boiled salmon sperm DNA (carrier DNA) and sample DNA (10µl if linear or 2µl if circular) was added to the cells and mixed vigorously. A typical transformation contained a total volume of 360µl. The tubes were incubated in a 42°C dH₂O bath for 40 minutes. Samples were then centrifuged for 30 seconds (13000rpm) and the supernatant discarded. Cells were resuspended in 500µl of sterile dH₂O and 200µl of this cell suspension was plated on the appropriate selective medium and incubated at 30°C for 2-3 days (Gietz and Woods, 2002).

2.5 Mating of *Saccharomyces cerevisiae*

Strains were crossed on YPD and grown overnight at 30°C and replica plated onto the appropriate double drop-out plates (to select for diploids) and grown overnight at 30°C. Diploids were streaked onto new plates to obtain single colonies and grown for 3 days at 30°C.

Alternatively, when markers were not available to select for diploids, strains of opposite mating types were patched one on top of the other to allow mating on YPD and grown for 5 hours at 30°C. Diploids were then isolated using a micromanipulator and

allowed to grow for 3 days on YPD at 30°C. MSY116 was crossed with MSY15a in this manner to ultimately generate MSY116.

2.6 Sporulation of *Saccharomyces cerevisiae*

Single colonies from diploids were patched onto sporulation (SPO) plates and grown for 5 to 9 days at 30°C. Once tetrads were observed under a light microscope, plates were removed from the 30°C incubator.

2.7 Dissection of *Saccharomyces cerevisiae* asci

Using a 3mm loop, cells from the patches on the SPO plate, a mixture of vegetative cells and asci, were placed into 100µl of tetrad juice (1M Sorbitol, 50mM Tris (hydroxymethyl) aminomethane (Tris) pH 7.5, 0.05mg/mL Zymolyase 100T). After 3 to 5 minutes of incubation at room temperature to lightly digest the spore wall, 5µl of the cell mixture was gently spread on a YPD plate. The cells of 10 to 12 tetrads were then separated and relocated in vertical rows using a micromanipulator and grown on rich medium (YPD) at 30°C for 2 days after which time the growth pattern of the haploids can be observed. Auxotrophic markers and mutations were assessed by replica plating onto selective media and observing growth after 1-2 days at 30°C or 38.5°C (Sherman and Hicks, 1991).

2.8 Marker Swap

In order to facilitate further characterization of synthetic lethal strains, a *leu2* auxotrophic marker was needed. The *LEU2* gene was disrupted with *LYS2* using the plasmid MS7 (D771) (Voth *et al.*, 2003). The plasmid was digested with *PstI* and *SacI* for 1 hour at 37°C, purified on a spin column (Qiagen) and resuspended in 50µl Tris ethylenediaminetetra acetic acid (EDTA) (TE) buffer. This linear DNA (10µl) was then transformed into the yeast strain MSY206 (see method above) and a *lys*⁺ *leu*⁻ colony was isolated. This strain is referred to as MSY207.

2.9 EMS mutagenesis and death rate calculation

Ethyl methane sulfonate (EMS), a volatile organic compound that is a carcinogen and a mutagen, was used to generate random mutations in DNA. Two 3ml cultures of MSY211a were grown in -URA drop out medium at 30°C for 3 days to a final OD₆₀₀ of 3.5 and then stored at 4°C. Two 1ml aliquots from one culture were used to test the death rate following EMS mutagenesis as follows. Cells were pelleted at 6500rpm in a microcentrifuge for 10 seconds and the supernatant discarded. Cells were resuspended in 1ml sterile dH₂O, pelleted and again washed with 1ml of sterile dH₂O. Cells were then resuspended in 1ml 0.1M sodium phosphate buffer pH 7.0. A 30µl aliquot of EMS was added to one of the two tubes, and sealed with parafilm. The untreated tube served as the control. The cells were vortexed vigorously and placed on a nutator in a 30°C incubator

for 1 hour. Cells were pelleted and the supernatant removed. The pellets were then resuspended in 200µl of 5% sodium thiosulfate (to inactivate the EMS) and transferred to new tubes. Cells were washed twice with 200µl of 5% sodium thiosulfate and finally resuspended in 1 ml of sterile dH₂O. Serial dilutions up to 10⁻⁴ were prepared and 150µl of each was plated on YPD plates and incubated at 30°C for 4 days. The death rate (colonies on treated plate divided by colonies on control plate) of MSY211a treated with 30µl of EMS for 1 hour was calculated by counting the number of colonies on the 1:1000 and 1:10000 plates. For the present screen a death rate of 62% was obtained where a death rate between 50 to 70% is considered ideal.

For the actual screen, mutagenesis was repeated under the same conditions with the second 3ml culture of MSY211a. A 150µl aliquot of a 1:500 dilution was plated on 60 plates and grown at 30°C for 4 days. It was calculated that this amount should correspond to ~48000 colonies (about an 8X coverage of the yeast genome).

Following the four day incubation at 30°C, the 60 plates were refrigerated at 4°C for 9 days to allow the red color in the mutated colonies to become more apparent. Red colonies were streaked onto new YPD plates and grown at 30°C for 3 to 4 days followed by storage at 4°C for 3-4 days. This was performed a total of three times to yield the final pool of synthetic lethal mutants studied.

2.10 Confirmation of genetic interaction

All 70 potential synthetic lethal mutants were crossed to a wild type strain (MSY20). Diploids were isolated and sporulated to obtain tetrads (see above section 2.6). Tetrads were dissected using a micromanipulator and grown on YPD for 3 days at 30°C. Dissection plates were then replica plated on –URA and YPD (at 38.5°C) to test the phenotype of the ascospores. Growth on the dissection and replica plates was scored and analyzed for a synthetic lethal phenotype.

2.11 Identification of TRAPP genes as synthetic lethal interactors

A library of yeast TRAPP subunits on individual low copy plasmids (DNA isolated from bacterial strains MSB299 (*BET5*), MSB300 (*TRS20*), MSB301 (*TRS31*), MSB302 (*TRS23*), MSB303 (*TRS85*), MSB304 (*TRS65*), MSB305 (*TRS120*), MSB306 (*TRS33*), with pRS313 backbones, was made with equal contribution from each plasmid DNA. This library as well as the individual plasmid in MSB470 (*YEL048c* with a pRS315 backbone) and MSB474 (*TRS130* with a pRS425 backbone), were transformed into all 70 synthetic lethal mutants. Transformation of these mutant strains with the plasmid from MSB297 (*BET3*, with a pRS313 backbone), was used as a control. Transformants were replica plated onto 5-FOA plates to counter-select against the *BET3*, *URA3* plasmid. Mutant strains which were not complemented by any genes present in the TRAPP library or *YEL048c* and *TRS130* are presumed to have a mutation in a non-

TRAPP gene. Colonies able to grow on 5-FOA were identified as having a mutation which can be complemented by a TRAPP subunit.

Further transformation with the individual TRAPP subunits helped identify the exact TRAPP subunit which could complement the unknown mutation in the synthetic lethal mutant.

2.12 Genomic DNA (gDNA) extraction

Genomic DNA was isolated using a standard yeast genomic extraction protocol. Initially, 1.5ml of overnight culture was pelleted at top speed (13000rpm) in a table top microcentrifuge for 1 minute. The pellet was washed with 750µl of dH₂O and then resuspended in 500µl of spheroplast medium (0.9M sorbitol, 0.1M EDTA, 30mM β-mercaptoethanol and 0.1mg zymolyase 100T) and incubated at 37°C for 1 hour. Cells were again pelleted at 13000rpm and gently resuspended in 200µl of Tris-EDTA (50mM Tris pH 7.5 and 20mM EDTA). A 30µl aliquot of 10% sodium dodecyl sulphate (SDS) was added and the cell mixture was incubated at 70°C. After 30 minutes, 80µl of 5M potassium acetate was added and tubes were incubated on ice for 30 minutes to one hour. Cells were centrifuged at 13000rpm for 10 minutes and the supernatant was transferred to a new tube. Tubes were centrifuged again for 1 minute (13000rpm) and the supernatant was transferred to a new tube avoiding the transfer of any debris. An equal volume of isopropanol was mixed with the supernatant and centrifuged for 1 minute. The pellet was

then washed with 500µl of 70% ethanol and air dried in a 37°C heat block for 15 minutes. The clear DNA pellet was then resuspended in TE buffer (10mM Tris pH 7.5 and 1mM EDTA).

2.13 Yeast plasmid library DNA maxiprep

The yeast plasmid library YEp213 with a *LEU2* backbone was kindly provided Doreen Marcus (Biotechnology Research Institute, Montreal). The cells were diluted up to 10^{-6} and plated on LB+amp to calculate the cell titer. Cell titer was calculated to be 4.32×10^9 cells/ml. Plasmid DNA was extracted from 2.16×10^{11} cells using a cesium chloride-based maxiprep. The concentration of the resulting DNA was 0.94µg/µl.

2.14 Yeast plasmid library screen and the identification of synthetic lethal mutants

To identify the mutated gene which was lethal in combination with *bet3-4*, the yeast plasmid library was transformed into the synthetic lethal mutants where the second mutation was shown not to be in one of the TRAPP subunit genes.

The yeast plasmid library DNA was transformed into synthetic lethal mutants on a small scale on one large 15cm plate of –LEU/low ADE (drop-out mix without leucine and only 6mg/L of adenine instead of 40mg/L) for each transformation. If a large number

of red transformants (2000 to 4000) and few (5) to no white or sectored transformants were observed, these strains were transformed on a large scale to obtain 50000 to 60000 transformants. White or sectored colonies were isolated by streaking out three consecutive times on fresh plates, allowing for three days of growth for each plate, and then grown in –LEU liquid media. Library plasmid DNA was extracted (see below) and amplified by transforming into *E. coli*. Bacterial plasmid DNA was extracted using a standard plasmid extraction kit (Fermentas) and re-transformed into the original mutant yeast strain to see if it could rescue the synthetic lethal phenotype again. Plates were replica plated onto 5-FOA. Those plasmids that were able to complement one of the mutations present (growth on 5-FOA) were sequenced to determine the boundaries of the yeast genomic DNA insert (see below).

2.15 Yeast plasmid extraction

The isolation of yeast plasmid DNA was performed using a user-developed protocol with the QIAprep Spin Miniprep Kit from Qiagen (Michael Jones from the Chugai Institute for Molecular Medicine in Ibaraki, Japan). Isolated white colonies from the synthetic lethal mutants complemented by plasmids from the YEp213 yeast plasmid library were grown overnight (16 to 24hrs) in 5ml of –LEU drop out medium at 30°C. Cells were then pelleted by centrifugation for 5 minutes at 3000 x g (gravity) in a table-top centrifuge and resuspended in 250µl of Resuspension buffer (Fermentas). Acid-washed glass beads (70mg) were added to the cells and the tubes were vortexed for 5

minutes. Beads were allowed to settle and the supernatant was removed. Lysis buffer (25 μ l) (Fermentas) was added to the supernatant and tubes were inverted gently 4-6 times and left at room temperature for 5 minutes. Neutralization buffer (Fermentas) (350 μ l) was added and the tubes were inverted gently 4-6 times. Lysates were centrifuged at 13000rpm for 10 minutes in a microcentrifuge. Supernatants were passed through QIAprep Spin Columns by centrifugation at 13000rpm for 1 minute. Flow-through was discarded and the column was washed with 750 μ l of PE buffer from the QIAprep spin miniprep kit and centrifuged at 13000rpm for 1 minute. Flow-through was again discarded and residual buffer was removed by an additional centrifugation step at 13000rpm for 1 minute. The DNA was eluted by the addition of 25 μ l of elution buffer (EB) (Fermentas). Following a 1 minute incubation at room temperature the eluted DNA was collected from the column by centrifugation at 13000rpm for 1 minute.

2.16 Sequencing of yeast plasmid insert

Complementing plasmids from the yeast plasmid library (YEp213) were checked for the presence of *BET3* by performing a polymerase chain reaction (PCR) with Taq DNA polymerase (Fermentas) and oligos Bet3BamHI-F and Bet3EcoRI-R. A 25 μ l amplification mix contained 1 Unit of Taq DNA polymerase (Fermentas) enzyme with 2.5 μ l of Taq Buffer (without magnesium chloride) (Fermentas), 2 μ l of 12.5X magnesium chloride, 2.5 μ l of each 5 μ M oligo, 2.5 μ l of 2mM dNTP and 1 μ l of a 1/5 dilution of the template DNA. Amplification conditions consisted of 95 $^{\circ}$ C for 3 minutes, 30 cycles of

95°C for 30 seconds, 55°C for 30 seconds and 72°C for 1 minute, and a final elongation step at 72°C for 7 minutes. If *BET3* was not detected in these samples, then they were sent for sequencing at the McGill Genome Centre with oligos P336 and P82. This generated plasmid and insert sequence information from both ends of the insert. Aligning the insert sequence to the *Saccharomyces cerevisiae* genome database (www.yeastgenome.org) allowed the coordinates of the beginning and end of the insert to be obtained. The chromosomal maps of these regions indicate the whole or partial genes present in the inserts.

2.17 Sequencing of TRAPP genes and potential novel interactors

Extracted genomic DNA from synthetic lethal mutants was subjected to PCR amplification using oligos to amplify the open reading frame (ORF) and 400bp upstream and downstream of the complementing gene (Table 2.4). A 50µl amplification mix contained 1.75 Units of High Fidelity Polymerase enzyme with 5µl of Buffer 1 (Roche), 5µl of each 5µM oligo, 5µl of 2mM dNTP and 1µl of a 1/5 dilution of the template DNA. Running conditions consisted of 95°C for 3 minutes, 30 cycles of 95°C for 30 seconds, 55°C for 30 seconds and 72°C for 1 minute/kb, and a final elongation step at 72°C for 7 minutes. For *TRS85* and *VPS16*, the extension temperature was lowered to 68°C. PCR products were purified using a PCR purification kit (Fermentas) and sequenced at the McGill University/ Genome Quebec Innovation Centre using sequencing oligos listed in Table 2.4.

2.18 Confirmation of potential novel suppressors

To help identify the potential novel suppressor in the insert in MSB736, fragments of this insert were cut out (removing certain genes) using restriction enzymes and the vector re-ligated together. MSB736 was digested with the restriction enzyme *PvuII* (New England Biolabs) and electrophoresed on a 1% agarose gel. Three fragments of 2380bp, 2628bp and 10286bp were observed as expected and the largest fragment was gel extracted using a gel extraction kit (Fermentas). This DNA was ligated together and transformed into competent DH5 α cells (as described in section 2.3). Plasmid DNA was extracted from a single colony using a miniprep kit (Fermentas) and digested with *PvuII* to verify the loss of part of the insert. As expected, since there should only be one *PvuII* restriction site in the new construct, only one fragment equal to the size of this plasmid (10286bp) was observed. This plasmid is referred to as MSB821.

3. Results

3.1 Synthetic lethal yeast screen

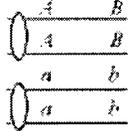
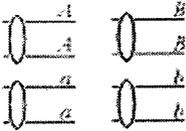
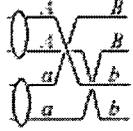
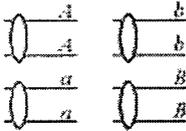
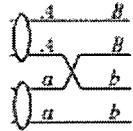
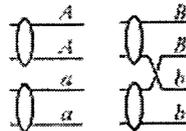
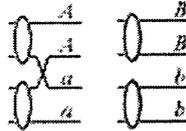
A synthetic lethal yeast screen was performed with a *bet3-4* mutant strain to help identify genetic interactors which may be linked to the ability of TRAPP to anchor itself specifically to Golgi membranes. The *bet3-4* mutant strain (MSY211a) was randomly mutagenized with EMS. Roughly 48000 colonies (8X the yeast genome) were screened for a uniformly red phenotype. A total of 187 such colonies, ranging from pink to dark red, were identified and re-streaked three times each, making sure to select colonies which were uniformly coloured following 3-4 days at 4°C. After re-streaking, only 70 mutants remained completely red and never showed any white sectoring. This uniform red colour indicates that all the cells need to retain the balancing plasmid in order to survive and that the mutations present in these mutants are synthetically lethal with *bet3-4*.

3.2 Confirmation of synthetic lethal phenotype

In order to confirm the genetic interaction visually observed by the red colony color, all 70 mutants identified in the synthetic lethal yeast screen were crossed with a wild type (wt) strain (MSY20), sporulated and dissected. Dissection plates were then replica plated onto YPD (grown at the restrictive temperature of 38.5°C) and -URA

(grown at 30°C). The *bet3-4* allele is temperature-sensitive at 38.5°C and so cells which have this mutation display slower growth at this temperature. The random mutation produced from the exposure to EMS is at an unknown locus and there is no direct way of tracking this mutation. In each ascus there are two wild type copies and two mutant alleles of each of these genes. In each ascus, the induced mutations either: do not co-segregate with the *bet3-4* allele (nonparental ditype), co-segregate in only one ascospore (tetratype), or co-segregate in two of the ascospores (parental ditype) (see Figure 3.1). Since we have no way of tracking the location of the unknown second mutation, we predict that if the cell does not grow (on YPD at 30°C) it has both mutations present and that the interaction is a confirmed synthetic lethal interaction. In this scenario, either all four or only three or two ascospores are able to grow from each tetrad. This pattern is referred to as a synthetic lethal growth pattern and indicated as 4,3,2 or 4,3 or 3,2 (see Figure 3.2). Fifty mutants were identified as having this synthetic lethal growth pattern (see Table 3.1). If the combination of these two mutations is in fact not lethal, then we would expect all four cells of all asci to be able to grow on the dissection plate. These strains would then have been rejected for not containing a synthetic lethal interaction. However, no such strains were identified.

If the two mutations co-segregate it is either that these mutations are found in the same gene (*BET3*) or in an adjacent gene tightly linked to *BET3*. In this case, two of the ascospores in all asci will not be able to grow on the dissection plate, while the other two would have no mutations and grow well. This is a 2 live: 2 dead growth pattern (indicated as 2:2) (see Figure 3.3). Eight mutants had this growth pattern and are presumed to

Tetrad type	Genes on homologous chromosomes	Genes on nonhomologous chromosomes
Parental ditype (PD) A B A B a b a b	No crossover 	No crossover 
Non-parental ditype (NPD) A b A b a B a B	Double crossover 	No crossover 
Tetratype (T) A B A b a B a b	Single crossover 	Single crossovers  or 

(©Fred Sherman)

Figure 3.1 Tetrad types. Different potential outcomes from the cross $AB \times ab'$ are listed whether the genes are on homologous chromosomes (center column) or on nonhomologous chromosomes (right column). Parental ditypes (PD): all four gametes are parental. Nonparental ditypes (NPD): all four gametes are recombinant. Tetratypes (T): two gametes are parental and two gametes are recombinant. When the $PD > NPD$ the genes are linked and are on homologous chromosomes. When genes are on nonhomologous chromosomes the $PD = NPD$ while the T are more common.

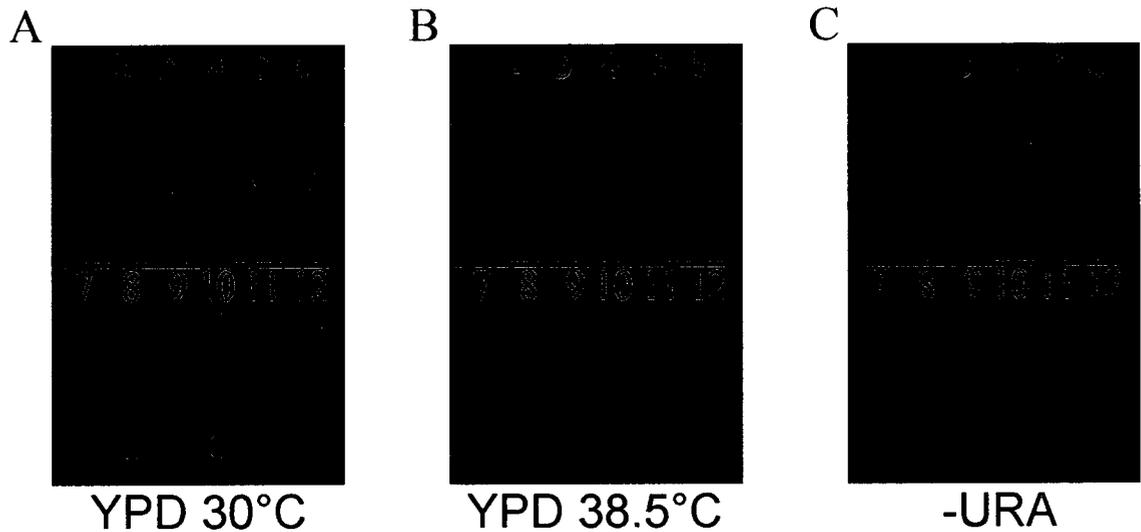


Figure 3.2 Independent segregation of mutant alleles. Dissection of mutant #98 crossed with MSY20 (wild type strain). All twelve dissected tetrads show a growth pattern indicative of independently segregating mutant alleles. (A) Dissection plate on YPD at 30°C. Three of the four ascospores grew in tetrads 3, 6, 7, 8 and 11, while only two grew in tetrads 1, 4, 9 and 12. This indicates a 3,2 growth pattern. There is a maximum of 2 red and 2 white colonies for each dissected tetrad. Although not shown here, it is possible for all four ascospores to be able to grow. This would be indicated as a 4,3,2 growth pattern. (B) Replica plate on YPD at 38.5°C. When only two ascospores grew on YPD at 30°C they were not temperature-sensitive at 38.5°C because they do not have the *bet3-4* mutation. When only three of the ascospores in a tetrad grow on YPD at 30°C, only one of these is temperature-sensitive at 38.5°C and grows slower. (C) The absence of growth on -URA at 30°C confirms the loss of the balancing *BET3 URA3* plasmid.

Table3.1 Observed growth pattern following dissection of diploids

Mutant	Colour	Cross to MSY20	Comments on dissection	Mutant	Colour	Cross to MSY20	Comments on dissection
3	red	2:2dead		85	dark red	4,3,2	
5	red	4,3,2	some small colonies	87	pink	4,3,2	
6	red	2:2dead		95	red	4,3,2	some small colonies
7	red	4,3,2		98	red	3,2	some small colonies
8	red	4,3,2		99	light red	4,3,2	
13	red	4,3,2		104	pink	4,3,2	
14	light red	3,2		112	red	---	some sectored colonies
15	red	4,3,2	some small colonies	113	dark red	4,3,2	
16	red	4,3,2		114	pink	4,3,2	some small colonies
17	pink	---	does not grow well	115	light red	4,3,2	
18	red	4,3,2		116	light red	4,3	
19	pink	3,2		121	red	4,3,2	
20	red	2:2dead		123	light red	3,2	
24	light red	---	does not grow well	125	red	4,3	
26	red	4,3		128	light red	4,3,2	
29	red	4,3,2		130	light red	---	some sectored colonies
30	red	2:2dead		132	light red	3,2	
31	light red	4,3,2		136	dark red	---	keeps URA plasmid
35	red	3,2		139	light red	4,3,2	
36	red	4,3,2		142	light red	4,3,2	
37	red	4,3		143	light red	4,3,2	some small colonies
38	red	3,2		144	red	---	does not grow well
43	light red	2:2dead		146	light red	4,3,2	

45	light red	----		does not grow well	148	light red	4,3	
48	light red	4,3,2		some small colonies	149	light red	3,2	
51	light red	2:2dead			153	red	----	keeps URA plasmid
52	light red	----		does not grow well	154	light red	4,3,2	
53	red	2:2dead			157	red	----	keeps URA plasmid
63	light red	----		does not grow well	159	dark red	4,3,2	
64	light red	----		some sector colonies	164	light red	2:2dead	
66	red	----		keeps URA plasmid	169	pink	4,3,2	
73	light red	4,3,2			172	red	4,3,2	
80	red	4,3,2			173	light red	4,3,2	
81	light red	4,3,2			177	light red	3,2	
82	dark red	4,3,2			179	red	4,3,2	

Table 3.1 Observed growth pattern following dissection of diploids. All 70 synthetic lethal mutants and their color are listed. All 70 mutants were crossed with the wild type strain MSY20, sporulated and dissected. A minimum of 12 tetrads were dissected for each mutant and the growth patterns observed are listed. Strains which behaved oddly (with the exception of the observation of small colonies) were disregarded and not further analysed.



YPD 30°C

Figure 3.3 Linked segregation of mutant alleles. Dissection of mutant #3 crossed with MSY20 (wild type strain) on YPD at 30°C. Tetrads 1 through 12 show a distinct 2 live: 2 dead pattern indicating linked segregation of the mutant alleles. The second mutation is either in or very close to the *BET3* gene. Dissected tetrads were also replica plated onto YPD and grown at 38.5°C and show no temperature sensitivity (not shown). Absence of growth on -URA confirmed the loss of the balancing *BET3 URA3* plasmid (not shown).

contain a second *bet3* mutation resulting in lethality (see Table 3.2).

Thirteen strains (mutants #17, 24, 45, 52, 63, 64, 66, 112, 130, 136, 144, 153 and 157) were disregarded as they either grew very poorly or they did not show conclusive growth patterns following dissection since they kept the wild type balancing plasmid (see Table 3.1).

3.3 *bet3-4* is synthetically lethal with genes encoding the TRAPP subunits *TRS23*, *TRS65* and *TRS85*

To determine whether any of the induced mutations were in known TRAPP genes, all confirmed synthetic lethal mutants were transformed with a library of plasmids containing all known TRAPP subunits (*BET5*, *TRS20*, *TRS23*, *TRS31*, *TRS33*, *TRS65*, *TRS85*, *TRS120*, *TRS130* and *TCA17*) on either *LEU2* or *HIS3* plasmids (see Table 2.2 and Figure 3.4). The synthetic lethal mutants which showed some complementation with the library of TRAPP subunits were further transformed with individual TRAPP subunits (see Figure 3.5). Transformants were replica plated onto 5-FOA plates to see if they could survive the loss of the *BET3* gene on the balancing *URA3* plasmid. If all transformants grew on 5-FOA then the specific TRAPP gene in question is able to complement either the *bet3-4* mutation or the second mutation found in that strain. To identify the second mutation, the DNA sequence of the TRAPP gene complementing the phenotype of the synthetic lethal mutant was compared to the sequenced genomic DNA

Table 3.2 Summary of TRAPP mutants

Mutant	Synthetic lethal phenotype when crossed to wt	TRAPP subunit	Identified mutation
7	4,3,2	Trs23	ORF wt, +/-300bp also wt
8	4,3,2	Trs23	1 base missing 29 bases after stop codon
16	4,3,2	Trs23	P194L, base 581 is a C changed to a T
26	4,3	Trs23	
95	4,3,2	Trs23	ORF wt, +/-300bp also wt
98	3,2	Trs23	G13S, base 37 is a G changed to an A
139	4,3,2	Trs23	
142	4,3,2	Trs23	
169	4,3,2	Trs23	
172	4,3,2	Trs23	
81	4,3,2	Trs23/Trs33	
104	4,3,2	Trs23/Trs33	
114	4,3,2	Trs23/Trs85	
148	4,3	Trs23/Trs31	
177	3,2	Trs23/Bet5	
37	4,3	Trs85	premature stop codon, 58a.a. truncation
159	4,3,2	Trs85	premature stop codon, 392a.a. truncation
146	4,3,2	Trs65	
149	3,2	Trs65/Trs120	
3	2:2	Bet3	
6	2:2	Bet3	
20	2:2	Bet3	
30	2:2	Bet3	
43	2:2	Bet3	
51	2:2	Bet3	
53	2:2	Bet3	
164	2:2	Bet3	

Table 3.2 Summary of TRAPP mutants. Synthetic lethal mutants which showed some complementation by a library of TRAPP subunits were classified as TRAPP hits. These were further tested with individual TRAPP subunits and the complementing subunit(s) are listed. *TRS23* was sequenced in mutants #7, 8, 16, 95 and 98. *TRS85* was sequenced in mutants #37 and 159. Mutants that had a 2:2 dissection pattern are presumed to contain mutations in *bet3* or a tightly linked gene.

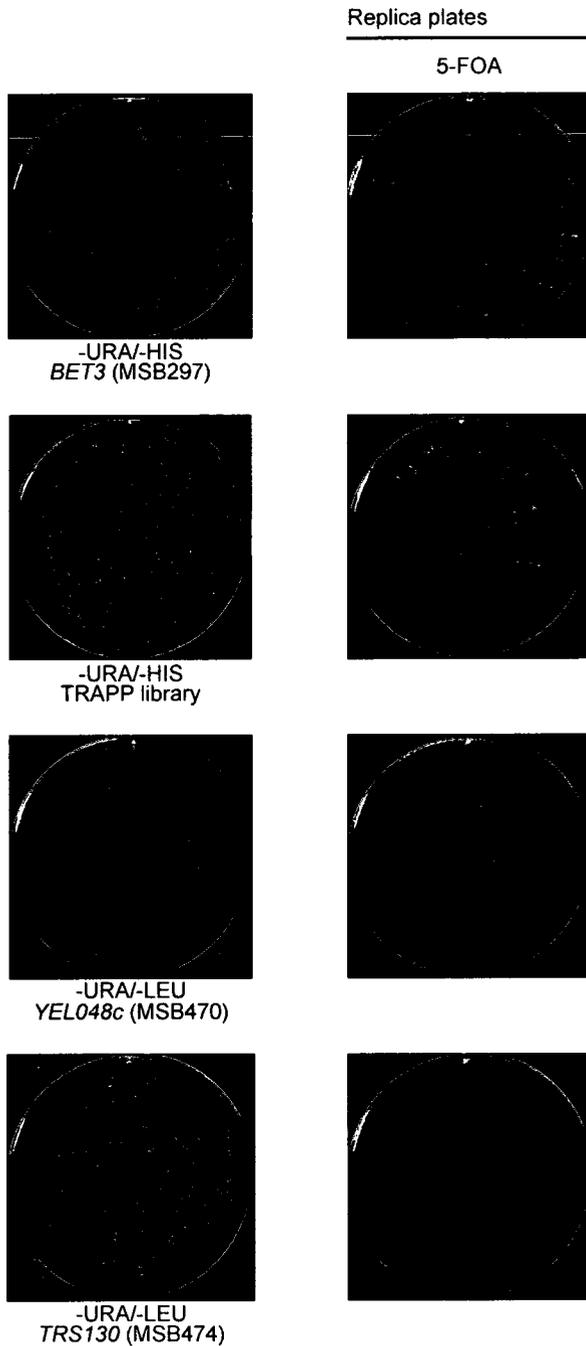
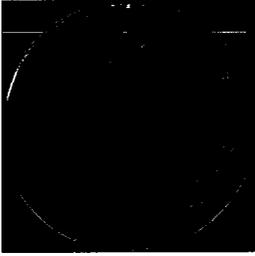


Figure 3.4 TRAPP library screen. Mutant #98 was transformed with the TRAPP library on plasmids containing either *HIS3* (*BET5*, *TRS20*, *TRS23*, *TRS31*, *TRS33*, *TRS65*, *TRS85*, *TRS120*) or *LEU2* (*TCA17*, *TRS130*) and replica plated onto 5-FOA (30°C) to counter select for the *BET3 URA3* balancing plasmid. Ability to grow on 5-FOA indicates complementation by a TRAPP gene. As seen by growth on 5-FOA, all colonies on the

control transformation plate with *BET3* (MSB297) were able to grow without the balancing plasmid. No growth is seen on the 5-FOA plates for the *TCA17* and *TRS130* transformations. Approximately 1/8th of the TRAPP library transformants were able to grow on 5-FOA. Therefore, mutant #98 most likely contains a mutation in one of the TRAPP genes tested on that plate.

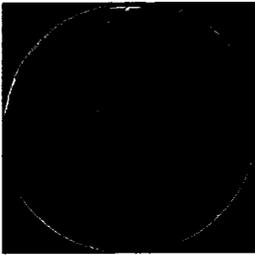
Replica plates

-URA/-HIS

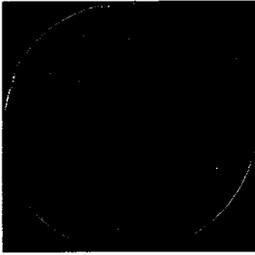
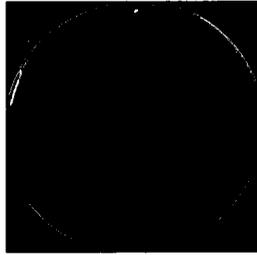


BET3 (MSB297)

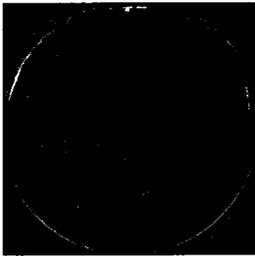
5-FOA



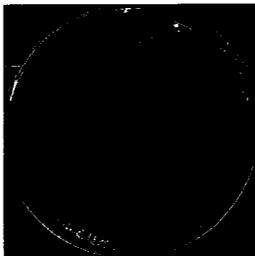
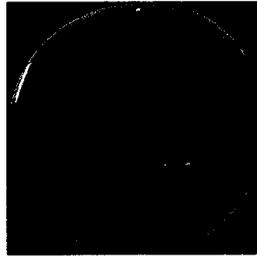
BET5 (MSB299)



TRS20 (MSB300)



TRS31 (MSB301)



TRS23 (MSB302)

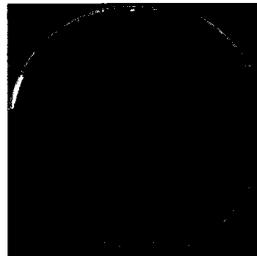


Figure 3.5 (continued) Complementation by a TRAPP subunit

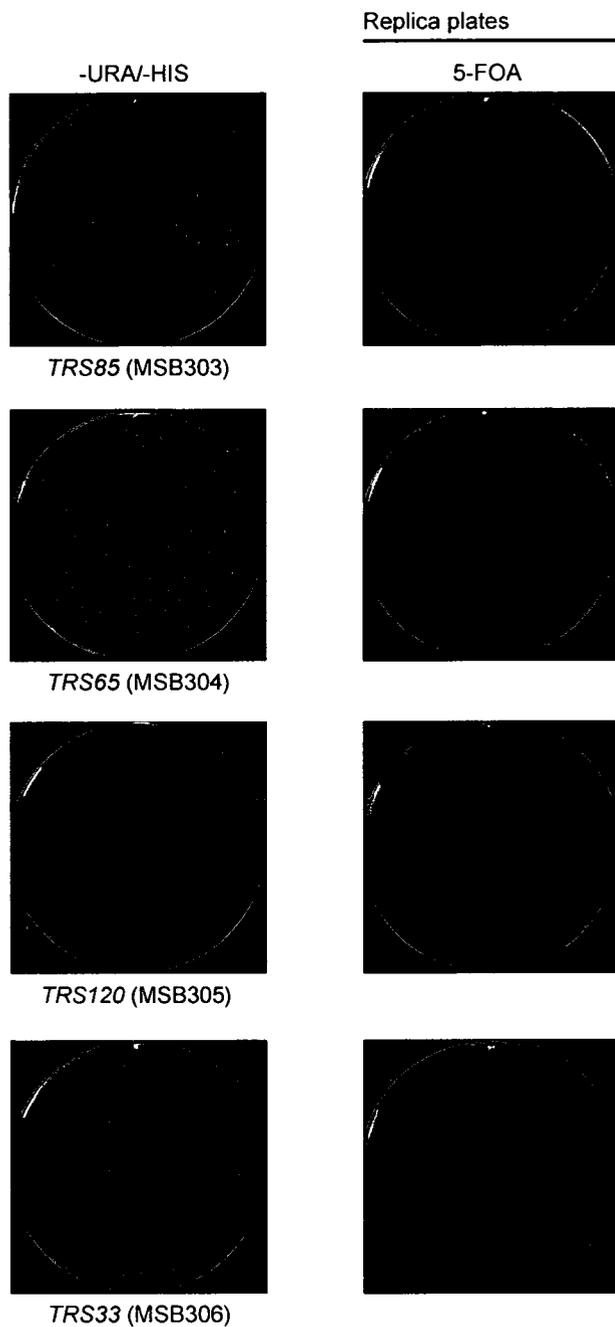


Figure 3.5 Complementation by a TRAPP subunit. Mutant #98 was transformed with individual TRAPP subunits (*HIS3* plasmids) and replica plated onto 5-FOA (30°C) to counter select for the *BET3 URA3* balancing plasmid. Ability to grow on 5-FOA indicates complementation by the TRAPP subunit. Only *TRS23* is able to complement one of the

mutations present in mutant #98 and allow growth on 5-FOA. Mutant #98 most likely contains a mutation in *TRS23*.

of the untreated (no EMS exposure) strain (MSY211a). *TRS23* and *TRS85* were confirmed as genetic interactors of the *BET3* allele *bet3-4*. While *TRS65*, *TRS31*, *TRS33*, *TRS120* and *BET5* complemented some of the synthetic lethal mutants (see Table 3.2), they were not sequenced to confirm the location of the mutation(s) present in the mutants since we were interested in non-TRAPP genes.

Potential *trs23* mutants: *TRS23* was able to complement the synthetic lethal phenotype in ten mutants generated from the screen (#7, 8, 16, 26, 95, 98, 139, 142, 169 and 172) (see Table 3.2). To verify that these strains indeed contained mutations in *TRS23*, five representatives of this pool (#7, 8, 16, 95 and 98) were sequenced with primers that covered only the ORF or primers that included the flanking 400 bases. Sequences were compared to that of untreated MSY211a (see Figure 3.6). Mutant #8 had no mutations present in the ORF but revealed a one base deletion 29 bases after the stop codon. Mutant #16 contained a C to T substitution at position 581 resulting in a P194L mutation in the protein. Mutant #98 contained a G to A substitution at position 37 resulting in a G13S mutation in the protein. No *TRS23* mutations were found in mutants #7 and #95.

There were five mutants (#18, 104, 114, 148 and 177) which showed complementation of the phenotype by *TRS23* as well as a second TRAPP subunit: #81 and #104 (*TRS33*), #114 (*TRS85*), #148 (*TRS31*) and #177 (*BET5*) (see Table 3.2). While both genes can complement the mutation, only one of these genes harbours the mutation. These mutants were not sequenced since we were interested in non-TRAPP

TTTTGGCAGAGATTGCTGGGGCGAGATGCAGTACTACACCCTTTAATGGAAAACTGAATAGGAAATGGTACAC
 GAGATTTTTCTTCCCATAAAATGGAAGTTATCAATATTGTAAAGAGAAGCATTTACAAGCTTTTATTTTTCTTTT
 TAATTTCCACTACTGGTTCTGCTTTAAAATGTTGTTTTATAATTTATGTACATTTAGGCCATAGAAGATTCTTT
 CAATAATATGCTACACATTCTTTTATTTTTCCATCATATGTTGGAGTTTATGCCCTCTCGGCAGGAGTTGGGCGG
 TGCGAAGAGAAGAAAAAGAGTGAAACTAAAAAAGGAATCTGCCTTTGCATAAGTTCAAAGTGCAATTTTAGTG
 TTGGATTTAAACGGGAAAAATTGAAATGGCCATCGAAACAATACTTGTAAATAAACAAATCAAGCGGACTAATCTA
 TCAGCGGAATTTTACCAACGACGAACAGAAATTGAACAGCAATGAATACTTAATTTCTTGTAGTACACTGCACGG
 TGTATTCCCATCGCGAGCCAGCTGACTCCGAAGGCATTACAGCTAACTCAACAAACGAACATCGAAAATACCAT
 CCCATATATACCTTACGTGGGCATGTCCAGCAATAGGAGCGATACAAGAAATGGAGGTGGCAATAACAACAACA
 CACTAATAATGAAAACTGGGCAGTTTTAAAGGAGATGATTTTTTCAAAGAACCATTTACGAACTGGAACAAGAG
 CGGATTGAGACAACATGACCGGATCAGTTCACGATGTTTATATATCAGACCCTAACCGGCTGAAGTTTGTGCGC
 TATCAGCTCCAGCGTCATGCCCCAGAGACAGCCAATATAGCCACCACCGACAAGCCTGACCGACCCAAGAGTAC
 ATCCAATTTGGCCATCCAAATAGCCGACAACCTCCTAAGGAAGGTTTACTGTTTGTATAGTACTACGTCATGAA
 AGACCCATCTTACTCAATGGAAATGCCTATAAGATCTAACCTGTTTCGACGAAAAAGTCAAAAAATGGTAGAAAA
 CCTACAAATAGACGCACGCGCCATCGGAAGATAAACGCATTCACACCAAGAAAATAAATACTACGTACTTCTAGAA
 CTAAGAAGAAGTTGCCGCTCTCCGTCTCCTCCTCATAAAATAGGGATAGCTTTTACTACTACGTACGAAAAAAGA
 CCACACTGCGACGGATCCTCTGCATCCCAACACCAGCGATAGAAAATGCGCCGCCATATATCGTCATCTTGCTTCC
 TTTTTCTTACTCCCTTCTGCCCTGGAAACACCATACAATCCATCACATCATATCCTGCCAATGCACATATTACGC
 AGCTTTTCGGCAGTGAGTACGGAAACACCAGTTCGGAACCGGGTACACAATAGGGATAGTCAAAGCTGTCCATTT
 TTTCCGCTGATGGATGACTGATACCAAAGTGTAAC

Figure 3.6 *trs23* mutants. The sequence of *TRS23* including 400 bases upstream and downstream of the ORF. Trs23p is 219 amino acids long. The start codon is highlighted in green while the stop codon is highlighted in red. Three synthetic lethal screen mutants were shown to have mutations in the *trs23* gene. Mutant #98 has the earliest mutation with a G to A base substitution at position 37 (highlighted in blue) which results in a G13S missense mutation. Mutant #16 has a C to T base substitution at position 581 (highlighted in grey) which results in a P194L missense mutation. In mutant #8 there are no changes in the ORF for *TRS23*. However, there is a deletion of the twenty ninth base after the stop codon (highlighted in purple).

genes and we would need to sequence several genes in each of these strains.

Potential *trs65* mutants: Mutants #146 and #149 could be complemented by either *TRS65* or both *TRS65* and *TRS120* (see Table 3.2). Genomic DNA from neither of these mutants was sequenced for reasons stated above.

Potential *trs85* mutants: *TRS85* was able to complement the second mutation found in two mutant strains: #37 and #159 (see Table 3.2 and Figure 3.7). PCR of the extracted genomic DNA was used to amplify the *TRS85* ORF and 400bp upstream and downstream. Mutant #37 contained a single base substitution resulting in a premature stop codon and a 58 amino acid truncation from the C-terminus of the protein. Mutant #159 also contained a single base substitution resulting in a premature stop codon and a 392 amino acid truncation.

3.4 Genetic interaction between *TRS85* and *BET3* is not allele specific

To test if the interaction between *TRS85* and *BET3* is specific to the allele used in the screen, a *TRS85* deleted strain was crossed with *bet3* mutants at our disposal to test for a synthetic genetic interaction. Since *TRS85* is not essential, it can be completely deleted from a strain while maintaining viability. A *trs85* Δ ::*HIS3* (MSY214) was crossed with three different mutant alleles of *BET3*: *bet3-3*, *bet3-4* and *bet3-5* (found in strains MSY14c, MSY15a and MSY146, respectively). Diploids were sporulated and dissected

TACCTTCCTCATCTTGATGCTGAAAGTAAATTGATGGAGTCAAGCGGTTACCAATAGCGGGATTTTCATCGCCTG
 AGTGATATGTTGTGGGAGATAGGCCTGGCAAGGAAAAATCCTTTAGTTAAAGTTGCATAGCAATAAATCAGCAACC
 AAACACCTCGTTTCATTACCGATTATTAATTATCAATATGTGTACTAATAATAATTGTCAAATTTATGATGTAAA
 TTTAGGGTCCCAACATATTTTACTCAACTGTAACAAGTCATAATTTCTCGGACAAAAATTAGGCAAAAATAACA
 GAAAAACCAATGGATGGGATGGGTAGGAAAATGAGTAAGTAACCCAAACAAACGGTACCTCTTTATTTCAGTCGGC
 TTTACAGATACTGAGGTAACCTATAATGGTTTTTCTTATGAGCACTATATGAATCTCCTTTTCCATTTGGATAA
 CAGTAAAGAAACGGTGCCTCCAGAGATTGCAAAAAGAATAATTTCAAATGCTATAGCTCCTGTAATAACAGTTAC
 TTCAACTCCTCTCTCGACAAACATATTCAAGAAACGTACAAGTAGATTCTCTATATGCTGCTGCGATTCTT
 TGGCGGTTGTGCTCTGATAGAGATCAAGCCAATGAAGCGAAGGTTGGACAGCATGAGCATGAGGTTTGTGATGC
 AAGTACTCGACGGATTCAATTTCCAAAAATAAAAAATTTGGAAGTGCCCAATTTATCAAAGAAAGGTAGTCGCAG
 TAGGTCGAATAGTCTTTTCCAGAGGGATTCAACGCAATCTCAATATATCAGGTTTACAAGGCCATTAGGTGACTT
 GATCGAAACAAGAGATGCAAATGATATGTTATTCAATTACCATTCTTTAGAGGTATTCTTAGATAAATTATTTGAA
 ATTGGTTGCAGCAAATACTGATGAAATGGTTCCTCATAATCTTCTTAAGAAATCCATTTATCATAGTTTCTTTTC
 ACTAGCAATTTTCATCCACAAATAAATTATCGCCCTATGAAACTTTTAATCACCCATTCTTTCCCTTGATTGCTTT
 AGATATATCAAATGGCGAAGTTTATGAGGATGCAAGAGATCTTTTAGTCAATTTCAAGAAATCTTAATCATAATAC
 TGAAAATTTTCTATCTTCATGAATACAAATGAAATGCTTCCAGTTTTCTTACTCTGCTACAATGACGATTCCCA
 AGAAGAATTCGAAAAATGCCAGGCGTTAGCTAAGAACTAAAGAAGCAGTTGTTTGTGAGAGTATCTTACTAGC
 ACTCTGGAAGGATTTCTTTTATTACGACGAAAAATTCAGTCATAAGTTACACCAACCAGTAATGTCATCGCTTGA
 AGAAATTTCTTCTTCTTCAAGCTCCAACCTCAACAACACTCTCTCTGGCTTTGATAAACTCGATCTATGATAT
 GCTTGATTATTTGGTTTATGATTTAATGATACCATTTCATGAAAAGAAAAGTGTCTTCTGGGAAGAGACAATTTT
 ACAGCCAAGAAAAGTCGCTATTTAATGGTGCAAAGTTTTTCAAAAAATTTATGAATAAAAAATCCTGTCAATGGTAA
 TCACCAACATAAATCTCTAACGAGAGACAGCCAGGGAATGAATACTTCGCATCGTCATCTTCTGAGTTTTTGTAT
 GAGAAAGTTAGCAGATTGGTCTATGATGCTATCCGACTTCAAACTGCTTATTTCCACATACGAATCGCTTATGGA
 TGACCTAGATGCATTTCCAAAGTACCTGGCCTCATGCATCGAATGGTGGCGGGTATCACTATTTGATGGGTGGCGA
 GAGCATAGTCACCGTGAATATGATCAAAAACGATATAAATCCTCTTATCGAAAGGGCATTAGCCACATACGAAAA
 CTGCTCACGAATACAACGTGGTAAAGGCAAAGAATCAAACCTTTTGGATGTTACAGAGCCAGTGGCTTCGATGA
 GACACGTTGTATGATTTTGGCATCTGAATTTGTTTTATCTTTAAGCAATACGTGGACATCTACCCCATACGCTAT
 CCAATATTTAGAAAACAATTCTAGACGAGTGCAAGTTGGGACCTTGTTCACAGATAATGGTTTGGGAAAGGCTTAG
 TGACTGCTATAAATTTGAGAGTTGACCCTAGAATCAAACATAGAGTTGGAGCAATGAAGAAGGACGCTAAAGACAC
 CGAAGATCTCCGAGGTGAGCATAAGTATAGCACAGATCAATTCACAGACGAGGACATATTTTCGGAAGGGTTAAC
 AAGAAGACGCAAGGCAGCTTTTTTTAGGTTAATAGCAGCTAAGAAGTGGGCAGAGCAAAAACAATGGAGAAGGTT
 TTCTTGGTGCCTAAAAGATATTGAAAGTACCTATTCAGAGATCAAATTTTTGCATGGTAACGGTTTAAATTTAAG
 CAAACTAAAAATCAACTCAATTTAAAGGACGTGGATTCTGCACCACGGCCCTCCGAAAAGAACTTACAAGAAC
 AAGTGTAGCTTTATTGGAATGACTTTATGGAAAAATCATGTTTTGAGTATAAATTATACGTACGAATCTTATAGA
 TATATATTTTTCTTTTAAAACCTCCATTTTCAGCTCATAAGCCGATACAAACACCTTCTATATATTTTCTCTAAC
 AGCTATGTTAACATGATTGCCTTTGTTTATCTACTAAAGGACCCTTCTACTTTATCTACCATACGCCTATATTTT
 CTCTGTGTTTCAATCATATCGAGAAAAATTTGGTACTTCGTGTCTAAAAGAATTCTATCTGGATGAGTTTTCTCA
 TTTGGATTGACAAATCTTGCATTACCCGTTAGCTCTGCATAACTTTCCATAGAAAACCTTGTCGGTTATATCTT
 CCCTCTCCTAGGCTCTCCTGTCCCACGGTCAATGAAGCATCCTTACT

Figure 3.7 *trs85* mutants. Trs85p is 698 amino acids. The sequence of *TRS85* including 400 bases upstream and downstream of the ORF. The start codon is highlighted in green while the stop codon is highlighted in red. Two synthetic lethal screen mutants were shown to have mutations in the *trs85* gene. Mutant #159 contained the earliest mutation with a C to T base substitution at position 919 (highlighted in purple) resulting in a premature stop codon and a 392 amino acid truncation of the protein. Mutant #37

contained a C to T base substitution at position 1921 (highlighted in blue) resulting in a premature stop codon and a 58 amino acid truncation of the protein.

onto YPD plates (grown at 30°C). The dissection plates of *trs85Δ::HIS3* x *bet3-3* and *trs85Δ::HIS3* x *bet3-4* were replica plated onto YPD (38.5°C), –LEU and –HIS. The dissection plate of *trs85Δ::HIS3* x *bet3-5* was incubated at 25°C and replica plated onto YPD (38.5°C) and –HIS (25°C). The growth patterns of these dissected tetrads were analysed for a synthetic genetic phenotype (see Table 3.3 rows 1 to 3). If a synthetic lethal interaction is observed there would be a combination of dissected asci which have 4, 3 or 2 ascospores growing at the permissive temperature. If no synthetic lethal phenotype is observed, all the ascospores from all asci should be able to grow. The crosses between *trs85Δ::HIS3* and *bet3-3* (Bet3p(K24/96E)), *bet3-4* (Bet3p(A94L)) or *bet3-5* (Bet3p(G64E)), all showed a synthetic lethal interaction (4, 3, 2 or 3, 2 growth pattern) indicating that the interaction is not allele specific (see Figure 3.8).

3.4.1 Genetic interaction between *TRS85* and *TRS33*, *TRS65*, *TRS130* and *BET5*

TRS85 was also tested for a genetic interaction with mutant alleles in genes encoding other TRAPP subunits: *TRS33*, *TRS65*, *TRS130* and *BET5*. A strain containing *trs85Δ::KanMX* (MSY362) was crossed with the following TRAPP mutants: *trs33Δ::HIS3*, *trs65Δ::HIS3*, *trs130-1::URA3* (temperature-sensitive at 38°C) and *bet5-1::URA3* (temperature-sensitive at 38°C). All sporulated diploids were then dissected on YPD plates (30°C) and replica-plated onto medium to reveal the markers linked to each mutation. (see Table 3.3 rows 4 to 7 and Figure 3.8). A synthetic lethal interaction was seen with *bet5* as indicated by the 4,3,2 growth pattern. No synthetic genetic interaction

Table 3.3 Genetic interaction between TRS85 and BET3, BET5, TRS33, TRS65 or TRS130

Strain	Diploid selection	Replica plates	Growth pattern following dissection
trs85Δ (MSY214) x bet3-3 (Bet3p(K24/96E)) (MSY14c)	-HIS/-TRP	-HIS, -LEU, YPD (38.5°C)	4,3,2
trs85Δ (MSY214) x bet3-4 (Bet3p(A94L)) (MSY15a)	-HIS/-TRP	-HIS, -LEU, YPD (38.5°C)	4,3,2
trs85Δ (MSY214) x bet3-5 (Bet3p(G64E)) (MSY146)	-HIS/-TRP	-HIS (25°C), YPD (38.5°C)	3,2
trs85Δ (MSY362) x trs33Δ (MSY21)	G418/-HIS	G418, -HIS	not lethal, sick
trs85Δ (MSY362) x trs65Δ (MSY22)	G418/-HIS	G418, -HIS	no genetic interaction
trs85Δ (MSY362) x trs130 ts mut (MSY174)	G418/-URA	G418, -URA, YPD (38.5°C)	no genetic interaction
trs85Δ (MSY362) x bet5 ts mut (MSY179)	G418/-URA	G418, -URA, YPD (38.5°C)	4,3,2

Table 3.3 Genetic interaction between TRS85 and BET3, BET5, TRS33, TRS65 or TRS130. (Rows 1-3) A trs85 deleted strain

(MSY214) was crossed with three temperature-sensitive *bet3* alleles. All show a synthetic lethal interaction indicating that this interaction is not allele specific. (Rows 4-8) A *trs85* deleted strain (MSY362) was crossed with five TRAPP mutants and the dissection patterns analysed. A synthetic lethal interaction was seen with *bet5-1*, while no synthetic genetic interaction was observed with *trs65Δ* or *trs130-1*. The *trs85Δ* and the *trs33Δ* mutants have a synthetic genetic interaction seen by a reduced ability to grow at 30°C.

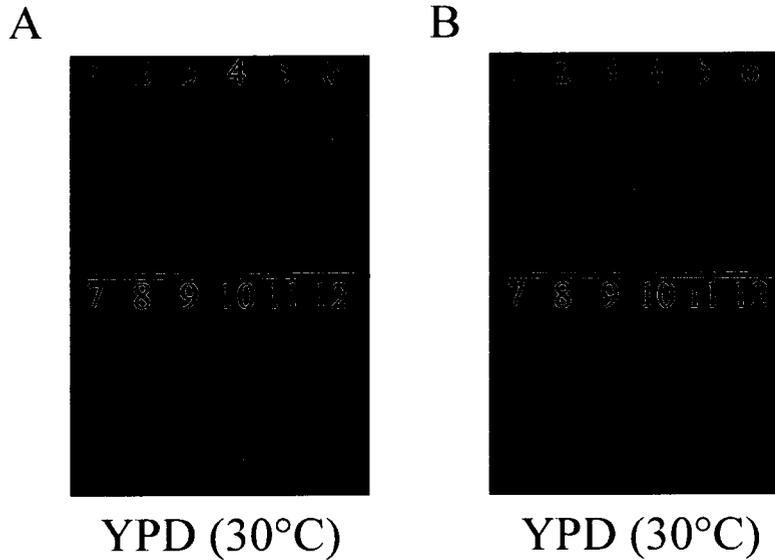


Figure 3.8 Synthetic lethal and non-synthetic lethal growth patterns. (A) Synthetic lethal growth. Dissection plate on YPD at 30°C of *bet5-1 x trs85Δ*. All twelve dissected tetrads show a growth pattern indicative of a synthetic lethal interaction (4,3,2). All four ascospores grew in tetrad 5. Three of the four ascospores grew in tetrads 2, 3, 4, 6, 8, 9, 10 and 11, while only two grew in tetrads 1, 7 and 12. (B) Non-synthetically lethal mutations. Dissection plate on YPD at 30°C of *trs65Δ x trs85Δ*. Ten of the twelve dissected tetrads show a growth pattern indicating no genetic interaction between the mutations present. All four ascospores grew in tetrads 1 to 4, 6, 7 and 9 to 12. To ensure the quality of the tetrads chosen and to track the location of the mutations present, dissection plates were replica plated onto selective media and the growth patterns analyzed (not shown).

was observed with *trs65* Δ or *trs130-1* since all four ascospores in the tetrads were able to grow even when both mutations were present in the same cell. In the *trs85* Δ cross with *trs33* Δ , while the mutations are not synthetically lethal the mutations have a synthetic genetic interaction seen by a reduced ability of the double mutants to grow at 30°C.

3.5 *BET3* genetically interacts with non-TRAPP genes

Synthetic lethal mutants (thirty) which showed no complementation by any TRAPP subunit tested were presumed to have a mutation in a non-TRAPP gene (mutants # 5, 13, 14, 15, 18, 19, 29, 31, 35, 36, 38, 48, 73, 80, 82, 85, 87, 99, 113, 115, 116, 121, 123, 125, 128, 132, 143, 154, 173 and 179) (see table 3.4). To identify these genes, a library (YEp213) containing all yeast genes was used. This library, on a *LEU2* plasmid, was transformed into a subset of these mutants (mutants # 5, 15, 29, 35, 36, 38, 80, 121, 128 and 132) which represented the range of mutants identified based on color intensity (red and light red) and dissection results (smaller colony size arising from a subset of ascospores). A full screen (mutants # 5, 15, 29, 80 and 121) was performed if less than five white colonies were observed in the small scale screen. Transformants were screened for complementation based on color change. There is a background of red colonies which did not receive the wild type copy of a gene which can complement one of the two mutations (either *bet3-4* or the unknown mutation). White or sectored colonies should be rare as few colonies receive a plasmid which can complement one of the mutations and no longer keeps selection for the balancing *BET3* plasmid. These white or sectored

Table 3.4 Summary of non-TRAPP mutants

mutant	# viable progeny per ascus	mutation	YEP213 library screen
15	4,3,2	not TRAPP	possible <i>VPS16</i> or <i>ELC1</i> mutant
5	4,3,2	not TRAPP	no rescue
29	4,3,2	not TRAPP	no rescue
80	4,3,2	not TRAPP	no rescue
121	4,3,2	not TRAPP	no rescue
13	4,3,2	not TRAPP	N/A
14	3,2	not TRAPP	N/A
18	4,3,2	not TRAPP	N/A
19	3,2	not TRAPP	N/A
31	4,3,2	not TRAPP	N/A
48	4,3,2	not TRAPP	N/A
73	4,3,2	not TRAPP	N/A
82	4,3,2	not TRAPP	N/A
85	4,3,2	not TRAPP	N/A
87	4,3,2	not TRAPP	N/A
99	4,3,2	not TRAPP	N/A
113	4,3,2	not TRAPP	N/A
115	4,3,2	not TRAPP	N/A
116	3,2	not TRAPP	N/A
123	3,2	not TRAPP	N/A
125	4,3	not TRAPP	N/A
143	4,3,2	not TRAPP	N/A
154	4,3,2	not TRAPP	N/A
173	4,3,2	not TRAPP	N/A
179	4,3,2	not TRAPP	N/A
35	3,2	not TRAPP	small scale only
36	4,3,2	not TRAPP	small scale only
38	3,2	not TRAPP	small scale only
128	4,3,2	not TRAPP	small scale only
132	3,2	not TRAPP	small scale only

Table 3.4 Summary of non-TRAPP mutants. Synthetic lethal mutants which were not complemented by any of the tested TRAPP subunits were classified as non-TRAPP hits. Small scale yeast plasmid library screens were carried out on mutants #5, 15, 29, 35, 36, 38, 80, 121, 128 and 132. Further large scale screens were done with mutants #5, 15, 29,

80 and 121. A *VPS16* containing YEp213 plasmid was able to complement a mutation found in mutant #15.

colonies were isolated three times on –LEU / low ADE plates and finally grown in liquid –LEU medium. Plasmid DNA was extracted from these cultures and amplified in bacteria.

Of the five strains subjected to a full screen, only mutant #15 yielded a plasmid that could complement the lethality when re-transformed into the original mutant strain (see Figure 3.9). Plasmids from the white colonies observed from mutant #15 that did not contain *BET3* as judged by PCR were sequenced. Sequencing of one such plasmid revealed it originated from chromosome XVI. The chromosomal map of this region (chromosome XVI coordinates 466119bp to 470749bp (see Figure 3.10) indicated the presence of two whole genes (*VPS16* and *ELC1*), the 5' end of a third gene (*NOP4*, 1-814bp out of 2058bp) and the 3' end of a fourth gene (Ingvarsdottir *et al.*, 2005) in the insert. Interestingly, Vps16p is a component of the multisubunit vesicle tethering complexes HOPS and CORVET which are involved in vesicle trafficking in the endosomal pathway (Peplowska *et al.*, 2007). *ELC1* is involved in transcription elongation and plays a role in excision repair. Of the more than 20 genes shown to interact with *ELC1*, it genetically interacts with *YPT6* and *SEC28*, two genes involved in membrane transport at the level of the Golgi. Nop4p is involved in ribosomal RNA processing (Sun and Woolford, Jr., 1994) while Sgf11p is a subunit of the Spt-Ada-Gcn5-acetyltransferase (SAGA) histone acetyltransferase complex (Pfeffer, 2001; Ingvarsdottir *et al.*, 2005).

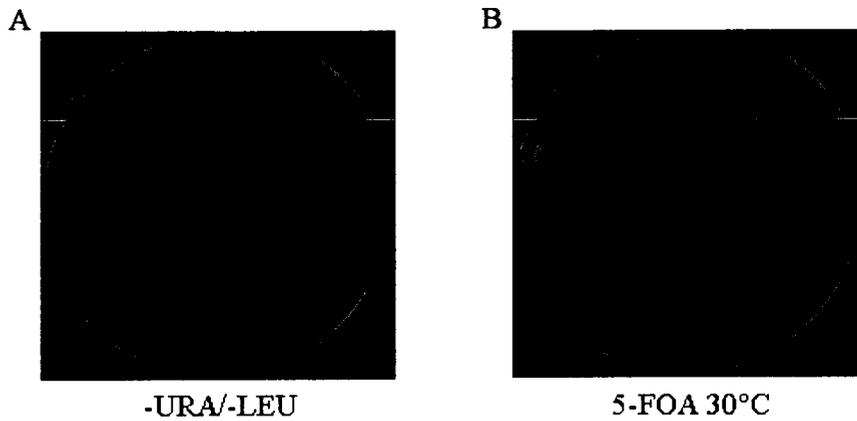


Figure 3.9 Complementation in mutant #15 by *VPS16* and *ELC1* containing library plasmid. (A) Mutant #15 was re-transformed with the *VPS16* and *ELC1* containing plasmid identified in the yeast plasmid library screen (saved under MSB736) and plated on -URA/-LEU and grown for 3 days at 30°C. (B) Transformants were replica plated onto 5-FOA and grown for 2 days at 30°C. Transformants show some re-growth on 5-FOA.

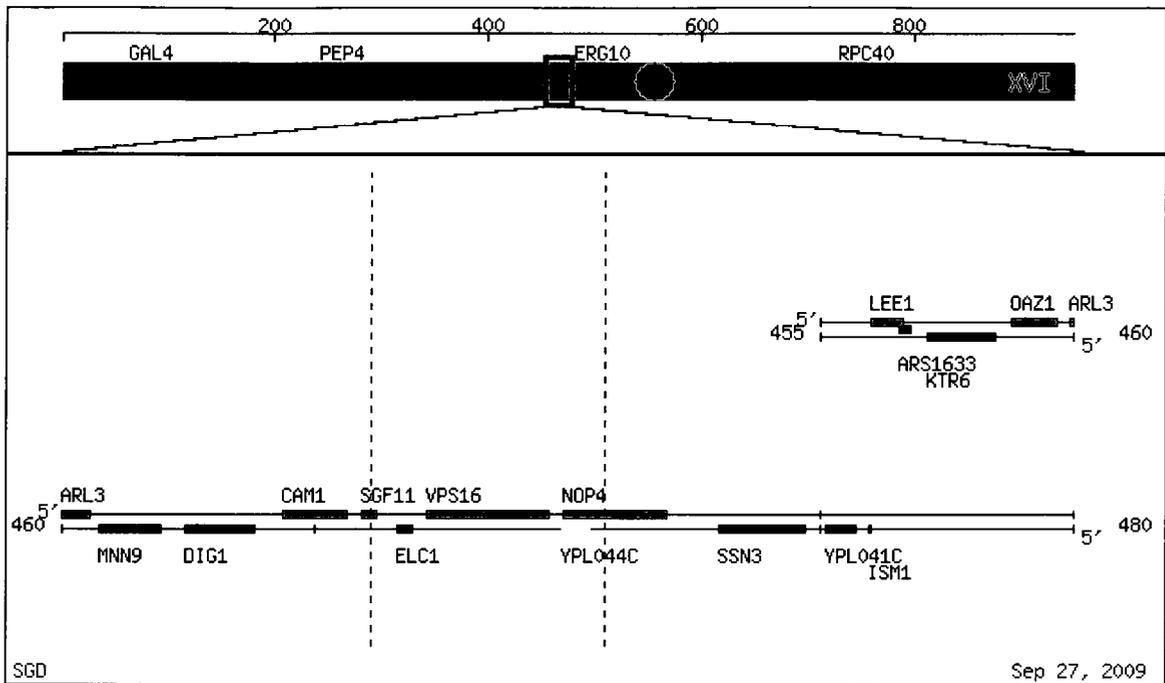


Figure 3.10 YEp213 yeast plasmid library insert which complemented mutant #15.

The chromosomal map of chromosome XVI coordinates 466119bp to 470749bp. There is a whole gene (*VPS16*) present and the N-terminal part of a second gene (*NOP4* 1-814bp out of 2058bp) in the insert.

3.6 *VPS16* appears to suppress an unidentified mutation in mutant #15

The extracted library plasmid containing *VPS16* and *ELC1* was able to complement one of the mutations in mutant #15. There are three scenarios to explain this: (i) *VPS16* or *ELC1* suppresses *bet3-4*, (ii) *VPS16* or *ELC1* complements a *vps16* or *elc1* mutation, respectively, (iii) *VPS16* or *ELC1* suppresses some other unidentified mutation.

To test the first possibility, the isolated plasmid was tested in a non-EMS treated *bet3-4* mutant (MSY207 temperature-sensitive at 38°C) and another *bet3* mutant, *bet3-5*, for suppression of the temperature-sensitive growth phenotype (see Figure 3.11). This latter allele was tested to see if suppression would be allele-specific. If any gene or partial gene present in this plasmid can complement the *bet3* mutation, then it should be able to suppress the temperature-sensitive growth at the restrictive temperature. As expected, *BET3* could suppress both *bet3* mutations and the transformed strains grew better at higher temperatures, similar to the wild type strain (panels B, C, F and G). In contrast, the library plasmid was not able to suppress the temperature-sensitive growth defect of either *bet3* mutant tested (panels D and H). These results suggest that the rescue of the synthetic lethality seen in mutant #15 is not due to suppression of the *bet3-4* mutation.

To test which gene is complementing the unidentified mutation in mutant #15, a plasmid construct was made where most of *VPS16* and all of *NOP4* was removed from the suppressing plasmid insert and the plasmid re-ligated together (MSB821; see Figure 3.12). This plasmid was then transformed back into mutant #15 along with the original

suppressing plasmid. A transformation with *BET3* (positive control) and an empty plasmid (negative control) was also performed. Transformants were plated on the appropriate selective medium (-LEU/low ADE or -HIS/low ADE) and grown at 30°C for 3 days. White colonies were observed with the addition of *BET3* or the original suppressing plasmid (MSB736) but not with the plasmid digested with *PvuII* (MSB821) or the empty negative control plasmid (see Figure 3.13). Therefore, *VPS16* or the *NOP4* fragment appears to be necessary for the suppression of the synthetic lethal phenotype in mutant #15.

To directly test if *VPS16* was altered in strain #15, the gene was sequenced in one direction using four oligonucleotides. Comparison to the sequenced wild type strain revealed no mutations in the ORF and the surrounding regions (125 bases upstream and 322 bases downstream). Overall, these results suggest that *VPS16* is suppressing an as yet unidentified mutation in strain #15.

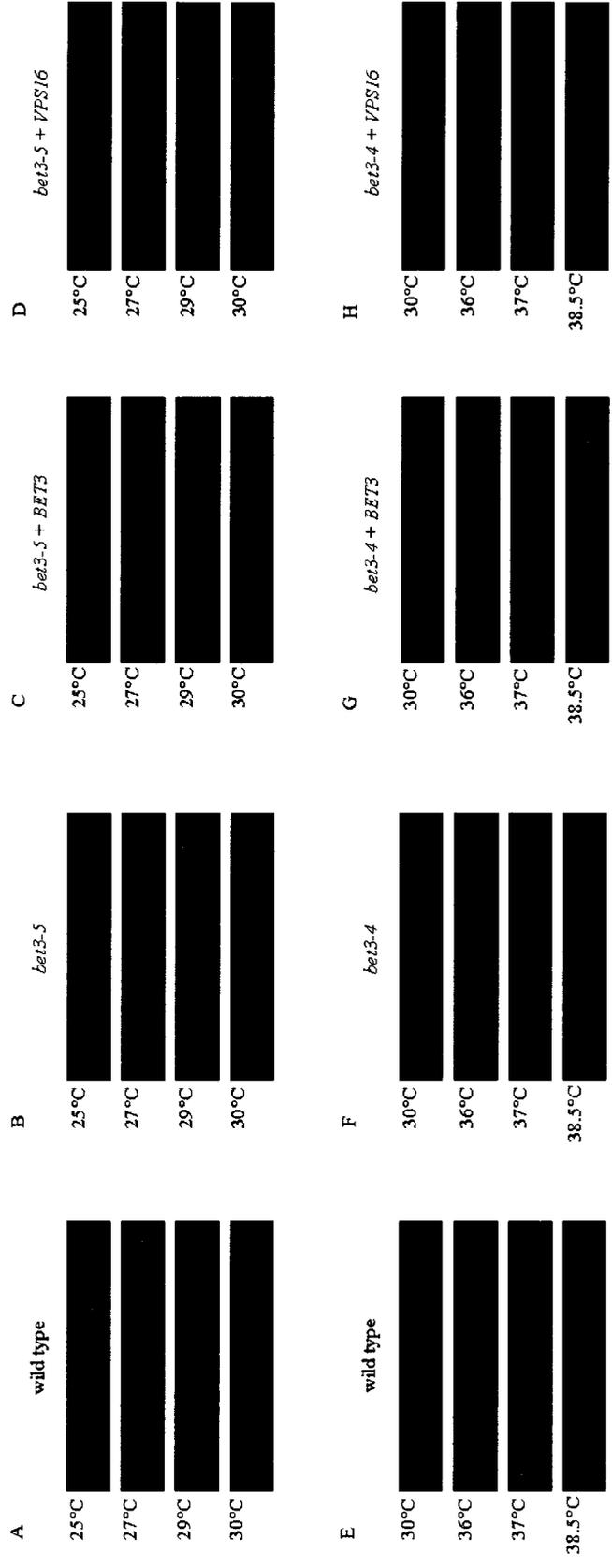


Figure 3.11 *VPS16* does not complement the *bet3-4* or *bet3-5* mutations. Yeast strains were grown overnight in –LEU liquid media and equalized according to their OD₆₀₀ measurements. 2µl of each tenfold serial dilution up to 1/10000 was plated on YPD for each sample and grown at various temperatures. (A) and (E) Wild type strain MSY116 transformed with an empty *LEU2* plasmid from MSB238 was grown at various temperatures: 25, 27, 29 and 30°C in (A) and 30, 36, 37 and 38.5°C in (E). (B) The *bet3-5* mutant strain MSY147 and (F) the *bet3-4* mutant strain MSY207 both transformed with an empty *LEU2* plasmid from MSB238 were grown at various temperatures: 25, 27, 29 and 30°C in (B) and 30, 36, 37 and 38.5°C in (F). (C) The *bet3-5* mutant strain MSY147 and (G)

the *bet3-4* mutant strain MSY207 both transformed with the *BET3 LEU2* plasmid from MSB290 were grown at various temperatures: 25, 27, 29 and 30°C in (C) and 30, 36, 37 and 38.5°C in (G). (D) The *bet3-5* mutant strain MSY147 and (H) the *bet3-4* mutant strain MSY207 both transformed with the *VPS16* carrying plasmid from MSB736 were grown at various temperatures: 25, 27, 29 and 30°C in (D) and 30, 36, 37 and 38.5°C in (H). There was no change in the growth phenotype of *bet3-4* or *bet3-5* with the addition of the *VPS16* carrying plasmid from MSB736. Therefore, it does not complement these mutations.

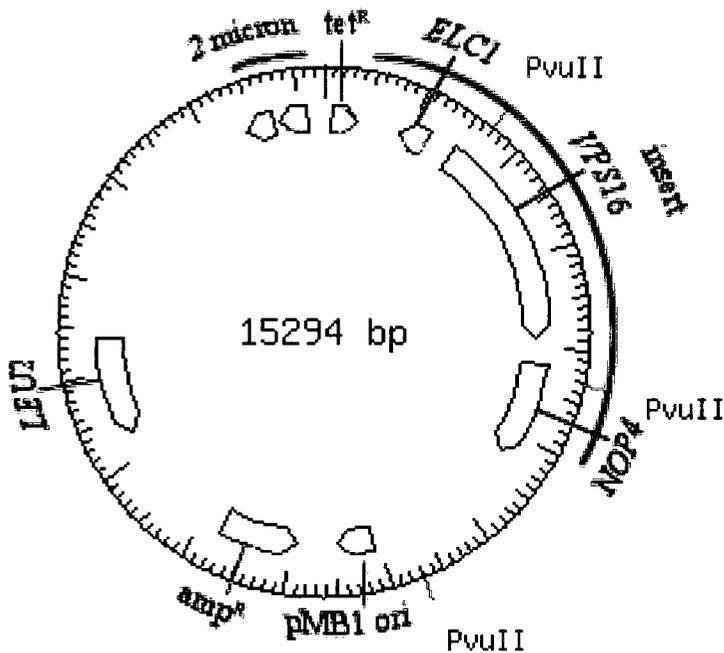


Figure 3.12 Mutant #15 suppressing plasmid. This plasmid (MSB736) from the yeast library (YE_p213) suppresses the synthetic lethal phenotype of mutant #15. The plasmid insert contains two whole genes (*VPS16* and *ELC1*) and two partial genes (*NOP4* and *SGF11*; not shown in diagram due to its small size). Restriction enzyme *PvuII* was used to remove most of *VPS16*, leaving only a few amino acids at the N-terminus, and all of *NOP4*. The re-ligated construct contains the short 3' end of *SGF11* from the original insert, all of *ELC1* and 177bp of the 5' end of *VPS16* (59 amino acids).

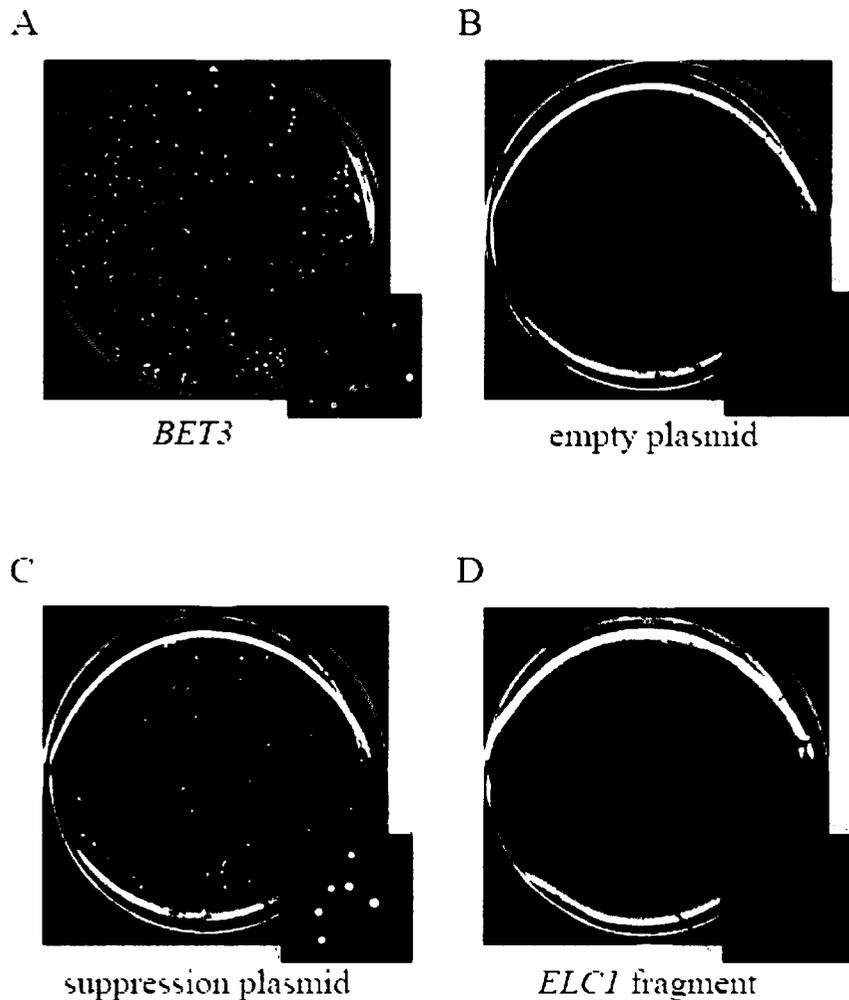


Figure 3.13 *VPS16* or *NOP4* suppresses the synthetic lethal phenotype of mutant #15. Synthetic lethal mutant #15 was transformed with (A) *BET3* plasmid (MSB297), (B) empty *LEU2* plasmid (MSB238), (C) the suppression plasmid (MSB736) and (D) the *ELC1* only containing plasmid (MSB821). *BET3* and MSB736 are both able to suppress the synthetic lethal phenotype of mutant #15 (A and C). However, the empty plasmid and MSB821, containing only *ELC1*, are not. These colonies retain the balancing plasmid and are red (B and D).

4. Discussion

4.1 The synthetic lethal screen

The synthetic lethal screen with *bet3-4* in the present study generated 70 mutants to analyse. The color of these mutants (red, light red and pink) was noted for a possible correlation to be made between colour intensity and genetic interaction. For example a pink colour may have indicated that the combination of the two mutations causes the cells to be sick resulting in a large number of cells in each colony losing the balancing plasmid. However, no such link has been made thus far and it is likely that none exists. Colour may be influenced by several different factors and, therefore, it is likely that there is no direct correlation with the kind of genetic interaction present (Hieter *et al.*, 1985; Koshland *et al.*, 1985). As would be expected since one of the mutations present is *bet3-4*, there were several mutants (Umezu *et al.*, 1971) which showed linked segregation when crossed to a wild type strain and dissected. This indicated that the second mutation is likely to be within *BET3* or in a gene that is very close and does not readily segregate away.

4.2 *trs23* and *trs85* mutants

As well as the *bet3* mutants, there were fifty mutants which showed a confirmed synthetic lethal interaction. Among these fifty mutants there were likely to be mutations

present in TRAPP genes. As expected, nineteen mutants were shown to be complemented by one or more TRAPP subunit genes. It remains formally possible that a mutation in another gene may be complemented by a TRAPP subunit gene. This notion is supported by the fact that mutants #7, #8 and #95 were suppressed by *TRS23* yet had no mutations in the ORF or the nearby flanking regions. The TRAPP genes in this class of mutants, therefore, need to be sequenced in order to confirm the location of the mutations.

Point mutations were identified in *trs23* in mutants #16 and #98, and in *trs85* in mutants #37 and #159. Since these were synthetically lethal with *bet3-4*, a mutation which causes the mislocalization of TRAPP, it is possible that, alone, they too would lead to the same phenotype. This can be tested by introducing these mutations into a yeast strain by homologous recombination or on a vector, thereby isolating the mutation from any other lesions present in the strains caused by the EMS treatment. Subcellular fractions on a sucrose gradient of the yeast cell lysates followed by western blotting for one of the TRAPP subunits (e.g. Trs33p) could be used to determine the localization of TRAPP within these mutant cells. In the case of *trs85* mutants, a *trs85* deleted strain can be used since it is a non-essential gene. If mislocalization of TRAPP is observed then these subunits are also involved in the anchoring of TRAPP to the Golgi membrane. This was in fact performed, however preliminary data for the subcellular fractionation of TRAPP in a *trs85* Δ mutant was inconclusive.

The *trs85* Δ mutant was crossed to three different *bet3* mutants and this showed that their synthetic lethal interaction was not allele specific. As opposed to the amino acid

substitutions found in *trs23* mutants #16 and #98, the *trs85* mutations in mutants #37 and #159 lead to stop codons and truncations of the protein. If an allele-specific interaction had been identified between these mutants and *bet3-4*, it would have indicated that the region conferring this function to *trs85* was located within the smallest of the two C-terminal truncations (the last 58 amino acids). However, no such allele-specific interaction was observed.

The two *trs23* mutations found in this study should also be tested for allele specificity with these three *bet3* mutants. An allele-specific synthetic lethal interaction with *bet3-4* would indicate that these specific amino acid changes are important for the ability of TRAPP to anchor itself to Golgi membranes. Furthermore, if more than one synthetic lethal mutant shows mutations in the same region of the same gene, it may be that the amino acids in this region are important for the function of this protein. If allele specificity is also observed between these mutants and *bet3-4*, then it would suggest that this region is involved in the ability of TRAPP to anchor itself to Golgi membranes.

4.3 Strains possibly harbouring multiple mutations

Thirteen strains did not conform to expected results for a mutation that is synthetically lethal with *bet3-4*. These thirteen strains may have many mutations present which may be affecting the cells in various ways including, in some cases, their ability to grow well. In addition, the six mutant strains that did not grow well at 30°C may have a

second mutation which itself is temperature-sensitive at this temperature and thus would require incubation at a lower temperature. Since the synthetic lethal screen was carried out at 30°C, all induced mutations analysed are able to grow at this temperature although they may grow better at a lower temperature and similarly may have slower growth at higher temperatures. To observe the potential temperature-sensitive phenotype of the induced mutations, mutant growth can be analysed at several temperatures spanning a wider range.

Strains that kept the wild type balancing plasmid after being crossed to a wild type strain (some colonies on the dissection plate were sectored or grew on -URA) were unexpected since in the diploid state there should be no selection for the plasmid. It is possible that although there is no selection for the plasmid, not all of the cells lose it. However, in such a case *BET3* on the plasmid masks the presence or absence of the *bet3-4* mutation and it becomes difficult to confirm the synthetic lethal interaction through the dissection growth pattern. Therefore, these mutants were classified as behaving oddly and not further analysed.

Strains (#64, #112 and #130) in which some colonies displayed red/white sectoring following dissection also indicated the presence of the balancing plasmid although this was not confirmed by replica plating onto -URA medium. Therefore, these strains were also unusual and not analysed further.

Preliminary analysis of the temperature sensitivity of the synthetic lethal mutants reveals no apparent temperature sensitivity (data not shown). However, for mutants which showed some small colonies following dissection or grew slowly at 30°C (and were classified as behaving oddly) it would be necessary to look at their possible temperature sensitivity further by comparing the growth of serial dilutions of each strain at the various temperatures listed above. It is possible that the temperature-sensitive phenotype was missed because the cell cultures may have been too concentrated to show a slight temperature-sensitive phenotype.

4.4 *trs85*Δ genetic interactions

As well as testing for allele specificity in the genetic interaction between *trs85*Δ and *bet3* mutants, *trs85*Δ was tested for genetic interactions with other TRAPP subunit genes. We found that *trs85*Δ genetically interacts with both *bet5* and *trs33*Δ. It is interesting to note that *bet3-4* was previously shown to be complemented by *TRS33* as seen by growth on YPD (Kim *et al.*, 2005a). In the present study, however, the *bet3-4* mutants were not complemented by *TRS33* on an 5-FOA plate. Taken together with the findings above showing a genetic interaction between *trs85*Δ and *bet3-4*, these findings may suggest that the two non-essential TRAPP subunits Trs85p and Trs33p help Bet3p in anchoring TRAPP to the Golgi or in other functions. Following these observations, it would be warranted to investigate the localization of TRAPP in a *trs33*Δ strain. A *trs33*Δ

mutant should be subjected to subcellular fractionation as described above and the localization of TRAPP assessed.

4.5 Non-TRAPP synthetic lethal interactions

The thirty mutants which had non-TRAPP mutations were a main focus for this study. Ten mutants were chosen as representatives for the small scale screen using the yeast plasmid library based, among other factors, on their various shades of red colour. The small scale screen revealed that the light red coloured strains (mutants #128 and #132) were unsuitable. The rare white colonies were virtually indistinguishable amongst a light red, almost pink, background of colonies. The five strains chosen for the large scale screens had the darkest red colour and the fewest white colonies.

An eight-fold coverage of the yeast genome was analyzed in the screen to ensure that even genes expressed at low levels would be represented. In all large scale screens, except for mutants #5 and #80, there were a surprisingly large number of white colonies (over forty). A large number of white colonies is unexpected unless the mutation is suppressed by multiple genes or is suppressed by genes that are highly expressed. The yeast plasmids from mutants #5 and #80 did not re-complement the mutations in these strains as seen by the lack of growth on 5-FOA medium. Therefore, systematic evaluation of the white colonies in strains #15, #29 and #121 was carried out. Isolated plasmids were tested to see if they could complement a mutation found in their respective strains by

replica plating onto 5-FOA and looking for growth. Only five isolated plasmids from mutant #15 (and none from any other mutant) were able to do so. The other white colonies were false positives, some of which contained empty plasmids as revealed by sequencing (data not shown). Of the five isolated plasmids, four contained *BET3* as expected in such a screen while one did not. This plasmid contains two whole genes (*VPS16* and *ELC1*), the 5' end of *NOP4* and the 3' end of a fourth gene *SGF11*. *SGF11* is unlikely to be of interest since the insert lacks the 5' untranslated region, the start codon and further sequences downstream. Although formally possible, *NOP4* is not believed to be the suppressing factor for several reasons. First, in the plasmid it is truncated to the extent that only ~39% of the gene is present. Second, Nop4p is involved in ribosomal RNA production (Patterson *et al.*, 2008) and, as seen in the *S cerevisiae* database (www.yeastgenome.org), does not display genetic or physical interactions with any known membrane trafficking components.

VPS16 seemed to be the likely suppressor in the plasmid since it is also involved in vesicle trafficking. Vps16p is part of both the HOPS and CORVET complexes which function in the late secretory pathway (Peplowska *et al.*, 2007). HOPS, found at the vacuole, is involved in vacuole-vacuole and vesicle-vacuole fusion (Haas *et al.*, 1995; Mayer and Wickner, 1997; Price *et al.*, 2000; Wurmser *et al.*, 2000). While the CORVET complex, at endosomes, mediates endosome-lysosome transport (Peplowska *et al.*, 2007). As a component of TRAPP II, which localizes and functions in the late Golgi/early endosome compartment (Cai *et al.*, 2005), Bet3p is also found in a portion of the secretory pathway that may be affected by HOPS and/or CORVET. Although Vps16p

may not have a direct interaction with Bet3p, *vps16* may aggravate an already defective late trafficking step in a *bet3* mutant. To address this, part of the insert in the suppressing plasmid containing most of *VPS16* and the entire *NOP4* fragment was removed and the plasmid re-ligated leaving *ELC1* intact. This plasmid was then tested for its ability to suppress the synthetic lethal phenotype. White colonies were observed when the entire insert is present (MSB736) but not when only *ELC1* is present (MSB821). Therefore, *VPS16* (or the N-terminal portion of *NOP4*) is complementing the as yet unidentified mutation in mutant #15. Sequencing analysis showed no mutations in *VPS16* in mutant #15. However, sequencing of *NOP4* remains to be carried out. A plasmid containing only *VPS16* should be used in the same experiment, described above, to conclusively show that *VPS16* is indeed the gene conferring suppression.

4.6 Future perspectives

This study suggests that an as yet unidentified mutation suppressed by either *VPS16* or the amino-terminal portion of *NOP4* genetically interacts with *bet3-4*. Since *VPS16* itself is not altered in mutant #15, and since the plasmid could not suppress any *bet3* alleles tested, it is likely that *VPS16* is complementing a defect in another gene. To identify this gene, another large scale yeast plasmid library screen could be done on mutant #15 in the hope that this gene could be identified in a complementing plasmid. However, by looking at known interactions of *VPS16* we may be able to make an educated guess as to this unidentified mutant gene. As part of the HOPS and CORVET

complexes, Vps16p interacts directly or indirectly with Vps11p, Vps18p and Vps33p (components common to both complexes), Vps39p and Vps41p (HOPS complex components), and Vps3p and Vps8p (CORVET complex components) (www.yeastgenome.org). It may be the case that mutant #15 has a mutation in one of the genes encoding these proteins. These genes should be sequenced in mutant #15 to look for a mutation. If a mutation is found, then this gene should then be tested for its ability to suppress the synthetic lethal phenotype in mutant #15. If no mutation is found in any of these genes then another yeast library screen would need to be carried out.

5. References

- Andag, U., Neumann, T., and Schmitt, H. D. (2001). The coatomer-interacting protein Dsl1p is required for Golgi-to-endoplasmic reticulum retrieval in yeast. *J.Biol.Chem.*, 276, 39150-39160.
- Andag, U. and Schmitt, H. D. (2003). Dsl1p, an essential component of the Golgi-endoplasmic reticulum retrieval system in yeast, uses the same sequence motif to interact with different subunits of the COPI vesicle coat. *J.Biol.Chem.*, 278, 51722-51734.
- Antonin, W., Fasshauer, D., Becker, S., Jahn, R., and Schneider, T. R. (2002). Crystal structure of the endosomal SNARE complex reveals common structural principles of all SNAREs. *Nat.Struct.Biol.*, 9, 107-111.
- Appling, D. R. (1999). Genetic approaches to the study of protein-protein interactions. *Methods*, 19, 338-349.
- Aridor, M., Weissman, J., Bannykh, S., Nuoffer, C., and Balch, W. E. (1998). Cargo selection by the COPII budding machinery during export from the ER. *J.Cell Biol.*, 141, 61-70.
- Banfield, D. K., Lewis, M. J., Rabouille, C., Warren, G., and Pelham, H. R. (1994). Localization of Sed5, a putative vesicle targeting molecule, to the cis-Golgi network involves both its transmembrane and cytoplasmic domains. *J.Cell Biol.*, 127, 357-371.
- Bard, F. and Malhotra, V. (2006). The formation of TGN-to-plasma-membrane transport carriers. *Annu.Rev.Cell Dev.Biol.*, 22, 439-455.
- Barlowe, C., Orci, L., Yeung, T., Hosobuchi, M., Hamamoto, S., Salama, N., Rexach, M. F., Ravazzola, M., Amherdt, M., and Schekman, R. (1994). COPII: a membrane coat formed by Sec proteins that drive vesicle budding from the endoplasmic reticulum. *Cell*, 77, 895-907.
- Barrowman, J., Sacher, M., and Ferro-Novick, S. (2000). TRAPP stably associates with the Golgi and is required for vesicle docking. *EMBO J.*, 19, 862-869.

- Behnia, R. and Munro, S. (2005). Organelle identity and the signposts for membrane traffic. *Nature*, 438, 597-604.
- Bender, A. and Pringle, J. R. (1991). Use of a screen for synthetic lethal and multicopy suppressor mutants to identify two new genes involved in morphogenesis in *Saccharomyces cerevisiae*. *Mol. Cell Biol.*, 11, 1295-1305.
- Boeke, J. D., LaCroute, F., and Fink, G. R. (1984). A positive selection for mutants lacking orotidine-5'-phosphate decarboxylase activity in yeast: 5-fluoro-orotic acid resistance. *Mol. Gen. Genet.*, 197, 345-346.
- Bonifacino, J. S. and Glick, B. S. (2004). The mechanisms of vesicle budding and fusion. *Cell*, 116, 153-166.
- Bourne, H. R., Sanders, D. A., and McCormick, F. (1990). The GTPase superfamily: a conserved switch for diverse cell functions. *Nature*, 348, 125-132.
- Boyd, C., Hughes, T., Pypaert, M., and Novick, P. (2004). Vesicles carry most exocyst subunits to exocytic sites marked by the remaining two subunits, Sec3p and Exo70p. *J. Cell Biol.*, 167, 889-901.
- Bruinsma, P., Spelbrink, R. G., and Nothwehr, S. F. (2004). Retrograde transport of the mannosyltransferase Och1p to the early Golgi requires a component of the COG transport complex. *J. Biol. Chem.*, 279, 39814-39823.
- Cai, H., Reinisch, K., and Ferro-Novick, S. (2007a). Coats, tethers, Rabs, and SNAREs work together to mediate the intracellular destination of a transport vesicle. *Dev. Cell*, 12, 671-682.
- Cai, H., Yu, S., Menon, S., Cai, Y., Lazarova, D., Fu, C., Reinisch, K., Hay, J. C., and Ferro-Novick, S. (2007b). TRAPP1 tethers COPII vesicles by binding the coat subunit Sec23. *Nature*, 445, 941-944.
- Cai, H., Zhang, Y., Pypaert, M., Walker, L., and Ferro-Novick, S. (2005). Mutants in *trs120* disrupt traffic from the early endosome to the late Golgi. *J. Cell Biol.*, 171, 823-833.
- Cai, Y., Chin, H. F., Lazarova, D., Menon, S., Fu, C., Cai, H., Sclafani, A., Rodgers, D. W., De La Cruz, E. M., Ferro-Novick, S., and Reinisch, K. M. (2008). The structural

basis for activation of the Rab Ypt1p by the TRAPP membrane-tethering complexes. *Cell*, 133, 1202-1213.

Carr, C. M., Grote, E., Munson, M., Hughson, F. M., and Novick, P. J. (1999). Sec1p binds to SNARE complexes and concentrates at sites of secretion. *J.Cell Biol.*, 146, 333-344.

Conibear, E., Cleck, J. N., and Stevens, T. H. (2003). Vps51p mediates the association of the GARP (Vps52/53/54) complex with the late Golgi t-SNARE Tlg1p. *Mol.Biol.Cell*, 14, 1610-1623.

Conibear, E. and Stevens, T. H. (2000). Vps52p, Vps53p, and Vps54p form a novel multisubunit complex required for protein sorting at the yeast late Golgi. *Mol.Biol.Cell*, 11, 305-323.

Dean, N. (1999). Asparagine-linked glycosylation in the yeast Golgi. *Biochim.Biophys.Acta*, 1426, 309-322.

Elsner, M., Hashimoto, H., and Nilsson, T. (2003). Cisternal maturation and vesicle transport: join the band wagon! (Review). *Mol.Membr.Biol.*, 20, 221-229.

Fasshauer, D., Sutton, R. B., Brunger, A. T., and Jahn, R. (1998). Conserved structural features of the synaptic fusion complex: SNARE proteins reclassified as Q- and R-SNAREs. *Proc.Natl.Acad.Sci.U.S.A*, 95, 15781-15786.

Finger, F. P., Hughes, T. E., and Novick, P. (1998). Sec3p is a spatial landmark for polarized secretion in budding yeast. *Cell*, 92, 559-571.

Fridmann-Sirkis, Y., Kent, H. M., Lewis, M. J., Evans, P. R., and Pelham, H. R. (2006). Structural analysis of the interaction between the SNARE Tlg1 and Vps51. *Traffic.*, 7, 182-190.

Gedeon, A. K., Colley, A., Jamieson, R., Thompson, E. M., Rogers, J., Sillence, D., Tiller, G. E., Mulley, J. C., and Gecz, J. (1999). Identification of the gene (SEDL) causing X-linked spondyloepiphyseal dysplasia tarda. *Nat.Genet.*, 22, 400-404.

Gietz, R. D. and Woods, R. A. (2002). Transformation of yeast by lithium acetate/single-stranded carrier DNA/polyethylene glycol method. *Methods Enzymol.*, 350, 87-96.

Guarente, L. (1993). Strategies for the identification of interacting proteins. *Proc.Natl.Acad.Sci.U.S.A*, 90, 1639-1641.

Guo, W., Roth, D., Walch-Solimena, C., and Novick, P. (1999). The exocyst is an effector for Sec4p, targeting secretory vesicles to sites of exocytosis. *EMBO J.*, 18, 1071-1080.

Haas, A., Scheglmann, D., Lazar, T., Gallwitz, D., and Wickner, W. (1995). The GTPase Ypt7p of *Saccharomyces cerevisiae* is required on both partner vacuoles for the homotypic fusion step of vacuole inheritance. *EMBO J.*, 14, 5258-5270.

Hieter, P., Mann, C., Snyder, M., and Davis, R. W. (1985). Mitotic stability of yeast chromosomes: a colony color assay that measures nondisjunction and chromosome loss. *Cell*, 40, 381-392.

Higashio, H., Sato, K., and Nakano, A. (2008). Smy2p participates in COPII vesicle formation through the interaction with Sec23p/Sec24p subcomplex. *Traffic.*, 9, 79-93.

Hsu, S. C., Hazuka, C. D., Roth, R., Foletti, D. L., Heuser, J., and Scheller, R. H. (1998). Subunit composition, protein interactions, and structures of the mammalian brain sec6/8 complex and septin filaments. *Neuron*, 20, 1111-1122.

Hsu, S. C., TerBush, D., Abraham, M., and Guo, W. (2004). The exocyst complex in polarized exocytosis. *Int.Rev.Cytol.*, 233, 243-265.

Ingvarsdottir, K., Krogan, N. J., Emre, N. C., Wyce, A., Thompson, N. J., Emili, A., Hughes, T. R., Greenblatt, J. F., and Berger, S. L. (2005). H2B ubiquitin protease Ubp8 and Sgf11 constitute a discrete functional module within the *Saccharomyces cerevisiae* SAGA complex. *Mol.Cell Biol.*, 25, 1162-1172.

Jackson, C. L. (2009). Mechanisms of transport through the Golgi complex. *J.Cell Sci.*, 122, 443-452.

Jahn, R. (2008). Some classic papers in the field of membrane fusion--a personal view. *Nat.Struct.Mol.Biol.*, 15, 655-657.

Jiang, Y., Scarpa, A., Zhang, L., Stone, S., Feliciano, E., and Ferro-Novick, S. (1998). A high copy suppressor screen reveals genetic interactions between BET3 and a new gene. Evidence for a novel complex in ER-to-Golgi transport. *Genetics*, 149, 833-841.

- Jones, S., Newman, C., Liu, F., and Segev, N. (2000). The TRAPP complex is a nucleotide exchanger for Ypt1 and Ypt31/32. *Mol. Biol. Cell*, *11*, 4403-4411.
- Kim, M. S., Yi, M. J., Lee, K. H., Wagner, J., Munger, C., Kim, Y. G., Whiteway, M., Cygler, M., Oh, B. H., and Sacher, M. (2005a). Biochemical and crystallographic studies reveal a specific interaction between TRAPP subunits Trs33p and Bet3p. *Traffic*, *6*, 1183-1195.
- Kim, Y. G., Raunser, S., Munger, C., Wagner, J., Song, Y. L., Cygler, M., Walz, T., Oh, B. H., and Sacher, M. (2006). The architecture of the multisubunit TRAPP I complex suggests a model for vesicle tethering. *Cell*, *127*, 817-830.
- Kim, Y. G., Sohn, E. J., Seo, J., Lee, K. J., Lee, H. S., Hwang, I., Whiteway, M., Sacher, M., and Oh, B. H. (2005b). Crystal structure of bet3 reveals a novel mechanism for Golgi localization of tethering factor TRAPP. *Nat. Struct. Mol. Biol.*, *12*, 38-45.
- Koshland, D., Kent, J. C., and Hartwell, L. H. (1985). Genetic analysis of the mitotic transmission of minichromosomes. *Cell*, *40*, 393-403.
- Kraynack, B. A., Chan, A., Rosenthal, E., Essid, M., Umansky, B., Waters, M. G., and Schmitt, H. D. (2005). Dsl1p, Tip20p, and the novel Dsl3(Sec39) protein are required for the stability of the Q/t-SNARE complex at the endoplasmic reticulum in yeast. *Mol. Biol. Cell*, *16*, 3963-3977.
- Kuehn, M. J., Herrmann, J. M., and Schekman, R. (1998). COPII-cargo interactions direct protein sorting into ER-derived transport vesicles. *Nature*, *391*, 187-190.
- Laage, R. and Ungermann, C. (2001). The N-terminal domain of the t-SNARE Vam3p coordinates priming and docking in yeast vacuole fusion. *Mol. Biol. Cell*, *12*, 3375-3385.
- Lanoix, J., Ouwendijk, J., Lin, C. C., Stark, A., Love, H. D., Ostermann, J., and Nilsson, T. (1999). GTP hydrolysis by arf-1 mediates sorting and concentration of Golgi resident enzymes into functional COP I vesicles. *EMBO J.*, *18*, 4935-4948.
- Letourneur, F., Gaynor, E. C., Hennecke, S., Demolliere, C., Duden, R., Emr, S. D., Riezman, H., and Cosson, P. (1994). Coatamer is essential for retrieval of dilysine-tagged proteins to the endoplasmic reticulum. *Cell*, *79*, 1199-1207.

Li, F., Pincet, F., Perez, E., Eng, W. S., Melia, T. J., Rothman, J. E., and Tareste, D. (2007). Energetics and dynamics of SNAREpin folding across lipid bilayers. *Nat.Struct.Mol.Biol.*, 14, 890-896.

Lowe, J. B. and Marth, J. D. (2003). A genetic approach to Mammalian glycan function. *Annu.Rev.Biochem.*, 72, 643-691.

Lupashin, V. and Sztul, E. (2005). Golgi tethering factors. *Biochim.Biophys.Acta*, 1744, 325-339.

Martinez-Menarguez, J. A., Prekeris, R., Oorschot, V. M., Scheller, R., Slot, J. W., Geuze, H. J., and Klumperman, J. (2001). Peri-Golgi vesicles contain retrograde but not anterograde proteins consistent with the cisternal progression model of intra-Golgi transport. *J.Cell Biol.*, 155, 1213-1224.

Mayer, A. and Wickner, W. (1997). Docking of yeast vacuoles is catalyzed by the Ras-like GTPase Ypt7p after symmetric priming by Sec18p (NSF). *J.Cell Biol.*, 136, 307-317.

McNiven, M. A. and Thompson, H. M. (2006). Vesicle formation at the plasma membrane and trans-Golgi network: the same but different. *Science*, 313, 1591-1594.

Morozova, N., Liang, Y., Tokarev, A. A., Chen, S. H., Cox, R., Andrejic, J., Lipatova, Z., Sciorra, V. A., Emr, S. D., and Segev, N. (2006). TRAPP II subunits are required for the specificity switch of a Ypt-Rab GEF. *Nat.Cell Biol.*, 8, 1263-1269.

Morre D.J. and Mollenhauer, H. H. (2007). Microscopic morphology and the origins of the membrane maturation model of Golgi apparatus function. *Int.Rev.Cytol.*, 262, 191-218.

Nakano, A. and Muramatsu, M. (1989). A novel GTP-binding protein, Sar1p, is involved in transport from the endoplasmic reticulum to the Golgi apparatus. *J.Cell Biol.*, 109, 2677-2691.

Nilsson, T., Jackson, M., and Peterson, P. A. (1989). Short cytoplasmic sequences serve as retention signals for transmembrane proteins in the endoplasmic reticulum. *Cell*, 58, 707-718.

Oka, T. and Krieger, M. (2005). Multi-component protein complexes and Golgi membrane trafficking. *J.Biochem.*, 137, 109-114.

Palade, G. (1975). Intracellular aspects of the process of protein synthesis. *Science*, 189, 347-358.

Patterson, G. H., Hirschberg, K., Polishchuk, R. S., Gerlich, D., Phair, R. D., and Lippincott-Schwartz, J. (2008). Transport through the Golgi apparatus by rapid partitioning within a two-phase membrane system. *Cell*, 133, 1055-1067.

Pelham, H. R. (1989). Heat shock and the sorting of luminal ER proteins. *EMBO J.*, 8, 3171-3176.

Pelham, H. R. and Rothman, J. E. (2000). The debate about transport in the Golgi--two sides of the same coin? *Cell*, 102, 713-719.

Peplowska, K., Markgraf, D. F., Ostrowicz, C. W., Bange, G., and Ungermann, C. (2007). The CORVET tethering complex interacts with the yeast Rab5 homolog Vps21 and is involved in endo-lysosomal biogenesis. *Dev. Cell*, 12, 739-750.

Pfeffer, S. R. (2001). Rab GTPases: specifying and deciphering organelle identity and function. *Trends Cell Biol.*, 11, 487-491.

Price, A., Seals, D., Wickner, W., and Ungermann, C. (2000). The docking stage of yeast vacuole fusion requires the transfer of proteins from a cis-SNARE complex to a Rab/Ypt protein. *J. Cell Biol.*, 148, 1231-1238.

Ram, R. J., Li, B., and Kaiser, C. A. (2002). Identification of Sec36p, Sec37p, and Sec38p: components of yeast complex that contains Sec34p and Sec35p. *Mol. Biol. Cell*, 13, 1484-1500.

Reggiori, F., Wang, C. W., Stromhaug, P. E., Shintani, T., and Klionsky, D. J. (2003). Vps51 is part of the yeast Vps fifty-three tethering complex essential for retrograde traffic from the early endosome and Cvt vesicle completion. *J. Biol. Chem.*, 278, 5009-5020.

Reilly, B. A., Kraynack, B. A., VanRheenen, S. M., and Waters, M. G. (2001). Golgi-to-endoplasmic reticulum (ER) retrograde traffic in yeast requires Dsl1p, a component of the ER target site that interacts with a COPI coat subunit. *Mol. Biol. Cell*, 12, 3783-3796.

Rossi, G., Kolstad, K., Stone, S., Palluault, F., and Ferro-Novick, S. (1995). BET3 encodes a novel hydrophilic protein that acts in conjunction with yeast SNAREs. *Mol.Biol.Cell*, 6, 1769-1780.

Sacher, M. (2003). Membrane traffic fuses with cartilage development. *FEBS Lett.*, 550, 1-4.

Sacher, M., Barrowman, J., Schieltz, D., Yates, J. R., III, and Ferro-Novick, S. (2000). Identification and characterization of five new subunits of TRAPP. *Eur.J.Cell Biol.*, 79, 71-80.

Sacher, M., Barrowman, J., Wang, W., Horecka, J., Zhang, Y., Pypaert, M., and Ferro-Novick, S. (2001). TRAPP I implicated in the specificity of tethering in ER-to-Golgi transport. *Mol.Cell*, 7, 433-442.

Sacher, M., Jiang, Y., Barrowman, J., Scarpa, A., Burston, J., Zhang, L., Schieltz, D., Yates, J. R., III, Abeliovich, H., and Ferro-Novick, S. (1998). TRAPP, a highly conserved novel complex on the cis-Golgi that mediates vesicle docking and fusion. *EMBO J.*, 17, 2494-2503.

Sato, K. and Nakano, A. (2004). Reconstitution of coat protein complex II (COPII) vesicle formation from cargo-reconstituted proteoliposomes reveals the potential role of GTP hydrolysis by Sar1p in protein sorting. *J.Biol.Chem.*, 279, 1330-1335.

Schekman, R. and Orci, L. (1996). Coat proteins and vesicle budding. *Science*, 271, 1526-1533.

Scrivens, P. J., Shahrzad, N., Moores, A., Morin, A., Brunet, S., and Sacher, M. (2009). TRAPPC2L is a novel, highly conserved TRAPP-interacting protein. *Traffic*, 10, 724-736.

Seals, D. F., Eitzen, G., Margolis, N., Wickner, W. T., and Price, A. (2000). A Ypt/Rab effector complex containing the Sec1 homolog Vps33p is required for homotypic vacuole fusion. *Proc.Natl.Acad.Sci.U.S.A.*, 97, 9402-9407.

Sega, G. A. (1984). A review of the genetic effects of ethyl methanesulfonate. *Mutat.Res.*, 134, 113-142.

Sherman, F. and Hicks, J. (1991). Micromanipulation and Dissection of Asci. In C.Guthrie and G. R. Fink (Eds.), *Guide to Yeast Genetics and Molecular Biology* (pp. 21-37). San Diego: Academic Press.

Shorter, J., Beard, M. B., Seemann, J., rac-Svejstrup, A. B., and Warren, G. (2002). Sequential tethering of Golgins and catalysis of SNAREpin assembly by the vesicle-tethering protein p115. *J.Cell Biol.*, 157, 45-62.

Smirnov, M. N., Smirnov, V. N., Budowsky, E. I., Inge-Vechtomov, S. G., and Serebrjakov, N. G. (1967). Red pigment of adenine-deficient yeast *Saccharomyces cerevisiae*. *Biochem.Biophys.Res.Commun.*, 27, 299-304.

Stroupe, C., Collins, K. M., Fratti, R. A., and Wickner, W. (2006). Purification of active HOPS complex reveals its affinities for phosphoinositides and the SNARE Vam7p. *EMBO J.*, 25, 1579-1589.

Sun, C. and Woolford, J. L., Jr. (1994). The yeast NOP4 gene product is an essential nucleolar protein required for pre-rRNA processing and accumulation of 60S ribosomal subunits. *EMBO J.*, 13, 3127-3135.

Sutton, R. B., Fasshauer, D., Jahn, R., and Brunger, A. T. (1998). Crystal structure of a SNARE complex involved in synaptic exocytosis at 2.4 Å resolution. *Nature*, 395, 347-353.

Suvorova, E. S., Duden, R., and Lupashin, V. V. (2002). The Sec34/Sec35p complex, a Ypt1p effector required for retrograde intra-Golgi trafficking, interacts with Golgi SNAREs and COPI vesicle coat proteins. *J.Cell Biol.*, 157, 631-643.

Suvorova, E. S., Kurten, R. C., and Lupashin, V. V. (2001). Identification of a human orthologue of Sec34p as a component of the cis-Golgi vesicle tethering machinery. *J.Biol.Chem.*, 276, 22810-22818.

Sweet, D. J. and Pelham, H. R. (1993). The TIP1 gene of *Saccharomyces cerevisiae* encodes an 80 kDa cytoplasmic protein that interacts with the cytoplasmic domain of Sec20p. *EMBO J.*, 12, 2831-2840.

Swennen, D. and Beckerich, J. M. (2007). *Yarrowia lipolytica* vesicle-mediated protein transport pathways. *BMC.Evol.Biol.*, 7, 219.

Teasdale, R. D. and Jackson, M. R. (1996). Signal-mediated sorting of membrane proteins between the endoplasmic reticulum and the golgi apparatus. *Annu.Rev.Cell Dev.Biol.*, 12, 27-54.

TerBush, D. R., Maurice, T., Roth, D., and Novick, P. (1996). The Exocyst is a multiprotein complex required for exocytosis in *Saccharomyces cerevisiae*. *EMBO J.*, 15, 6483-6494.

Tripathi, A., Ren, Y., Jeffrey, P. D., and Hughson, F. M. (2009). Structural characterization of Tip20p and Dsl1p, subunits of the Dsl1p vesicle tethering complex. *Nat.Struct.Mol.Biol.*, 16, 114-123.

Turnbull, A. P., Kummel, D., Prinz, B., Holz, C., Schultchen, J., Lang, C., Niesen, F. H., Hofmann, K. P., Delbruck, H., Behlke, J., Muller, E. C., Jarosch, E., Sommer, T., and Heinemann, U. (2005). Structure of palmitoylated BET3: insights into TRAPP complex assembly and membrane localization. *EMBO J.*, 24, 875-884.

Umez, K., Amaya, T., Yoshimoto, A., and Tomita, K. (1971). Purification and properties of orotidine-5'-phosphate pyrophosphorylase and orotidine-5'-phosphate decarboxylase from baker's yeast. *J.Biochem.*, 70, 249-262.

Ungar, D. and Hughson, F. M. (2003). SNARE protein structure and function. *Annu.Rev.Cell Dev.Biol.*, 19, 493-517.

Ungar, D., Oka, T., Brittle, E. E., Vasile, E., Lupashin, V. V., Chatterton, J. E., Heuser, J. E., Krieger, M., and Waters, M. G. (2002). Characterization of a mammalian Golgi-localized protein complex, COG, that is required for normal Golgi morphology and function. *J.Cell Biol.*, 157, 405-415.

VanRheenen, S. M., Cao, X., Lupashin, V. V., Barlowe, C., and Waters, M. G. (1998). Sec35p, a novel peripheral membrane protein, is required for ER to Golgi vesicle docking. *J.Cell Biol.*, 141, 1107-1119.

VanRheenen, S. M., Cao, X., Sapperstein, S. K., Chiang, E. C., Lupashin, V. V., Barlowe, C., and Waters, M. G. (1999). Sec34p, a protein required for vesicle tethering to the yeast Golgi apparatus, is in a complex with Sec35p. *J.Cell Biol.*, 147, 729-742.

VanRheenen, S. M., Reilly, B. A., Chamberlain, S. J., and Waters, M. G. (2001). Dsl1p, an essential protein required for membrane traffic at the endoplasmic reticulum/Golgi interface in yeast. *Traffic.*, 2, 212-231.

Voth, W. P., Jiang, Y. W., and Stillman, D. J. (2003). New 'marker swap' plasmids for converting selectable markers on budding yeast gene disruptions and plasmids. *Yeast*, 20, 985-993.

Wang, W., Sacher, M., and Ferro-Novick, S. (2000). TRAPP stimulates guanine nucleotide exchange on Ypt1p. *J.Cell Biol.*, 151, 289-296.

Wang, Y., Dulubova, I., Rizo, J., and Sudhof, T. C. (2001). Functional analysis of conserved structural elements in yeast syntaxin Vam3p. *J.Biol.Chem.*, 276, 28598-28605.

Waters, M. G. and Pfeffer, S. R. (1999). Membrane tethering in intracellular transport. *Curr.Opin.Cell Biol.*, 11, 453-459.

Weisman, L. S., Bacallao, R., and Wickner, W. (1987). Multiple methods of visualizing the yeast vacuole permit evaluation of its morphology and inheritance during the cell cycle. *J.Cell Biol.*, 105, 1539-1547.

Whyte, J. R. and Munro, S. (2001). The Sec34/35 Golgi transport complex is related to the exocyst, defining a family of complexes involved in multiple steps of membrane traffic. *Dev.Cell*, 1, 527-537.

Wiederkehr, A., De Craene, J. O., Ferro-Novick, S., and Novick, P. (2004). Functional specialization within a vesicle tethering complex: bypass of a subset of exocyst deletion mutants by Sec1p or Sec4p. *J.Cell Biol.*, 167, 875-887.

Wurmser, A. E., Sato, T. K., and Emr, S. D. (2000). New component of the vacuolar class C-Vps complex couples nucleotide exchange on the Ypt7 GTPase to SNARE-dependent docking and fusion. *J.Cell Biol.*, 151, 551-562.

Yoshihisa, T., Barlowe, C., and Schekman, R. (1993). Requirement for a GTPase-activating protein in vesicle budding from the endoplasmic reticulum. *Science*, 259, 1466-1468.

Zolov, S. N. and Lupashin, V. V. (2005). Cog3p depletion blocks vesicle-mediated Golgi retrograde trafficking in HeLa cells. *J.Cell Biol.*, 168, 747-759.