THE PENNSYLVANIA STATE UNIVERSITY
THE SCHREYER HONORS COLLEGE

DEPARTMENT OF BIOCHEMISTRY AND MOLECULAR BIOLOGY

YEAST 2-HYBRID TESTING OF β_{HEAVY}-SPECTRIN (GEFLONG) WITH CANDIDATE RHO AND RAB GTPASES AND THE RE-EVALUATION OF PREVIOUS YEAST 2-HYBRID POSITIVES

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SPRING 2012

A thesis
submitted in partial fulfillment
of the requirements
for a baccalaureate degree
in BIOTECHNOLOGY
with honors in BIOTECHNOLOGY

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ABSTRACT

The spectrin-based membrane skeleton (SBMS) is a network of membrane-associated proteins that also functions as a scaffold for association with other proteins at the plasma membrane. The roles of the SBMS in multicellular animals were initially thought to be restricted to determining cell shape and maintaining membrane integrity. However, current analyses on the SBMS have suggested that the SBMS has a more dynamic role in the cell such as modulating the process of endocytosis, endosomal trafficking and participating in the determination of apical basal polarity. Recently, a study has shown that Rac1 (a Rho GTPase) could be modulated by β_H-spectrin, which is in turn regulated by Pak1, an effector for Rac1. In a separate experiment, β_H-spectrin was demonstrated to have an epistatic interaction with Rab5. It has also been shown that β_H-spectrin plays a role in regulating the progression of the early endosome and decision to either transport protein back to the membrane (recycling), or to lysosomes (degradation).

Novel sequence analysis software (GDDA-BLAST) suggests that β_H-spectrin contains a Dbl homology domain in its C-terminus. Hence, it is possible that the Dbl homology domain in β_H-spectrin may have GEF-like properties including the ability to bind GTPases. So, one hypothesis suggests that βH33 may function as a guanine nucleotide exchange factor (GEF) mimic that binds GTPase proteins and prevents them from being activated by other GEFs. To test this hypothesis, several candidate members from the Rho GTPase family and Rab GTPase family were selected to be tested for direct interaction with GEFlong (a fragment of β_H-spectrin C-terminus that contains the predicted Dbl homology domain through the PH domain) by using the yeast 2-hybrid system. In addition, some genes identified in a previous yeast 2-hybrid screen were critically reevaluated.

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ACKNOWLEDGMENTS

I would like to express my gratitude to Dr. Graham Thomas for his invaluable guidance and support throughout my two years in Thomas lab. Special thanks to all my graduate lab mates: Seung-Kyu Lee, Mansi Khanna, Monika Tjota, and Juan Wu. Your companionship has made my work experience very enjoyable.

Also, I would like to thank my beloved family for their continuing support and encouragements throughout the duration of my studies in Pennsylvania State University.

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INTRODUCTION

Spectrin Based Membrane Skeleton

The spectrin-based membrane skeleton (SBMS) is a network of membrane-associated proteins that also function as a scaffold for association with other proteins at the plasma membrane (Bennett and Baines, 2001). The SBMS was first discovered in the erythrocyte plasma membrane as a major determinant of erythrocyte cell shape and is needed for erythrocyte survival during circulation (Marchesi and Steers, 1968; Yu *et al*., 1973; Greenquist *et al*., 1978; Bodine *et al*., 1984). To date, SBMS have been identified in most other cell types including brain, muscle and epithelial cells of many metazoans, including *Drosophila melanogaster* (Bennett *et al*., 1982; Burridge *et al*., 1982; Glenney *et al*., 1982; Goodman *et al*., 1981; Levine and Williard, 1981; Appleyard *et al*., 1984; Dubreuil *et al*., 1987).

The roles of the SBMS in multicellular animals were initially thought to be restricted to determining cell shape and maintaining membrane integrity (Elgsaeter *et al*., 1986). Hence, the SBMS was regarded as a structural component of the plasma membrane that is also involved in the morphogenesis of epithelial tissues (Zarnescu and Thomas, 1999), targeting and stabilization of ion channels and adhesion molecules (Zhou *et al*., 1998), and Ca^{2+} homeostasis protein compartmentalization (Tuvia *et al*., 1999). However, current analyses on the SBMS have suggested that the SBMS has a more dynamic role in the cell such as modulating the process of endocytosis, endosomal trafficking and participating in the determination of apical basal polarity (Williams *et al*., 2004; Tjota *et al*., 2011; Phillips and Thomas, 2006; Johansson *et al*., 2007;

Zarnescu and Thomas, 1999). These discoveries changed our understanding on the roles of spectrin and paved the way for further research.

Spectrin Subunits and Structure

Spectrins are elongated fibrous proteins comprising of two α subunits and two β subunits (Bennett and Baines, 2001; Dubreuil *et al*., 1989, 1990). Each α and β subunit contains many 106 amino acids spectrin repeats, each of which is folded into three alpha helices (Speicher and Marchesi, 1984; Yan *et al*., 1993). The three helices are arranged in such a way that two of them are parallel while the other one is in antiparallel orientation (Yan *et al*., 1993). This triple-helical structure is further stabilized by the interaction between hydrophobic residues found in the helices (Figure 1) (Yan *et al*., 1993).

Generally, α-spectrin isoforms include twenty 106-residue triple-helical repeats (Speicher and Marchesi, 1984), a Src-homology 3 (SH3) domain (Musacchio *et al*., 1992), and a calcium-binding EF hand domain related to calmodulin (Trave *et al*., 1995) (Figure 2). Vertebrate α_2-spectrin has an additional Ca^{2+}-activated protease cleavage site and a calmodulin-binding site (Harris *et al*., 1988; Leto *et al*., 1989). *Drosophila* α-spectrin also contains a calmodulin-binding site, but located at a different position from α_2-spectrin (Dubreuil *et al*., 1989). In general, α-spectrins can form dimers with either β-spectrin or β_H-spectrin (Dubreuil *et al*., 1997; Thomas and Williams, 1999).

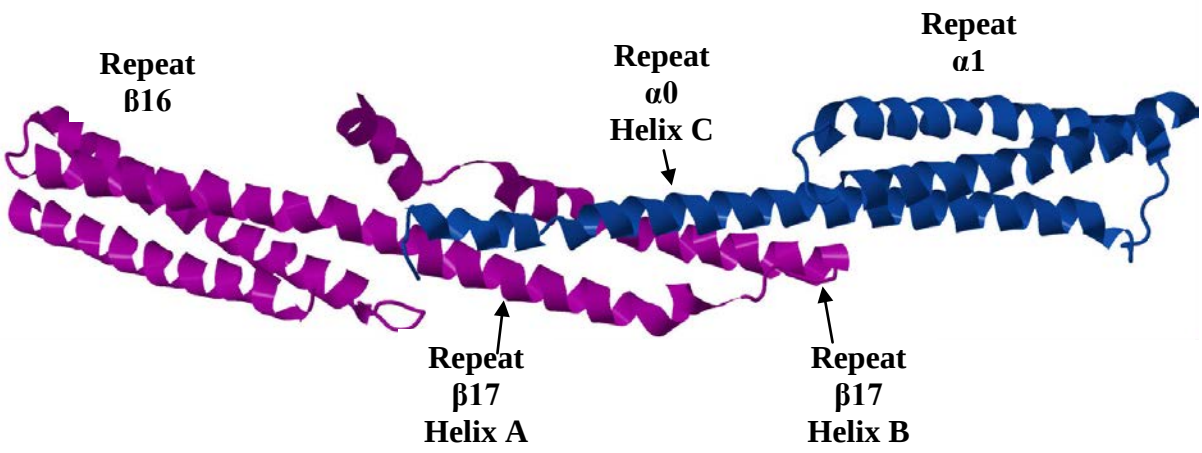


Figure 1: Ribbon diagram illustrating two spectrin repeats. One complete repeat from each of α-spectrin (blue) and β-spectrin (purple) are shown interacting at the tetramerization site. The tetramerization site are consisted of helix A and helix B of repeat β17 and helix C of repeat α0. Triple-helical structure can be observed in repeat β16 and repeat α1.

Image obtained from the Protein Database at
http://www.rcsb.org/pdb/explore/explore.do?structureId_3LBX

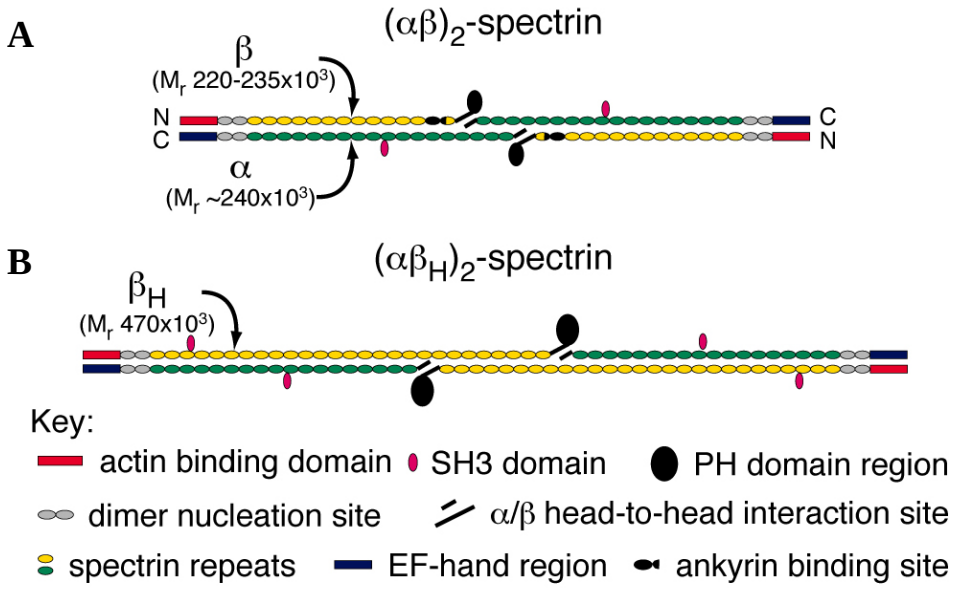


Figure 2: Schematic representation of $(\alpha\beta)_2$ and $(\alpha\beta_H)_2$ tetramers and their respective domains. The location of actin binding domain, SH3 domain, PH domain, dimer nucleation site, head-to-head interaction site, spectrin repeats, EF-hand region, and ankyrin binding site are mapped on the diagrams. (A) An $(\alpha\beta)_2$ spectrin tetramer composed of two α subunits and two β subunits. (B) An $(\alpha\beta_H)_2$ tetramer composed of two α subunits and two β_H subunits.

Figure courtesy of Dr. Graham H. Thomas

β-spectrin contains an N-terminal actin-binding domain (Frappier *et al*., 1992; Karinch *et al*., 1990), 17 triple-helical repeats (Yan *et al*., 1993), an ankyrin binding domain (Kennedy *et al*., 1991), and a C-terminal plekstrin homology (PH) domain (Macias, *et al*., 1994; Zhang, *et al*., 1995) (Figure 2). The 17^{th} spectrin repeat is a partial repeat and forms only two helices instead of the usual triple-helix (Desilva *et al*., 1992; Speicher *et al*., 1993; Tse *et al*., 1990). The N-terminus of α-spectrin can interact with repeat 17 of β-spectrin which leads to the formation of a complete triple-helical structure (Desilva *et al*., 1992; Speicher *et al*., 1993; Tse *et al*., 1990). This noncovalent interaction mediates the formation of a tetrameric spectrin from two α-β dimers (Ipsaro *et al*., 2010).

β_H-spectrin contains a N-terminal actin-binding domain, 30 triple-helical repeats, and a C-terminal PH domain (Figure 2) (Dubreuil *et al*.,1990; Thomas and Kiehart, 1994; Thomas *et al*., 1997). The structure of β_H-spectrin closely resembles β-spectrin with several exceptions: Unlike β-spectrin, β_H-spectrin has 13 more triple helical repeats and it does not have an ankyrin binding domain (Stabach and Morrow, 2000; Thomas *et al*., 1997). In *Drosophila*, β_H-spectrin has an SH3 domain located in its 5^{th} spectrin repeat (Thomas *et al*., 1997).

These α and β subunits align themselves in an antiparallel orientation to form heterodimers through the dimer nucleation site and by curling around one another (Speicher *et al*., 1992). The ends of two α-β or α-β_H dimers then interact *via* the head-to-head interaction and assemble into the heterotetrameric spectrin protein (Kennedy *et al*., 1994; Kotula *et al*., 1993; Tse *et al*., 1990; Speicher *et al*., 1992). Head-to-head contacts between the subunits resembles a triple helical structure of a spectrin repeat, and is mediated by interaction between one alpha helix from N-terminus of the α subunit with two antiparallel alpha helices from the C-terminus of the β subunit (Figure 1) (Desilva *et al*., 1992; Speicher *et al*., 1993; Tse *et al*., 1990; Ipsaro *et al*.,

2010). Additionally, the ends of the tetramers have actin binding sites that allow crosslinking of actin filaments at the plasma membrane (Karinch *et al*., 1990; Frappier *et al*., 1992). The interaction between spectrin tetramers and actin is mediated by a pair of calponin homology (CH) domains in the N-terminal actin-binding domain of the β subunit (Banuelos *et al*., 1998).

Spectrin is classified as part of a protein superfamily that includes other proteins such as α-actinin, dystrophin and utrophin (Thomas *et al*., 1997). Proteins in this superfamily share similarities in their structural domains: all have an N-terminal actin-binding domain, a central rod domain, and a C-terminal calcium-binding domain (Broderick and Winder, 2005). Furthermore, the triple-helical repeats of α-actinin closely resembles the last spectrin repeats of α-spectrin and the first spectrin repeats of β-spectrin (Byers *et al*., 1989). This has led to speculations that α-actinin may be an evolutionary precursor of spectrin (Byers *et al*., 1992; Thomas *et al*., 1997; Viel, 1999).

Currently, two isoforms of the α subunit (α_1, α_2) (Sahr *et al*., 1990; Wasenius, *et al*., 1989), four isoforms of the β subunits (β_1, β_2, β_3, β_4) (Berghs *et al*., 2000; Hu *et al*., 1992; Ma *et al*., 1993; Mishra *et al*., 1998; Ohara *et al*., 1998; Stankewich *et al*., 1998; Winkelmann *et al*., 1990) and one β_H (β_5) (Stabach and Morrow, 2000) have been characterized in vertebrates. In *Drosophila melanogaster*, just one α subunit (Dubreuil et al., 1989), one β subunit (Byers et al., 1992) and one β_H subunit (Dubreuil *et al*., 1990; Thomas *et al*., 1997) are encoded by their genome.

Spectrin Localization on the Membrane

Recruitment of spectrin to a membrane is central to spectrin function. In fly epithelial cells, spectrin dimers are present in two forms; α-β and α-β_H. α-β dimers are localized to the basolateral membrane whereas the α-β_H dimers are segregated to the apical membrane where they associate with the *zonula adherens* and also with the brush border (Dubreuil *et al*., 1997; Phillips and Thomas, 2006). All data to date suggests that spectrin association with the membrane is mediated by its β subunit and is independent of α subunit (Hülsmeier *et al*., 2007). The exclusive distribution of these dimers to their respective membrane domains is a response to pathways establishing apicobasal polarity in epithelial cells (Pellikka *et al*., 2002). Generation of apicobasal polarity does not require β_H-spectrin as apicobasal polarity can be maintained without β_H-spectrin (Zarnescu and Thomas, 1999; Phillips and Thomas, 2006). Nevertheless, β_H-spectrin is essential for epithelial cell morphogenesis and border cell morphogenesis (Zarnescu and Thomas, 1999).

The ankyrin binding domain in β-spectrin allows it to bind to this adapter protein, which is in turn associated with integral membrane proteins (Bennett and Stenbuck, 1980). Another adaptor protein, protein 4.1, forms a complex at the spectrin/F-actin junction, where other protein such as adducin and dematin are also found (Derick *et al*., 1992). Interaction between this protein complex and integral membrane proteins allows its localization on the basolateral membrane, forming a network of spectrins, actins and spectrin-associated proteins at the membrane (Figure 3) (Derick *et al*., 1992). Besides that, ankyrin can crosslink ion channels (e.g. anion exchanger, and Na/K ATPase) (Davis *et al*., 1989; Ding and Kopito, 1996; Willardson *et al*., 1989; Jordan *et al*., 1995; Zhou *et al*., 1998) and cell adhesion molecules (e.g. L1 CAM family of proteins) (Hortsch, 2000; Zhang *et al*., 1998) to spectrins, allowing their assembly on SBMS.

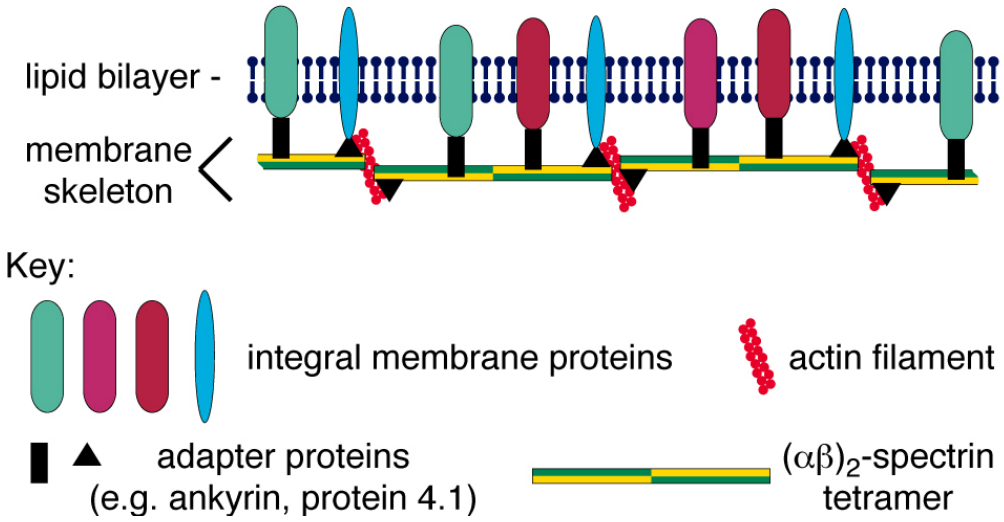


Figure 3: Schematic representation of an hypothetical α-β spectrin network on the basolateral membrane. α-β spectrin tetramers bind actin and protein 4.1 at the spectrin/F-actin junction. The adaptor protein ankyrin binds to α-β spectrin and the integral membrane proteins, allowing α-β spectrin localization to the basolateral membrane.

Figure Courtesy of Dr. Graham H. Thomas

Unlike β-spectrin, β_H-spectrin does not bind ankyrin which suggests that its membrane association is mediated by other factors (Stabach and Morrow, 2000; Thomas *et al*., 1997). Recent studies on *Drosophila* β_H-spectrin have identified Crumbs as a key protein in recruiting β_H-spectrin to the apical membrane (Figure 4) (Pellikka *et al*., 2002). Crumbs is an integral membrane protein that is found at the apical membrane of an epithelial cell (Pellikka *et al*., 2002). Its extracellular segments contain 30 EGF-like and 4 laminin A G-domain-like repeats which could be involved in protein-protein interactions (Tepass *et al*., 2002). It is suggested that Crumbs may be involved in binding to PDZ protein scaffold such as Stardust (Sdt) and Disc-lost (Dlt), forming a complex that has been implicated as an essential component in the establishment of polarized epithelia (Figure 4) (Roh *et al*., 2003).

The expression and localization of Crumbs is required to specify the apical polarity of the epithelial cells (Wodzarz *et al*., 1995). This is supported by the fact that overexpression of Crumbs causes a significant reduction of the basolateral membrane and an enlargement of the apical membrane (Médina *et al*., 2002; Wodzarz *et al*., 1995). Because Crumbs recruits β_H-spectrin to the membrane, Crumbs overexpression also causes the redistribution of β_H-spectrin to the expanded apical membrane (Wodzarz *et al*., 1995).

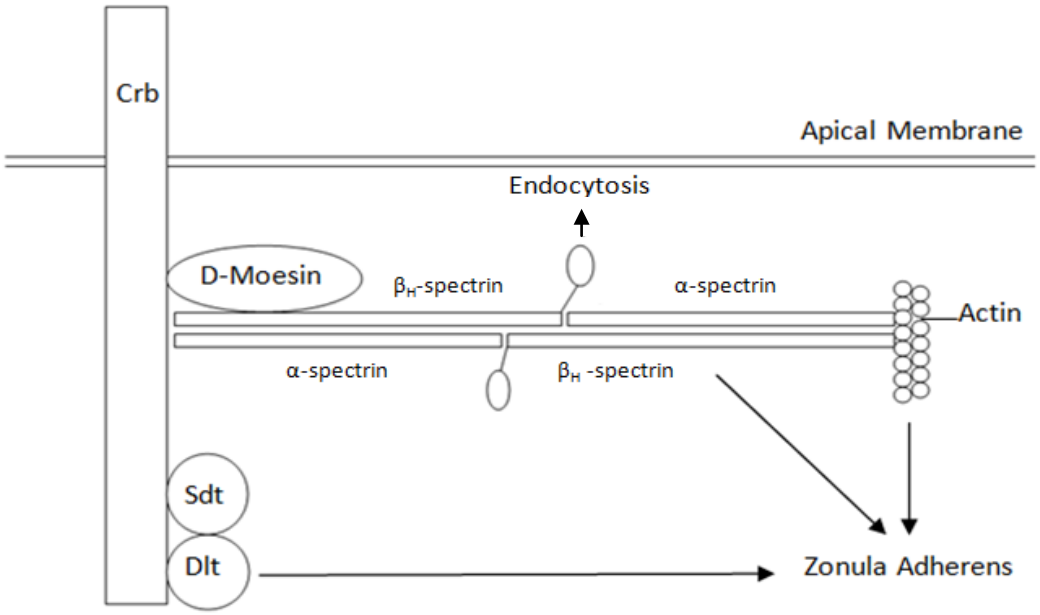


Figure 4: Schematic model of α-β_H spectrin interaction at the apical membrane. Crumbs recruits β_H-spectrin, possibly *via D*-Moesin, to the apical membrane. Crumbs also recruits the PDZ scaffold proteins: Stardust (Sdt) and Discs-lost (Dlt). The C-terminus of β_H-spectrin regulates endocytosis. Spectrin, Dlt and actin bound to spectrin play a role in the establishment of *zonula adherens*.

(Figure adapted from Médina et. al., 2002)

The cytoplasmic domain of Crumbs associates indirectly with β_H-spectrin, possibly *via* *D*-Moesin and its FERM domain binding site to form a protein complex (Médina *et al*., 2002). Thus, *D*-Moesin may be another key player in maintaining cell polarity and was suggested to function as a bridge between Crumbs and the SBMS (Médina *et al*., 2002). However, mutations in *D*-Moesin do not release β_H-spectrin from the membrane, perhaps because β_H-spectrin can also directly associate with the membrane independently of Crumbs (Graham Thomas, personal communication). This interaction is mediated by the PH domain and other sequences within segment 33 at the C-terminus of β_H-spectrin (βH33) (Williams *et al*., 2004; Lee *et al*., 2010). The PH domain can bind the phospholipids at the membrane (Lee *et al*., 2010) but this domain by itself is insufficient to provide stable membrane association: Protein sequences at the N-terminal side of the PH domain are also found to be required in establishing a stable βH33-membrane association (Williams *et al*., 2004).

Roles of β_H-spectrin in Drosophila

We are particularly interested in studying *Drosophila* β_H-spectrin. Previous studies have suggested possible roles of β_H-spectrin in epithelial cell morphogenesis and the modulation of endocytotic and endosomal trafficking (Williams *et al*., 2004; Tjota *et al*., 2011; Phillips and Thomas, 2006; Zarnescu and Thomas, 1999; Lee and Thomas, 2011). One of my research goals is to evaluate several candidate GTPases from these pathways as possible binding partners of β_H-spectrin (see below).

A. β_H-spectrin Role in Cell Morphogenesis

Specific coordination of various proteins and cytoskeletal factors are required in the process of cell morphogenesis and the establishment of epithelial polarity. (Watanabe *et al*., 2009; Yamada and Nelson, 2007). During *Drosophila* gastrulation, the embryo forms mesoderm and endoderm which undergo invagination, producing multiple tissue layers (Leptin and Grunewald, 1990; Sweeton *et al*., 1991). After the epithelial-mesenchymal transition stage, cells may reform epithelia or remain mesenchymal (Knust and Bossinger, 2002; Nelson, 2003). Epithelial cells are known to exhibit apicobasal polarity whereas mesenchymal cells do not (Knust and Bossinger, 2002; Nelson, 2003). Polarity is important for morphogenesis because morphogenetic machinery must be correctly located in the cell. For example, the apical constriction of epithelial tissues requires the recruitment of myosin-II and F-actin to the apical cell cortex and the activation of small GTPases from the Rho family in that region (Young et al., 1991; Winter et al., 2001).

The Rho family of GTPases has been recognized as key players in epithelial morphogenesis (Van Aelst and Symons, 2002). Rac1 (a Rho GTPase) have been previously

implicated in modulating the process of invagination, actin rearrangement, assembly and disassembly E-cadherin-mediated adhesion complexes at cell junction, apicobasal polarity and also to provide support in maintaining epithelial polarity (Gulli and Peter, 2001; Kaibuchi *et al*., 1999; Luo *et al*, 1994; Watanabe *et al*., 2009). Recently, a study has shown that Rac1 could be modulated by β_H-spectrin which is in turn regulated by Pak1, an effector for Rac1 (Lee and Thomas, 2011). The ability of β_H-spectrin to modulate Rac1 indicates that β_H-spectrin is also an essential player in cell morphogenesis.

The phenotype arising from the expression of Trio (a GEF for Rac1, leads to the activation of Rac1 by converting Rac1-GDP (inactive form) to Rac1-GTP (active form)), was found to be greatly enhanced when β_H-spectrin levels were reduced, but was suppressed when the C-terminus of β_H-spectrin (βH33) is co-expressed (Lee and Thomas, 2011). Additionally, a strong dominant eye phenotype was induced in *Drosophila* when βH33 was extensively expressed in the eye but was suppressed by the co-overexpression of Trio (Lee and Thomas, 2011). It was also shown that a reduction in Rac1 signalling by loss-of-function *pak* (Pak1) mutations suppresses larval lethality that normally arose in *karst* mutants, whereas the expression of a constitutively active *pak* mutant induces apical membrane expansion and apical protein destabilization in larval salivary glands (Lee and Thomas, 2011). All these observations led to the conclusion that Rac1 signaling is suppressed by the C-terminal domain of β_H-spectrin, and that β_H-spectrin is in turn negatively regulated by Rac1 *via* Pak1 (Lee and Thomas, 2011). However, the mechanism by which Rac1 and β_H-spectrin interact with one another has yet to be characterized. Both proteins may be involved in either direct or indirect interactions.

B. A Role for β_H-spectrin in Modulating Endocytosis and Endosomal Trafficking

Endocytosis is a cellular process in which cells take up external molecules, membranes and integral membrane proteins into their cytoplasm. This process allows cells to obtain large molecular weight molecules which normally would not be able to enter the cell by diffusing through the membrane. It also enables cells to internalize its surface receptors and recycle cellular components back to the membrane. Like all important cellular process, it is subjected to regulatory pathways that modulate its activity. β_H-spectrin has been implicated in modulating the process of endocytosis but the exact mechanisms have not yet been elucidated (Williams *et al*., 2004).

The clathrin-mediated endocytotic pathway is triggered when surface receptors (e.g. EGF-receptors) bind to their ligands, which can be growth factors, enzymes, hormones, etc (McMahon and Boucrot, 2011). This leads to the formation of coated pits on the membrane by the protein Clathrin (Pearse, 1976). The coated pits then invaginate to form coated buds. At this time, the GTPase protein Dynamin accumulates and polymerizes into a ring-like structure around the regions where the coated vesicles will be pinched off from the membrane (Kosaka and Ikeda, 1983). GTP hydrolysis induces a conformational change in Dynamin ring structure, effectively pinching off the vesicles (Hinshaw and Schmid, 1995). After that, Dynamin disassembles from the membrane. Subsequent dissociation of clathrin coat from the vesicles allows the vesicles to progress into early endosomes (Mayor *et al*., 1993).

The process of endocytosis generates a membrane-based compartment known as the endosome. The main roles of endosomes are to facilitate the transport of surface receptors and internalized extracellular components to the lysosomes where they will be degraded (Straus, 1954). The endosomes progress from endocytotic vesicles to the early endosome to the late

endosome (MVB) to the lysosome (Draye *et al*., 1987). Alternatively, the early endosome can also progress into a recycling endosome which will be directed back to the plasma membrane (Draye *et al*., 1987).

Recent studies have shown that the overexpression of C-terminal βH33 constructs in *Drosophila* salivary gland cells produces a bi-membrane structure that is associated with Dynamin (a GTPases involved in the scission of endocytotic vesicles) (Marks et al., 2001; Damke et al. 1994; Williams *et al*., 2004). These bi-membrane structures are observed as membrane sheet protrusions that roll or fold into the cytoplasm from the plasma membrane (Williams *et al*., 2004). Co-staining of βH33 and Dynamin has detected high levels of Dynamin in these structures (Williams *et al*., 2004). It was suggested that the βH33-induced bi-membrane structures sequester Dynamin, inhibiting endocytosis and therefore causing the membrane expansion (Williams *et al*., 2004). The bi-membranes, however, are considered to be an exaggeration of a normal β_H function or gain-of-function phenotype caused by the overexpression of βH33 (Graham Thomas, personal communication). However, these are an indication of what β_H-spectrin may normally do. The dimensions of these bi-membranes closely resemble that of annexin-mediated membrane junctions (Tjota *et al*., 2011). Members of the annexin family have been known to be involved in endocytosis, its downstream endosomal targeting and the formation of multi-vesicular bodies (Tjota *et al*., 2011). It is known that some vertebrate Annexins bind to Dynamin and it was recently shown that Annexin B9 is also sequestered by βH33 (Tjota *et al*., 2011). This suggests the possibility that spectrin may be working closely with annexins in regulating endocytosis and other annexin-related processes.

The involvement of β_H-spectrin in endosomal trafficking was discovered following the overexpression studies of βH33, and in studies of *karst* (encodes β_H-spectrin) mutant of brush

border epithelial cells (Williams *et al*., 2004; Phillips and Thomas, 2006). Subsequent studies provide evidence that βH33 physically interacts and co-localizes with Annexin B9 in βH33-induced bi-membranes (Tjota *et al*., 2011). Both βH33 and Annexin B9 are needed for cellular cargo progression *via* the MVB as the reduction or absence of either protein will disrupt normal endosomal trafficking in the cell (Tjota *et al*., 2011). Additional studies utilizing the β_H-derived protein Minikarst and loss-of-function *pak* mutation have confirmed that β_H-spectrin does not strictly localizes at the apical membrane but can also be found in endosomal compartments (Tjota *et al*., 2006). Also, it was found that the *karst* mutation causes the loss of Rab5-positive early endosomes and an increase in lysosomal compartments (Phillips and Thomas, 2006). This shows that β_H-spectrin plays a role in regulating the progression of the early endosome and decision to either transport protein back to the membrane (recycling), or to lysosomes (degradation).

karst and Rab5 show a curious genetic interaction. Rab5-positive endosomes can be detected in *karst* mutant cells in first and second-instar middle midguts but not in the third-instar (Phillips and Thomas, 2006). Loss of early endosome Rab5 staining was observed when Rab5S43N, a dominant-negative Rab5 that is constitutively bound to GDP, was expressed in the midgut (Phillips and Thomas, 2006). Interestingly, the expression of Rab5S43N in first and second instar *karst* mutants did not cause any loss of Rab5-positive endosomes (Phillips and Thomas, 2006). This epistatic relationship between Rab5 and β_H-spectrin implicated both proteins in the same pathway maintaining the integrity of endosomes (Phillips and Thomas, 2006). The exact relationship or interaction between Rab5 and β_H-spectrin has yet to be characterized and this poses an area of interests for further research.

Possible binding partners of βH33 based on previous Yeast 2-hybrid screen

Previously, a yeast 2-hybrid library screen was employed to find possible binding partners of βH33 (Klipfell, 2006). Using the colony lift assay for LacZ expression, several proteins were identified as positive interactors of βH33 which include Eps15, Fax and Nemy. These three proteins have specific functions that are relevant to β_H-spectrin but the extent of their relationship has yet to be characterized. A second goal of my research is to re-evaluate Eps15, Fax and Nemy as possible binding partners of βH33 using a yeast spotting assay, a yeast 2-hybrid screening approach different than the one previously employed.

Eps15

Epidermal growth factor receptor pathway substrate clone 15 (*eps15*) encodes for an endocytotic protein that serves as a substrate for EGF receptor (EGFR) tyrosine kinase (Salcini *et al*., 1999). Eps15 is localized to the edges of budding Clathrin-coated pits in the cell, implicating its role as an endocytotic regulator (Torrisi *et al*., 1999). Furthermore, this protein has been found to bind clathrin-dependent endocytotic-related proteins such as EGFR, AP-2, AP180, Stonin, Synaptojanin, Epsin, Dynamin and Clathrin (Salcini *et al*., 1999; Majumdar *et al*., 2006). Two post-translational modifications are found to be required for Eps15 function in the internalization of EGFR: tyrosine phosphorylation by EGFR and monoubiquitination by an unidentified protein (Confalonieri, *et al*., 2000; Van Delft, *et al*., 1997). Recently, Eps15 has been shown to be essential for synaptic vesicle recycling in *Drosophila* (Majumdar *et al*., 2006).

Like Eps15, β_H-spectrin has been implicated as an important modulator of endocytosis (Williams *et al*., 2004). Co-localization of β_H-spectrin with Dynamin (an essential endocytotic protein that also binds Eps15) seems to support this notion (Williams *et al*., 2004). Both proteins

could be involved in the same endocytotic pathway, but the nature of their relationship is not clear. Hence, we consider Eps15 as a possible binding partner of β_H-spectrin.

Fax

Failed axon connection (fax) encodes for a 47-kDa neurofilament protein in *Drosophila* (Hill *et al*., 1995). Its C-terminus shares certain homology with rat NF-M (neurofilament intermediate protein M) and bovine NF-L (neurofilament intermediate protein L) (Hill *et al*., 1995). Fax is localized to the plasma membrane where it could be involved in protein-protein interaction (Hill *et al*., 1995). However, the exact mechanism in which Fax is recruited to the membrane is not well understood. Genetic analysis had shown that Fax may function downstream of the Abelson tyrosine kinase (Abl) pathway, although Fax itself is not a substrate for Abl (Hill *et al*., 1995). Furthermore, other proteins such as Dab (*disabled*), Ena (*enabled)* and Trio also exhibited genetic interaction with Abl (Liebl *et al*., 2000). This suggests that these proteins are likely to be in the same pathway, probably related to the modulation of axon guidance (Liebl *et al*., 2000). In vertebrates, the Abl pathway is modulated by spectrin bound proteins ((Ziemnicka-Kotula *et al*., 1998). However, the relationships between Fax, Dab, Ena and Abl have not been elucidated.

Interestingly, spectrin has also been implicated to be an essential modulator of axon guidance in *Drosophila* nervous system (Hülsmeier *et al*., 2007). Although both α-spectrin and β-spectrin are needed for neural development, β-spectrin appears to be the key determinant in the control of axon patterning (Hülsmeier *et al*., 2007). Mutation in β-spectrin but not α-spectrin was found to produce structural defects in neuronal growth cones (Hülsmeier *et al*., 2007). Hence, we

consider the possibility that SBMS may be somehow related with Fax as both are implicated to have a role in axon pathfinding.

Nemy

No-extended memory (*nemy*) encodes a *Drosophila* homolog of cytochrome b561 (CytB561) which is present in animals and plants but not in fungi and prokaryotes (Verelst and Asard, 2003). CytB561 is an integral membrane protein that functions as an electron transporter (Srivastava *et al*., 1984). Generally, this protein localizes in small synaptic vesicles and large dense core vesicles but may vary depending on its isoform types (Fischer von Mollard *et al*., 1990). To date, several isoforms of CytB561 have been identified: chromaffin granule CytB561 (CGCytb), duodenal CytB561 (DCytb), and lysosomal CytB561 (LCytb) (McKie *et al*., 2001; Zhang *et al*., 2006). CGCytb is specifically expressed in chromaffin granules of neuroendocrine tissues and other cells secreting peptide or catecholamine (Perin *et al*., 1988). DCytb expression is restricted to the apical brush border plasma membrane and vesicular membrane of duodenal epithelial cells (McKie *et al*., 2001). LCytb, on the other hand, is expressed primarily in the membranes of late endosome-lysosome of certain cell types such as alveolar macrophages and Sertoli cells (Zhang *et al*., 2006). This particular CytB561 isoforms can also be found in thymus, white pulp of spleen and lamina propria of intestinal villi (Zhang *et al*., 2006).

All known isoforms of CytB561 are known to facilitate the shuttling of electrons across vesicular membrane. In general, CytB561 utilizes extravesicular ascorbate as its electron donor and intravesicular monodehydro-ascorbate (oxidized form of ascorbate) and/or ferric ion as its electron acceptor (Verelst and Asard, 2003). For CGCytb, the reduction of monodehydro-ascorbate regenerates the ascorbate in the vesicle which is then used as cofactor for the enzymes

dopamine-β-hydroxylase (DbH) and peptidyl α-hydroxylating monooxygenase (PHM) (Kent and Fleming, 1987). Both DbH and PHM are mixed function oxidases involved in neurotransmission pathways: DbH hydrolyzes dopamine to norepinephrine whereas PHM α-amidates neuroendocrine peptides, converting them into their active forms (Kent and Fleming, 1987). As for other CytB561 isoforms, DCytb is not involved in DbH or PHM pathways whereas LCytb is predicted to be involved in those pathways (Zhang *et al*., 2006). Aside from that, CGCytb, DCytb and maybe LCytb also exhibited ferric reductase activity; they mediates the transfer of electrons from ascorbate to ferric ion (Vargas *et al*., 2003; Zhang *et al*., 2006). This facilitates the transfer of iron from endosomal vesicles to the cytoplasm.

The *Drosophila* genome encodes two splice variants of CytB561 homologs: CG1275 and CG8776 (now known as *nemy*) (Verelst and Asard, 2003). Similar to the CGCytb isoform, Nemy is found to be primarily expressed in the neuroendocrine tissues in *Drosophila* larval and adult brain (Iliadi *et al*., 2008). Partial co-expression with Nemy with PHM also suggested a role for Nemy in regulating the activity of PHM (Iliadi *et al*., 2008). It was found that defects in Nemy impair *Drosophila* learning and memory retention, possibly because PHM-mediated production of amidated neuropeptides involved in memory processes is greatly disrupted in the absence of Nemy (Iliadi *et al*., 2008). Roles for Nemy in the gut and/or lysosome have yet to be defined.

Hypothesis

Previous studies have suggested that the C-terminal domain of β_H-spectrin may be involved in regulating the function of key GTPases (Williams *et al*., 2004; Tjota *et al*., 2011; Phillips and Thomas, 2006; Zarnescu and Thomas, 1999; Lee and Thomas, 2011). One hypothesis suggests that βH33 may function as a guanine nucleotide exchange factor (GEF) mimic that binds GTPase proteins and prevents them from being activated by other GEFs. This hypothesis was suggested by a number of observations: First, βH33 suppresses Rac1 signaling, perhaps by preventing Trio from activating Rac1. Co-expression of Trio and βH33 produces normal levels of Rac1 activity but expression of only Trio causes an increase in the levels of Rac1-GTP (Lee and Thomas, 2011). We hypothesize that βH33 may be competing with Trio for the same binding site on Rac1, implicating that β_H-spectrin might be interacting directly with Rac1.

This hypothesis is further supported by sequence analysis of β_H-spectrin: Novel sequence analysis software (GDDA-BLAST; Chang *et al*., 2008) suggests that β_H-spectrin contains a Dbl homology domain in its C-terminus. Dbl homology domains are key structural domains of GEFs for Rho family GTPases (Graham Thomas, personal communication). Hence, it is possible that the Dbl homology domain in β_H-spectrin may have GEF-like properties including the ability to bind GTPases. There is also a striking parallel between the structure of Trio and β_H-spectrin: both have spectrin repeats, Dbl homology domain and PH domain (Figure 5). To date, any such role for the putative Dbl homology domain in β_H-spectrin has not been identified. It is unsure whether the domain still retains its function or has loss its function during the course of β_H-spectrin evolution or if this function was gained by Trio. This region is also the tetramerization domain of β_H-spectrin and would have to have a dual function if it is a GEF (Graham Thomas,

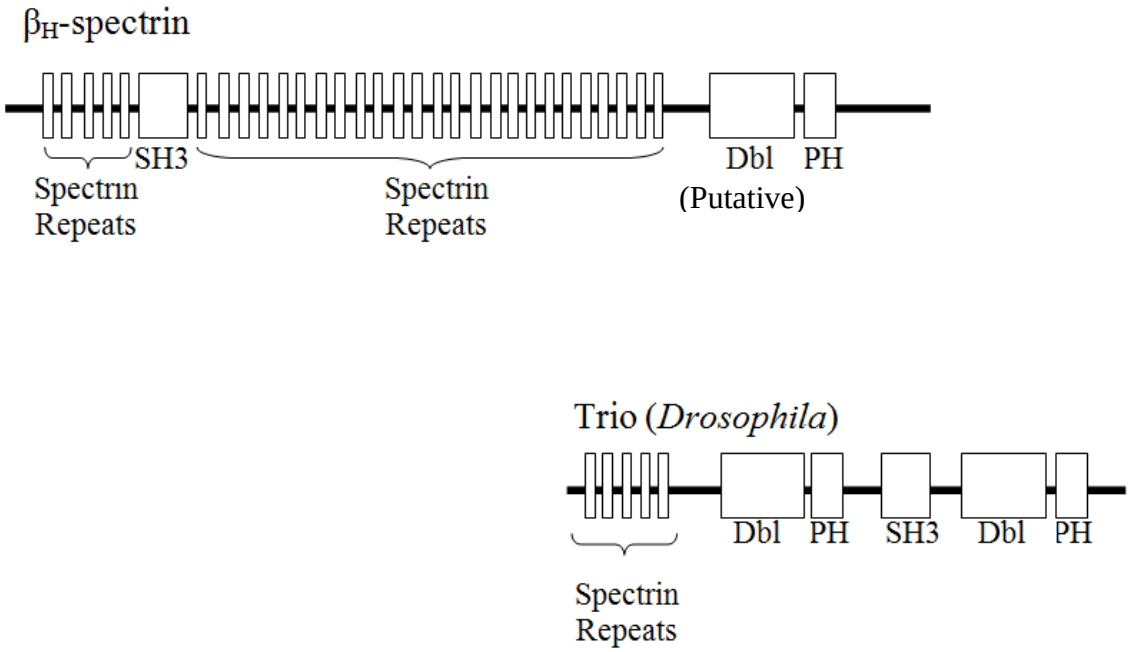


Figure 5: Schematic representation of β_H-spectrin and Trio. The location of spectrin repeats, SH3 domain, Dbl homology domain, and PH domain are mapped on the diagram. β_H-spectrin is predicted to have a Dbl homology domain at amino acid position 3412-3919.

(Figure adapted from Bateman and Van Vactor, 2001)

personal communication). Hence, β_H-spectrin may have to dissociate itself from α-spectrin in order to function as a dominant negative GEF, and this function may be inactive when β_H-spectrin is in its tetrameric form. However, this notion has yet to be validated.

To test the hypothesis, several candidate members from the Rho GTPase family were selected to be tested for direct interaction with GEFlong (a fragment of β_H-spectrin C-terminus that contains the predicted Dbl homology domain through the PH domain) by using the yeast 2-hybrid system. Candidate Rho GTPases are Rho1, RhoL, Rac2, and Mtl.

In a separate experiment, βH33 was demonstrated to have an epistatic interaction with Rab5 (Phillips and Thomas, 2006). It was observed that expression of dominant negative Rab5S43N disrupts early endosome in *Drosophila* first instar larvae. However, the lack of β_H-spectrin prevents the disruption of early endosome by dominant negative Rab5. Currently, it is unclear what exactly is going on in these observations. Nevertheless, it is possible that β_H-spectrin and Rab5 may be somehow interacting with one another. If so, the Dbl homology domain of β_H-spectrin could be mediating this interaction. To test this notion, Rab5 along with several other candidates of the Rab GTPase family were also selected to be screened for direct interaction with GEFlong *via* the yeast-2-hybrid system. In general, Rab GTPases function in coordinating endocytosis and the downstream endosomal trafficking pathways. The tested members were Rab4 (early endosome), Rab5 (early endosome), Rab7 (late endosome), and Rab11 (recycling endosome).

Overview of Yeast 2-hybrid System

The yeast 2-hybrid (Y2H) system is a very useful genetic assay for detecting *in vivo* protein-protein interactions (Fields and Song, 1989). This system can also be used to identify domains or amino acid residues that are involved in the protein-protein interactions (Fields and Sternglanz, 1994).

As outlined in the Y2H protocol supplied by (Fields and Song, 1989), the Y2H system requires the insertion and expression of two hybrid constructs in yeast: bait and prey. Bait construct encodes for a DNA binding domain fused to a protein/protein fragment of interest whereas the prey construct encodes for an activation domain fused to a candidate binding partner of the former protein. In our case, the bait protein is GEFlong and the prey proteins are the candidate GTPases. The physical interaction between bait and prey proteins will bring the DNA binding domain and the activation domain together, forming a functional transcription factor that activates several reporter genes. The strength of the bait-prey interaction can be determined based on the ability of the bait-prey complex in activating certain reporter genes (activation of gene for adenine biosynthesis requires stronger bait-prey interaction than the activation of gene for histidine biosynthesis) (Fields et. al., 1994).

In the Y2H tests, we will carry out the Y2H protocol (Fields and Song, 1989) specified by Matchmaker™ GAL4 Two-Hybrid System 3 & Libraries User Manual (Clontech, Mountain View, CA). The yeast will be grown in three different amino acid deficient media: SD/-Leu/-Trp, SD/-Leu/-Trp/-His, and SD/-Leu/-Trp/-His/-Ade plates. The ability of yeasts to grow on SD/-Leu/-Trp/-His, and SD/-Leu/-Trp/-His/-Ade plates indicate weak and strong bait-prey interaction respectively. Yeast grown on SD/-Leu/-Trp indicate that both bait and prey constructs are expressed properly (viability control).

MATERIALS AND METHODS

DNA Constructs, Transformation and Cell Culture for GEFlong and Candidate GTPases

GEFlong/bait DNA construct was obtained by cloning GEFlong into a pGBKT7 vector (Clontech, Mountain View, CA). The GEFlong fragment digested with EcoR1 and Xho1 has been previously prepared by Graham Thomas. GEFlong is a β_H spectrin fragment containing Dbl homology domain through the PH domain of the β_H spectrin C-terminus (amino acids 3412-3919) (Figure 6). For cloning, pGBKT7 vector was digested with EcoR1 and Sal1 followed by DNA purification using spermine precipitation protocol. Both digested vector and GEFlong insert were joined together *via* sticky end ligation.

Rho1/prey, RhoL/prey, Rac2/prey, Mtl/prey, Rab4/prey, Rab5/prey, Rab7/prey and Rab11/prey DNA constructs were obtained by respectively amplifying the Rho1, RhoL, Rac2, Mtl, Rab4, Rab5, Rab7 and Rab11 transgenes from cDNAs in pGEX clones (A gift from Arno Mueller, Dundee University, UK) and cloning them into pACT2 vector (Clontech, Mountain View, CA) respectively.

To amplify the Rho1 transgene for cloning, I used:

top primer: Rho1top (5'-TACCCGGGTACGACGATTCGCAAGAAA-3')

bottom primer: pgexbot (5'-GCAGATCGTCAGTCAGTCACGATGAATTC-3')

To amplify the RhoL, Rac2, and Mtl transgene for cloning, I used:

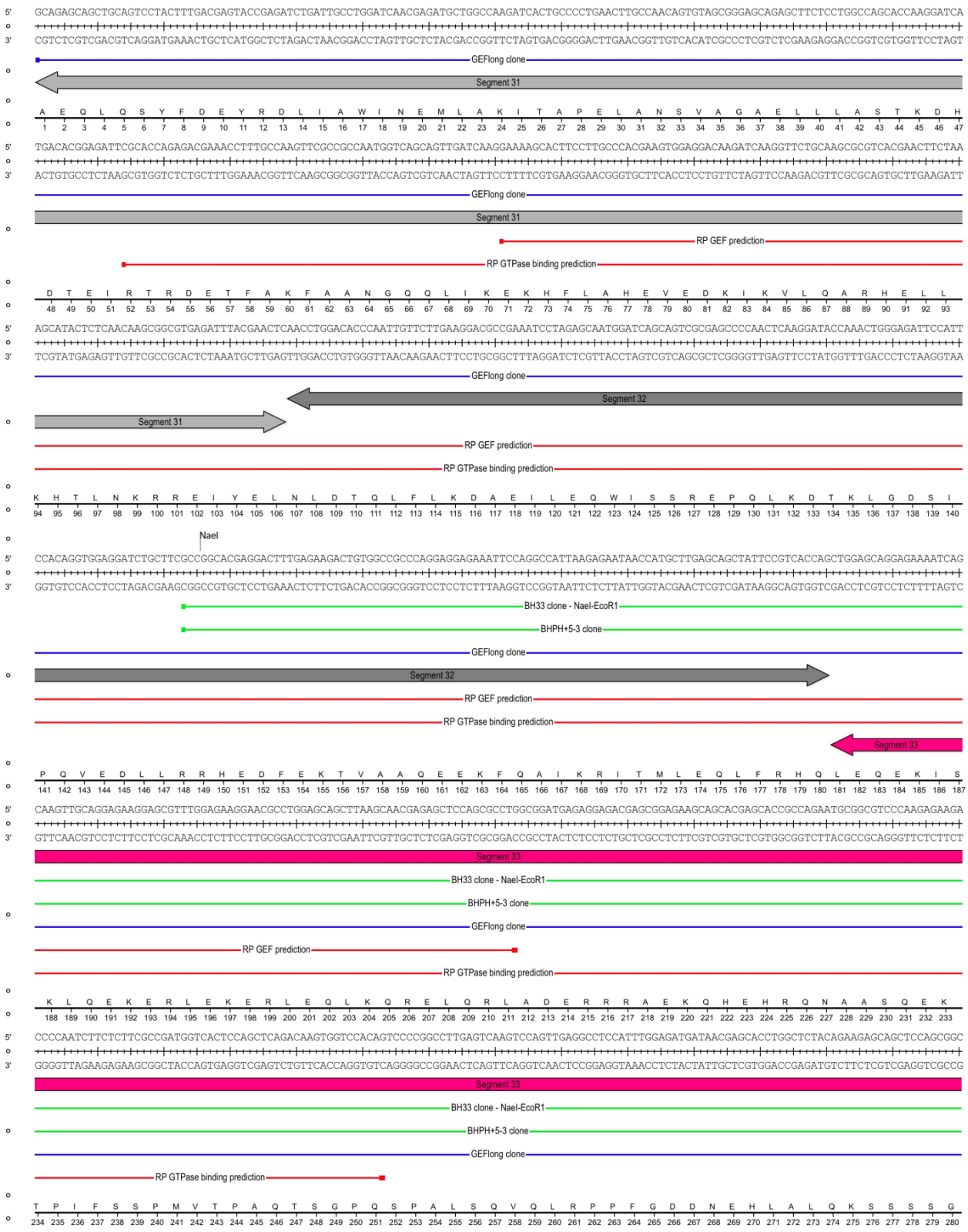
top primer: pgxunivf (5'-TACCATGGTAGATCTGGTTCCGCGTG-3')

bottom primer: pgexbot (5'-GCAGATCGTCAGTCAGTCACGATGAATTC-3')

To amplify the Rab4, Rab5, Rab7 and Rab11 transgene for cloning, I used:

top primer: pgxunivf (5'-TACCATGGTAGATCTGGTTCCGCGTG-3')

bottom primer: Rabclone (5'-CGGGGCGAAAACTCTCAAGGAT-3')


5' GCAGAGCAGCTGCAGTCCTACTTTGACGAGTACCGAGATCTGATTGCCTGGATCAACGAGATGCTGGCCAAGATCACTGCCCCTGAACTTGCCAACAGTGTAGCGGGAGCAGAGCTTCTCCTGGCCAGCACCAAGGATCA
3' CGTCTCGTCGACGTCAGGATGAAACTGCTCATGGCTCTAGACTAACGGACCTAGTTGCTCTACGACCGGTTCTAGTGACGGGGACTTGAACGGTTGTCACATCGCCCTCGTCTCGAAGAGGACCGGTCGTGGTTCCTAGT
140
GEFlong clone
Segment 31
A E Q L Q S Y F D E Y R D L I A W I N E M L A K I T A P E L A N S V A G A E L L L A S T K D H
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47
5' TGACACGGAGATTCGCACCAGAGACGAAACCTTTGCCAAGTTCGCCGCCAATGGTCAGCAGTTGATCAAGGAAAAGCACTTCCTTGCCCACGAAGTGGAGGACAAGATCAAGGTTCTGCAAGCGCGTCACGAACTTCTAA
3' ACTGTGCCTCTAAGCGTGGTCTCTGCTTTGGAAACGGTTCAAGCGGCGGTTACCAGTCGTCAACTAGTTCCTTTTCGTGAAGGAACGGGTGCTTCACCTCCTGTTCTAGTTCCAAGACGTTCGCGCAGTGCTTGAAGATT
280
GEFlong clone
Segment 31
RP GEF prediction
RP GTPase binding prediction
D T E I R T R D E T F A K F A A N G Q Q L I K E K H F L A H E V E D K I K V L Q A R H E L L
48 49 50 51 52 53 54 55 56 57 58 59 60 61 62 63 64 65 66 67 68 69 70 71 72 73 74 75 76 77 78 79 80 81 82 83 84 85 86 87 88 89 90 91 92 93
5' AGCATACTCTCAACAAGCGGCGTGAGATTTACGAACTCAACCTGGACACCCAATTGTTCTTGAAGGACGCCGAAATCCTAGAGCAATGGATCAGCAGTCGCGAGCCCCAACTCAAGGATACCAAACTGGGAGATTCCATT
3' TCGTATGAGAGTTGTTCGCCGCACTCTAAATGCTTGAGTTGGACCTGTGGGTTAACAAGAACTTCCTGCGGCTTTAGGATCTCGTTACCTAGTCGTCAGCGCTCGGGGTTGAGTTCCTATGGTTTGACCCTCTAAGGTAA
420
GEFlong clone
Segment 32
Segment 31
RP GEF prediction
RP GTPase binding prediction
K H T L N K R R E I Y E L N L D T Q L F L K D A E I L E Q W I S S R E P Q L K D T K L G D S I
94 95 96 97 98 99 100 101 102 103 104 105 106 107 108 109 110 111 112 113 114 115 116 117 118 119 120 121 122 123 124 125 126 127 128 129 130 131 132 133 134 135 136 137 138 139 140
NaeI
5' CCACAGGTGGAGGATCTGCTTCGCCGGCACGAGGACTTTGAGAAGACTGTGGCCGCCCAGGAGGAGAAATTCCAGGCCATTAAGAGAATAACCATGCTTGAGCAGCTATTCCGTCACCAGCTGGAGCAGGAGAAAATCAG
3' GGTGTCCACCTCCTAGACGAAGCGGCCGTGCTCCTGAAACTCTTCTGACACCGGCGGGTCCTCCTCTTTAAGGTCCGGTAATTCTCTTATTGGTACGAACTCGTCGATAAGGCAGTGGTCGACCTCGTCCTCTTTTAGTC
560
BH33 clone - NaeI-EcoR1
BHPH+5-3 clone
GEFlong clone
Segment 32
RP GEF prediction
RP GTPase binding prediction
Segment 33
P Q V E D L L R R H E D F E K T V A A Q E E K F Q A I K R I T M L E Q L F R H Q L E Q E K I S
141 142 143 144 145 146 147 148 149 150 151 152 153 154 155 156 157 158 159 160 161 162 163 164 165 166 167 168 169 170 171 172 173 174 175 176 177 178 179 180 181 182 183 184 185 186 187
5' CAAGTTGCAGGAGAAGGAGCGTTTGGAGAAGGAACGCCTGGAGCAGCTTAAGCAACGAGAGCTCCAGCGCCTGGCGGATGAGAGGAGACGAGCGGAGAAGCAGCACGAGCACCGCCAGAATGCGGCGTCCCAAGAGAAGA
3' GTTCAACGTCCTCTTCCTCGCAAACCTCTTCCTTGCGGACCTCGTCGAATTCGTTGCTCTCGAGGTCGCGGACCGCCTACTCTCCTCTGCTCGCCTCTTCGTCGTGCTCGTGGCGGTCTTACGCCGCAGGGTTCTCTTCT
700
Segment 33
BH33 clone - NaeI-EcoR1
BHPH+5-3 clone
GEFlong clone
RP GEF prediction
RP GTPase binding prediction
K L Q E K E R L E K E R L E Q L K Q R E L Q R L A D E R R R A E K Q H E H R Q N A A S Q E K
188 189 190 191 192 193 194 195 196 197 198 199 200 201 202 203 204 205 206 207 208 209 210 211 212 213 214 215 216 217 218 219 220 221 222 223 224 225 226 227 228 229 230 231 232 233
5' CCCCAATCTTCTCTTCGCCGATGGTCACTCCAGCTCAGACAAGTGGTCCACAGTCCCCGGCCTTGAGTCAAGTCCAGTTGAGGCCTCCATTTGGAGATGATAACGAGCACCTGGCTCTACAGAAGAGCAGCTCCAGCGGC
3' GGGGTTAGAAGAGAAGCGGCTACCAGTGAGGTCGAGTCTGTTCACCAGGTGTCAGGGGCCGGAACTCAGTTCAGGTCAACTCCGGAGGTAAACCTCTACTATTGCTCGTGGACCGAGATGTCTTCTCGTCGAGGTCGCCG
Segment 33
BH33 clone - NaeI-EcoR1
BHPH+5-3 clone
GEFlong clone
RP GTPase binding prediction
T P I F S S P M V T P A Q T S G P Q S P A L S Q V Q L R P P F G D D N E H L A L Q K S S S S G
234 235 236 237 238 239 240 241 242 243 244 245 246 247 248 249 250 251 252 253 254 255 256 257 258 259 260 261 262 263 264 265 266 267 268 269 270 271 272 273 274 275 276 277 278 279 280


5' ATGTTCGGTGACCGACTCCGTCGCGGATCTGCGGATGCCAATGTGAAGCGGGCGGAGAGCATGAAGGTACAGCCCAAGCAGGCCAAGCGTACACCATCGTTCACCACCAGAAGACGTGCTCAGTCCTTCAGGAAGAACCA
3' TACAAGCCACTGGCTGAGGCAGCGCCTAGACGCCTACGGTTACACTTCGCCCGCCTCTCGTACTTCCATGTCGGGTTCGTCCGGTTCGCATGTGGTAGCAAGTGGTGGTCTTCTGCACGAGTCAGGAAGTCCTTCTTGGT
980
Segment 33
BH33 clone - NaeI-EcoR1
BHPH+5-3 clone
GEFlong clone
M F G D R L R R G S A D A N V K R A E S M K V Q P K Q A K R T P S F T T R R R A Q S F R K N Q
281 282 283 284 285 286 287 288 289 290 291 292 293 294 295 296 297 298 299 300 301 302 303 304 305 306 307 308 309 310 311 312 313 314 315 316 317 318 319 320 321 322 323 324 325 326 327
5' GAAGGGTGAAGGATTCGACCTGCCACCCGTTGAGATTCAGGGCAGTCTGGAGCGTAAGCATGGTTTGCAATCTGGCGGCAAGAAGGCTCCTGTACGATCCTGGAAGCAGTTCCACACTGTCCTCTGTGGCCAGTTGGTGT
3' CTTCCCACTTCCTAAGCTGGACGGTGGGCAACTCTAAGTCCCGTCAGACCTCGCATTCGTACCAAACGTTAGACCGCCGTTCTTCCGAGGACATGCTAGGACCTTCGTCAAGGTGTGACAGGAGACACCGGTCAACCACA
1120
Segment 33
BH33 clone - NaeI-EcoR1
BHPH+5-3 clone
GEFlong clone
PH domain
K G E G F D L P P V E I Q G S L E R K H G L Q S G G K K A P V R S W K Q F H T V L C G Q L V
328 329 330 331 332 333 334 335 336 337 338 339 340 341 342 343 344 345 346 347 348 349 350 351 352 353 354 355 356 357 358 359 360 361 362 363 364 365 366 367 368 369 370 371 372 373
5' GCTTCTTTAAGGACGAAAATGATTTCCTGCAACAGAAGACGGCCACTGCACCTGTTAATATCCTCGGTGCCAAATGCGAACGTGCTGATGATTACACGAAGAAGAAGTACGTGTTCAGGCTAAAACTACCCGACGGATCG
3' CGAAGAAATTCCTGCTTTTACTAAAGGACGTTGTCTTCTGCCGGTGACGTGGACAATTATAGGAGCCACGGTTTACGCTTGCACGACTACTAATGTGCTTCTTCTTCATGCACAAGTCCGATTTTGATGGGCTGCCTAGC
1260
PH domain
Segment 33
BH33 clone - NaeI-EcoR1
BHPH+5-3 clone
GEFlong clone
C F F K D E N D F L Q Q K T A T A P V N I L G A K C E R A D D Y T K K K Y V F R L K L P D G S
374 375 376 377 378 379 380 381 382 383 384 385 386 387 388 389 390 391 392 393 394 395 396 397 398 399 400 401 402 403 404 405 406 407 408 409 410 411 412 413 414 415 416 417 418 419 420
5' GAGTTCCTGTTCGAGGCTCCCTCGCTGGACATTCTAAATGACTGGGTGCGCAAGATATCCTTCCACGCCAGTCTGCCGCCGAACATGCAACTGCTTAGCTACGACGAGTCCATGAAGCAACAGTCGAGCAGTTCGCCGGA
3' CTCAAGGACAAGCTCCGAGGGAGCGACCTGTAAGATTTACTGACCCACGCGTTCTATAGGAAGGTGCGGTCAGACGGCGGCTTGTACGTTGACGAATCGATGCTGCTCAGGTACTTCGTTGTCAGCTCGTCAAGCGGCCT
1400
PH domain
Segment 33
BH33 clone - NaeI-EcoR1
BHPH+5-3 clone
GEFlong clone
E F L F E A P S L D I L N D W V R K I S F H A S L P P N M Q L L S Y D E S M K Q Q S S S S P D
421 422 423 424 425 426 427 428 429 430 431 432 433 434 435 436 437 438 439 440 441 442 443 444 445 446 447 448 449 450 451 452 453 454 455 456 457 458 459 460 461 462 463 464 465 466 467
5' CATCAAGGTTACTAGCAGTGTGGAATCACCAGTTAGTTCTCGTAACTCATCTCCCGACTCCCAGCGACGTACTAGCGGAGCCCAGGTCCTTGACGGAACAGCCACTCCCCAAATGGCATTCCTTCAACGACAAATGCAAC
3' GTAGTTCCAATGATCGTCACACCTTAGTGGTCAATCAAGAGCATTGAGTAGAGGGCTGAGGGTCGCTGCATGATCGCCTCGGGTCCAGGAACTGCCTTGTCGGTGAGGGGTTTACCGTAAGGAAGTTGCTGTTTACGTTG
1540
Segment 33
BH33 clone - NaeI-EcoR1
BHPH+5-3 clone
GEFlong clone
OPA
I K V T S S V E S P V S S R N S S P D S Q R R T S G A Q V L D G T A T P Q M A F L Q R Q M Q
468 469 470 471 472 473 474 475 476 477 478 479 480 481 482 483 484 485 486 487 488 489 490 491 492 493 494 495 496 497 498 499 500 501 502 503 504 505 506 507 508 509 510 511 512 513
5' AACAACAACAGCAGCAACAATCCCAGCCCAGTTCACCCACCGGCGGATTCGACCAGAAGCCACCGATCCCGCCCAGAGGAGCTGCCCCAGTGGCCAGCCATCGCCAAAGCCAGGAGAATCTAGTTGTGATGCGCAATCGC
3' TTGTTGTTGTCGTCGTTGTTAGGGTCGGGTCAAGTGGGTGGCCGCCTAAGCTGGTCTTCGGTGGCTAGGGCGGGTCTCCTCGACGGGGTCACCGGTCGGTAGCGGTTTCGGTCCTCTTAGATCAACACTACGCGTTAGCG
Segment 33
BH33 clone - NaeI-EcoR1
OPA
Q Q Q Q Q Q Q S Q P S S P T G G F D Q K P P I P P R G A A P V A S H R Q S Q E N L V V M R N R
514 515 516 517 518 519 520 521 522 523 524 525 526 527 528 529 530 531 532 533 534 535 536 537 538 539 540 541 542 543 544 545 546 547 548 549 550 551 552 553 554 555 556 557 558 559 560

Figure 6: DNA sequence and the corresponding amino acid sequence of C-terminus of β_H-spectrin. The sequence for segments 31-33 is shown. Double arrow indicate the section boundaries. The constructs mentioned in this thesis are: βH33: 3560-4097; GEFlong: 3412-3919; Segment 31: 3412-3517; Segment 32: 3518-3591; Segment 33: 3591-4097. The numbers are for full-length β_H, but the diagram labels the C terminus only (1 to 686).

For cloning, the Rho1 PCR product and its corresponding pACT2 vector were digested with Sma1 and EcoR1. RhoL, Rac2, Mtl, and their pACT2 vectors were digested with Nco1 and EcoR1. Rab4, Rab5, Rab7, Rab11 and their pACT2 vectors were digested with Nco1 and Xho1. All the insert and vector fragments were purified using Qiagen QIAEX II gel extraction kit (Qiagen, Valencia, CA) and then ligated together *via* blunt end joining for Rho1/prey construct and sticky ends joining for the rest of the prey constructs (Figure 7).

All DNA constructs were transformed into *Escherichia coli* XL-1 blue cells using electroporation (Bio-Rad, Hercules, CA). Cells transformed with GEFlong/bait were selected on LB media containing 50µg/mL kanamycin. Cells transformed with Rho1/prey, RhoL/prey, Rac2/prey, Mtl/prey, Rab4/prey, Rab5/prey, Rab7/prey and Rab11/prey were selected on LB media containing 100µg/mL ampicillin. The growth conditions of cultures were set at the temperature of 37ºC and incubation time of 16-24hrs. Cell cultures were placed on shaker at 175-200 rpm when liquid LB media was used. DNA constructs were isolated from their respective cells using the CTAB miniprep protocol (Del Sal *et al.*, 1989). All constructs were verified with diagnostic digestion followed by DNA sequencing at Genomic Core Facility (Pennsylvania State University).

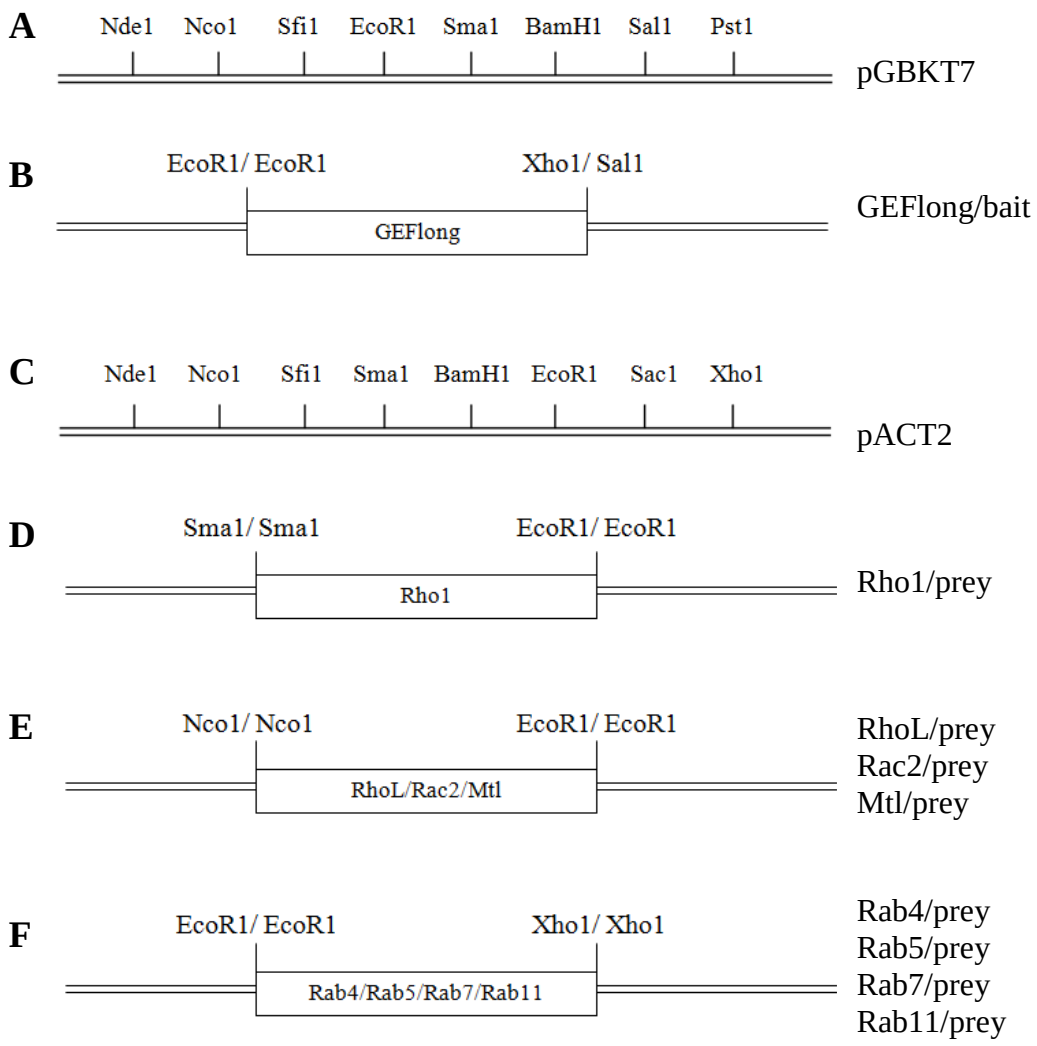


Figure 7: Structures of bait and prey construct inserts. (A) Multiple cloning site of the DNA binding domain vector pGBKT7 (bait). (B) GEFlong was cloned into pGBKT7 at the EcoR1 and Sal1 sites. (C) Multiple cloning site of the activation domain vector pACT2 (prey). (D) Rho1 was cloned into pACT2 at the Sma1 and EcoR1 sites. (E) RhoL, Rac2, and Mtl were separately cloned into pACT2 at the Nco1 and EcoR1 sites. (F) Rab4, Rab5, Rab7, and Rab11 were separately cloned into pACT2 at the EcoR1 and Xho1 sites.

Preparation of Competent Yeast Cells

The following protocol is supplied by (Fields and Song, 1989) and is based on the Matchmaker™ GAL4 Two-Hybrid System 3 & Libraries User Manual (Clontech, Mountain View, CA). *Saccharomyces cerevisiae* AH107 was inoculated into YPDA media and then incubated overnight at 30°C on a shaker set at 250rpm. On the following day, the overnight culture was sub-inoculated into a new YPDA media to make a culture with final OD_{600} of 0.3. The culture was incubated at 30°C on 250 rpm until the OD_{600} reaches 0.5. Cells were then transferred to a 50mL tube and centrifuged at 1000 x g for 5 minutes at room temperature. Supernatant was discarded and the cell pellet was resuspended in sterile H_2O. Cells were again centrifuged at 1,000 x g for 5 minutes at room temperature. Supernatant was discarded and the pellet was resuspended in sterile 1X TE/LiAc.

Yeast Co-Transformation

The following protocol is supplied by (Fields and Song, 1989) and is based on the Matchmaker™ GAL4 Two-Hybrid System 3 & Libraries User Manual (Clontech, Mountain View, CA). Reaction samples were prepared by adding 0.1µg of bait construct, 0.1µg of prey construct and 0.1mg of pre-boiled salmon sperm carrier DNA (Rockland, Gilbertsville, PA) into 0.1mL of AH107 competent cells. The samples were mixed well with vortexing. 0.6mL sterile PEG/LiAc solution was added to each samples and mixed by vortexing. Samples were incubated at 30ºC for 30 minutes on a shaker set at 200 rpm. 70µL of DMSO was then added to each samples and mixed by gentle inversion. Samples were subjected to heat shock for 15 minutes in a 42°C water bath and then chilled on ice for 2 minutes. All samples are centrifuged for 5 seconds at room temperature at 14,000 rpm. The supernatant was discarded and cells were

resuspended in 0.5mL 1X TE. 100µL from each samples were plated on SD/-Leu/-Trp plates. Plates were incubated at 30°C for 3 days and stored at 4°C.

Rescue of Frozen Yeast Cultures

Pun12/Eps15, Pun12/Fax, and Pun12/Nemy frozen yeast clones were obtained from Klipfell's archives. Using an inoculation loop, the cells were streaked on SD/-Leu/-Trp plates. The plates were incubated at 30°C for 3 days and then stored in 4°C.

Smash and Grab Yeast DNA Prep

The following Smash and Grab protocol was carried out as specified in the paper, "Preparation of Yeast DNA" (Hoffman, 2001). Yeast were grown overnight in SD/-L/-T media at 30^0C with shaking at 250 rpm. 1.5mL of culture transferred into an Eppendorf tubes and was centrifuged for 1 minute. Supernatant was aspirated. Then, 200µL of SnG Lysis buffer (2% Triton X-100, 1% SDS, 100mM NaCl, 10mM Tris-Cl (pH 8), 1mM EDTA) was added to the tube. 0.3g of sterile glass beads were added to the tube. Tube was vortexed for 5 minutes and centrifuged for 10 minutes at 14000 rpm. Clear supernatant was recovered and transferred into a new tube. DNA sample was purified using phenol/chloroform extraction followed by ethanol precipitation and washed with 70% ethanol. Sample was centrifuged, and the supernatant was discarded. Pellet was resuspended in 100µL TE.

Sub-culturing of Yeast Cells

Several colonies from previous SD/-Leu/-Trp plates were selected and transferred to a new SD/-Leu/-Trp plates using an inoculation loop. The plates were incubated at 30°C for 3 days and then stored in 4°C.

Yeast Spotting Assay

The following protocol is supplied by (Fields and Song, 1989) and is based on the Matchmaker™ GAL4 Two-Hybrid System 3 & Libraries User Manual (Clontech, Mountain View, CA). Transformed yeast were grown overnight in 2mL SD/-Leu/-Trp media at 30°C. On the following day, three overnight cultures for each plasmid combination were sub-inoculated into a new 3mL SD/-Leu/-Trp media to make cultures with final OD_{600} of 0.3. To bring the OD_{600} up to 0.5, the cultures were incubated for 4 hours at 30°C on a shaker set at 250rpm. Subsequently, 0.5 OD/mL samples from the cultures were transferred into Eppendorf tubes and centrifuged for 10 seconds at 14,000 rpm. The supernatants were discarded and the pellets were resuspended in 500µL sterile H_2O. Tubes were centrifuged for 10 seconds at 14,000 rpm. The supernatants were discarded and the pellets were resuspended in 1.5mL sterile H_2O. 10µL of resuspended yeast cells was spotted on SD/-Leu/-Trp, SD/-Leu/-Trp/-His, and SD/-Leu/-Trp/-His/-Ade plates. The plates were incubated at 30°C for 2 days and then stored in 4°C.

Preparation of Nemy Construct

Nemy/prey construct was obtained by amplifying the Nemy gene from total RNA pool and cloned into pACT2 vector. To initially amplify the Nemy gene from total RNA, I used:

top primer: topUTR (5'-GTCACACGCCACACGAACTTG-3')

bottom primer: botUTR (5'-GCGACTCGCTTACCCTCCTG-3')

After that, I did a nested PCR using the following primers:

top primer: nemytop (5'-TAGAATTCATATGGACACGAACTCGGTGA-3')

bottom primer: nemybot (5'-ATCTCGAGCTAAATGCGCTCCGTG-3')

For cloning, the Nemy PCR product and its corresponding pACT2 vector were digested with EcoR1 and Xho1. The insert and vector fragments were purified using Qiagen QIAEX II gel extraction kit (Qiagen, Valencia, CA) and then ligated together *via* sticky end joining. DNA constructs were transformed into *Escherichia coli* XL-1 blue cells using electroporation (Bio-Rad, Hercules, CA). Cells transformed with Nemy/prey were selected on LB media containing 100µg/mL ampicillin. The growth conditions of cultures were set at the temperature of 37ºC and incubation time of 16-24hrs. Cell cultures were placed on shaker at 175-200 rpm when liquid LB media was used. DNA constructs were isolated from their respective cells using the CTAB miniprep protocol (Del Sal *et al*., 1989). DNA construct was verified with DNA sequencing at Genomic Core Facility (Pennsylvania State University). The sequence indicated a single nucleotide change (C-> T) resulting in an alanine to valine amino acid substitution at position 206.

Mapping of β_H site that interacts with Nemy

A set of βH33 deletion (bait) constructs was obtained from Seung-kyu, Lee. These constructs are: Pun12/bait, βH33-5/bait, and PH+5-3/bait (Figure 7). Nemy/prey was co-transformed with each of the deletion constructs and tested using yeast spotting assay according to the methods described earlier (see above).

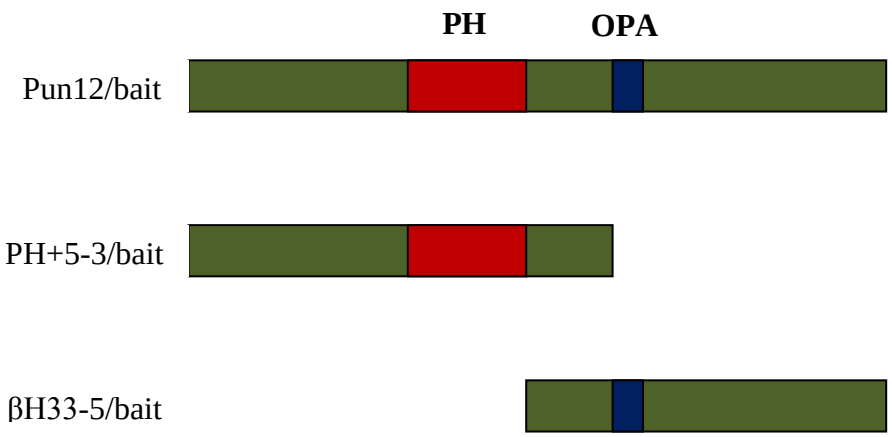


Figure 8: Schematic representation of βH33 deletion constructs. Pun12/bait encodes βH33 (codon 3560-4097 of full-length β_H); PH+5-3/bait encodes βH33 (codon 3560-3920 of full-length β_H) with deletion at its C-terminus up to and including the OPA region (short polyglutamine repeat; blue); βH33-5/bait encodes βH33 (position 3858-4097 of full-length β_H) with deletion at its N-terminus down to and including the PH domain (red).

RESULTS

Rab4, Rab5, Rab7 and Rab11 were chosen for interaction tests because of their known roles with the endosomal/recycling system. Rho1, RhoL, Rac2, Mtl were chosen for similar testing because of their known roles in epithelial cell morphogenesis.

Rab5, Rho1, RhoL, Rac2, and Mtl do not physically interact with GEFlong

Yeast-2-hybrid screening on yeast clones expressing both the GEFlong (bait) and the Rab5 Rho1, RhoL, Rac2, and Mtl (prey) did not detect any direct interaction between the protein pairs (Figure 9). GEFlong seems to be self-activating when expressed in yeasts growing on SD/-L/-T/-H media (FIgure 9). Self-activation of GEFlong presumably arose from interaction between GEFlong and the activation domain encoded by the pACT2 vector. This observation is not unusual as self-activation of expressed protein is quite common in yeast 2-hybrid system. However, this self activation is weak because no self activation was detected on SD/-L/-T/-H/-A (Quad) medium and so, for the purpose of our analysis, we consider only the results from the Quad plates. On Quad medium, no interaction is seen between GEFlong and Rab5 while the positive control (CT17989 + RC1) shows strong growth (Figure 9).

Rab4, Rab7 and Rab11 do not physically interact with GEFlong

Identical results to that of Rab5, Rho1, RhoL, Rac2, and Mtl were seen when yeast 2-hybrid testing had used to test for direct interaction between GEFlong (bait) and Rab4, Rab7 and Rab11 (prey) (Figure 10). As before, GEFlong is observed to be self-activating on SD/-L/-T/-H plate, but not on Quad medium (Figure 10). On Quad medium only the positive control grew (Figure 10). In addition, a test with Rac1 was also negative (Seung-kyu Lee, personal communication).

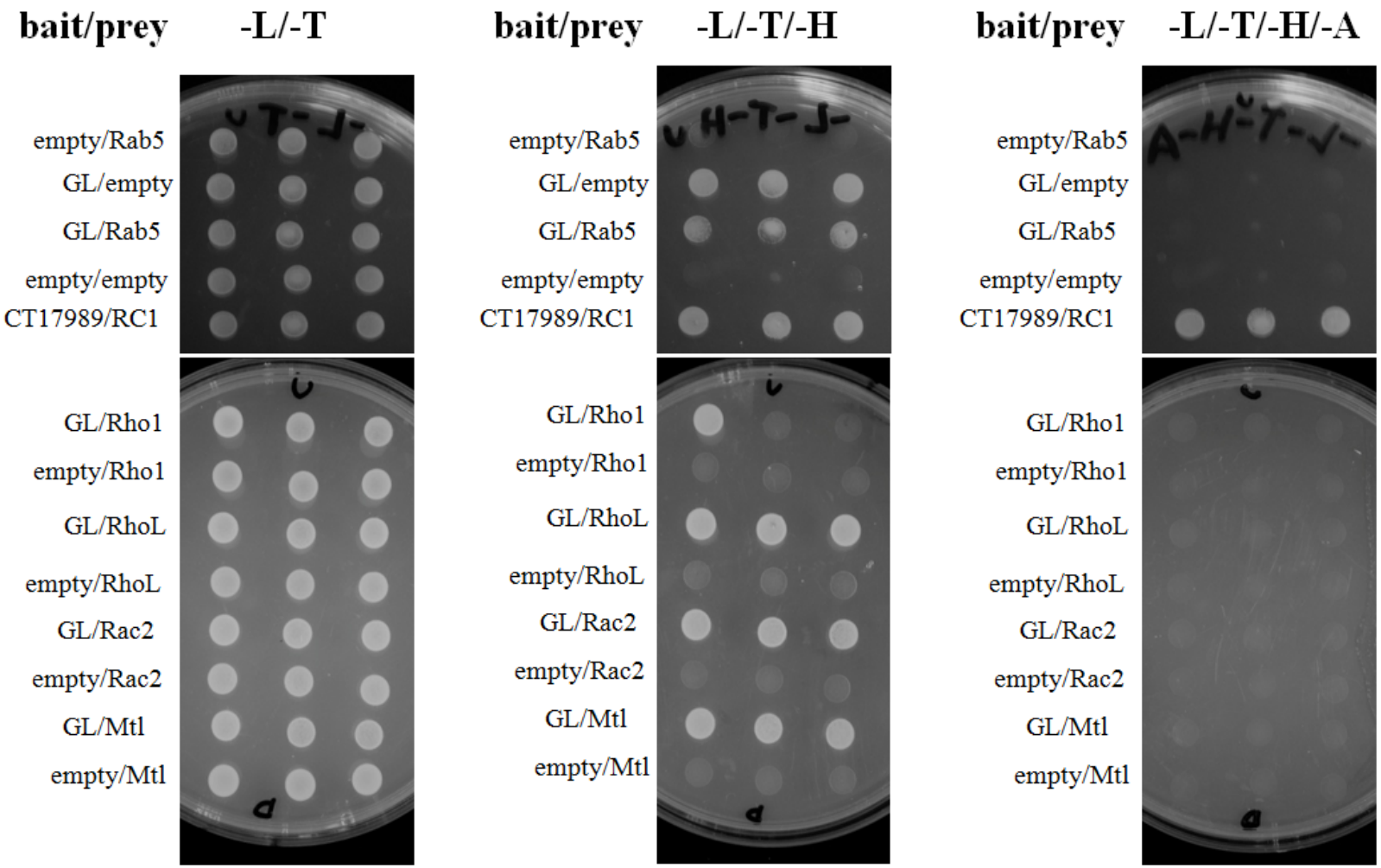


Figure 9: Yeast 2-hybrid interaction analysis of GEFlong with Rab5, Rho1, RhoL, Rac2 and Mtl. Labels indicate bait/prey combinations: empty = empty bait or prey vector, GL = GEFlong. CT17989/RC1 is the positive control. -L/-T indicate medium deficient in leucine and tryptophan (viability control). -L/-T/-H indicate medium deficient in leucine, tryptophan and histidine (interaction-dependent growth). -L/-T/-H/-A indicate medium deficient in leucine, tryptophan, histidine and adenine (interaction-dependent growth). GEFlong is observed to be self-activating on -L/-T/-H plate. No physical interaction is detected between GEFlong and Rab5, Rho1, RhoL, Rac2 and Mtl on -L/-T/-H/-A plate. Only the positive control grew on -L/-T/-H/-A plate.

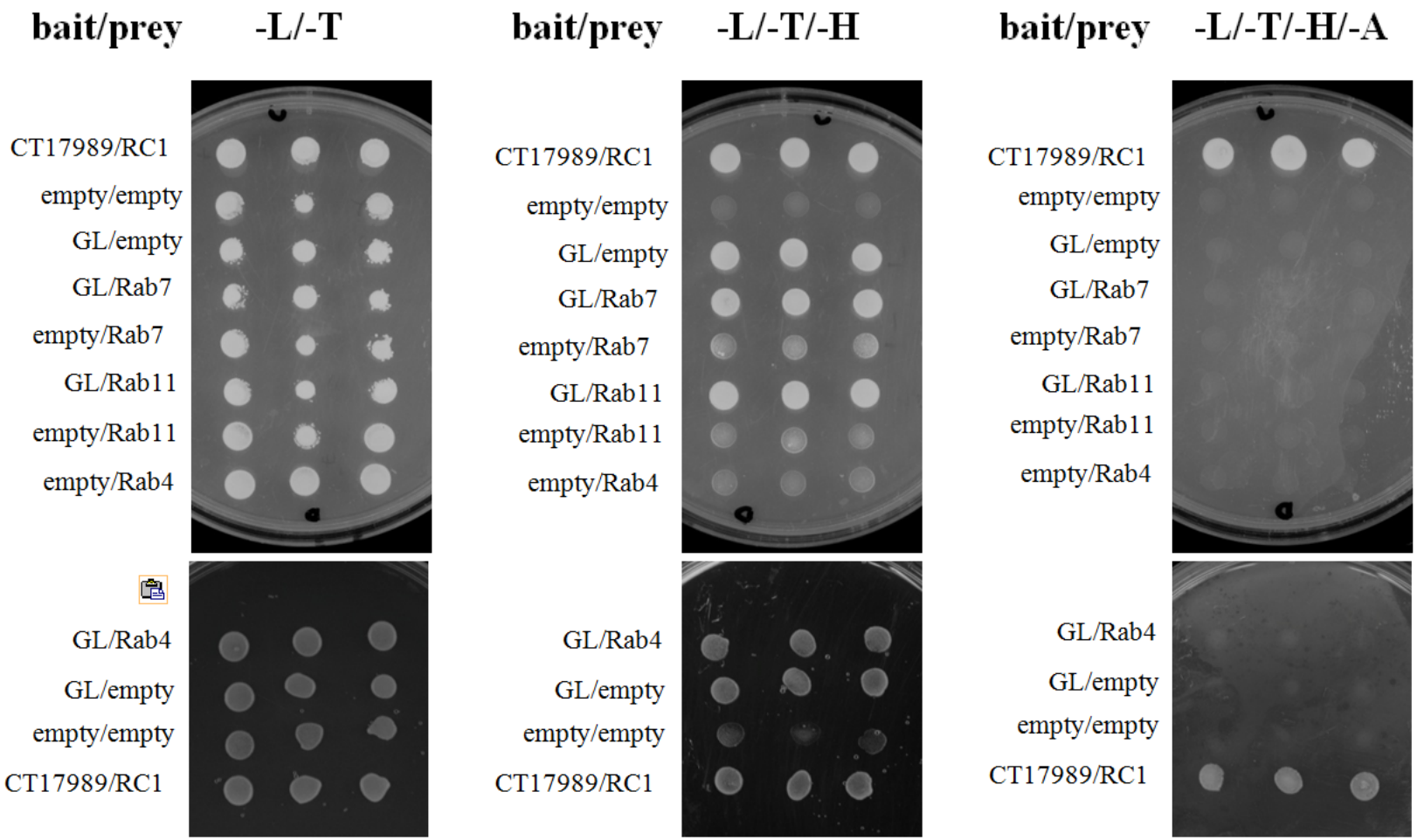


Figure 10: Yeast 2-hybrid interaction analysis of GEFlong with Rab4, Rab7, and Rab11. Labels indicate bait/prey combinations: empty = empty bait or prey vector, GL = GEFlong. CT17989/RC1 is the positive control. -L/-T indicate medium deficient in leucine and tryptophan (viability control). -L/-T/-H indicate medium deficient in leucine, tryptophan and histidine (interaction-dependent growth). -L/-T/-H/-A indicate medium deficient in leucine, tryptophan, histidine and adenine (interaction-dependent growth). GEFlong is observed to be self-activating on -L/-T/-H plate. No physical interaction is detected between GEFlong and Rab4, Rab7, and Rab11 on -L/-T/-H/-A plate. Only the positive control grew on -L/-T/-H/-A plate.

Having tested all the most obvious G-proteins, I decided to instead use the same technology to follow up on some positive interactions found by Elizabeth Klipfell, a former graduate student in Thomas lab (Klipfell, 2006).

βH33 physically interacts with Nemy (Klipfell's clone)

Yeast 2-hybrid assay was carried out using Klipfell's clone that I had rescued from frozen cultures (Figure 11). Assay on yeast clones expressing βH33 and Fax did not detect any phisical interaction between the two proteins. As for the clone expressing Eps15, one out of three turned out positive for physical interaction whereas the other two were negative. It is unclear on what is causing such inconsistency. Positive interaction was detected for Nemy and βH33 as yeast expressing these proteins grew on Quad plates. To evaluate Nemy further, a new Nemy clone was constructed (due to our inability to isolate the Nemy DNA from Klipfell's clone) and tested with Y2H using several βH33 deletion constructs.

No Interaction was detected between βH33 and the new Nemy construct

By using several βH33 deletion constructs, I attempted to map the site of interaction on βH33 in a Y2H assay (Figure 12). βH33-5/bait and βH33+5-3/bait seems to be slightly self-activating in my hands on -L/-T/-H plate. Thus for the purpose of our analysis, we again consider only the results from the Quad plates. The assay gave negative results for every combination of βH33 deletion constructs and Nemy (Pun12/Nemy, βH33-5/Nemy, βH33+5-3/Nemy) on Quad plate. It should be noted that the new Nemy construct contain a single amino acid change (alanine to valine) at position 206 which could not be fixed before thesis submission and that could be responsible for the unexpected loss of physical interaction between βH33 and Nemy.

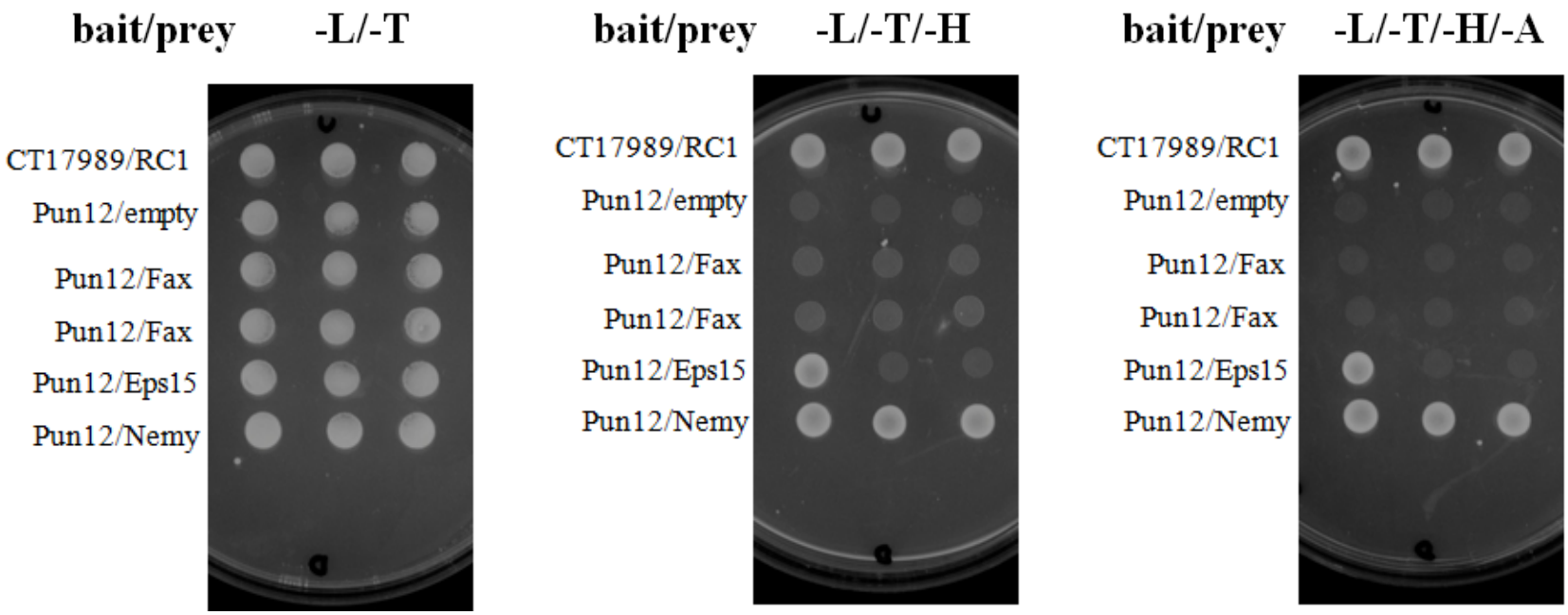


Figure 11: Yeast 2-hybrid interaction analysis of βH33 with Fax, Eps15, and Nemy. Labels indicate bait/prey combinations: empty = empty bait or prey vector, Pun12 = βH33. CT17989/RC1 is the positive control. -L/-T indicate medium deficient in leucine and tryptophan (viability control). -L/-T/-H indicate medium deficient in leucine, tryptophan and histidine (interaction-dependent growth). -L/-T/-H/-A indicate medium deficient in leucine, tryptophan, histidine and adenine (interaction-dependent growth). Pun12/Fax and Pun12/Eps15 (lane 1 and 2) did not grow on -L/-T/-H and -L/-T/-H/-A medium. The positive control, Pun12/Nemy, and Pun12/Eps15 (lane 3) grew on -L/-T/-H/-A plate.

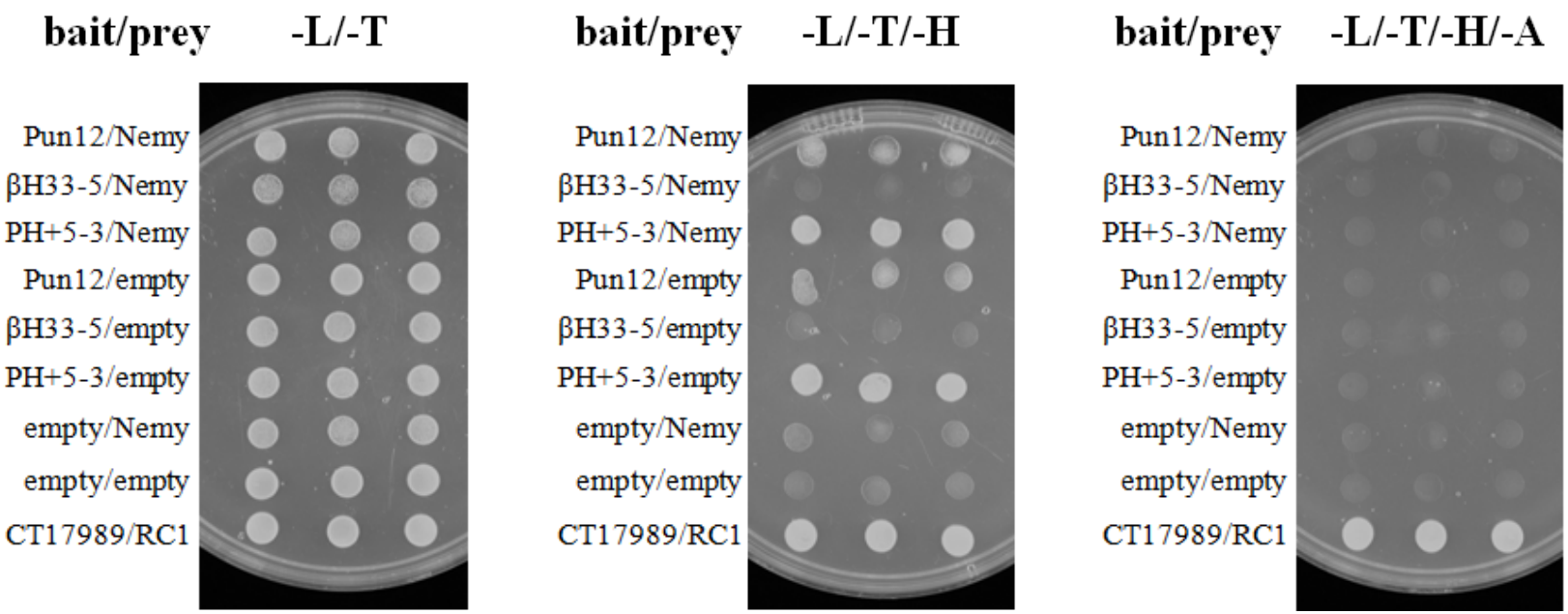


Figure 12: Yeast 2-hybrid interaction analysis of Nemy with Pun12, βH33-5, and PH+5-3. Labels indicate bait/prey combinations: empty = empty bait or prey vector, Pun12 = βH33, βH33-5 = βH33 with deletion at its N-terminus, βH33+5-3 = βH33 with deletion at its N-terminus (see Figure 8), CT17989/RC1 is the positive control. -L/-T indicate medium deficient in leucine and tryptophan (viability control). -L/-T/-H indicate medium deficient in leucine, tryptophan and histidine (interaction-dependent growth). -L/-T/-H/-A indicate medium deficient in leucine, tryptophan, histidine and adenine (interaction-dependent growth). Pun12 and βH33+5-3 observed to be self-activating on -L/-T/-H plate. Only the positive control grew on -L/-T/-H/-A plate.

DISCUSSION

Previous studies have identified a novel genetic interaction between β_H-spectrin and Rac1 in *Drosophila* epithelial cell (Lee and Thomas, 2011). Those experiments demonstrated that Rac1 activity increases when Trio is expressed, but is suppressed when βH33 is co-expressed. Reduction in β_H-spectrin levels also resulted in an increase of Rac1 activity. Additionally, overexpression of Trio was found to suppress a dominant eye phenotype induced by the expression βH33 in *Drosophila*. All these observation indicate a strong relationship between β_H-spectrin, Trio and Rac1 in the same pathway. One hypothesis suggested that β_H-spectrin could physically interact with Rac1 which would prevent Trio from binding to Rac1. I selected Rho1, RhoL, Rac2, and Mtl for yeast 2-hybrid testing with GEFlong as these GTPases are related to Rac1; like Rac1, these proteins belonged to the Rho GTPase family and are implicated as important regulators of cell morphogenesis.

In other studies, an epistatic relationship has been demonstrated between Rab5 and β_H-spectrin (Phillips and Thomas, 2006). Those experiments showed that the expression of dominant negative Rab5 causes dissociation of Rab5 from the early endosome in midgut of wild-type *Drosophila*, but not in *karst* mutant guts. The *karst* mutation alone was also found to cause the loss of Rab5-positive early endosomes while increasing the amount of lysosomes. These results have led to the hypothesis that β_H-spectrin could be associated with Rab5, Rab4, Rab7, or Rab11. This analysis is further supported by staining experiments that demonstrated that β_H-spectrin can localizes in endosomal compartments in addition to the apical membrane (Tjota *et al.*, 2011).

The yeast 2-hybrid tests strongly suggest that GEFlong does not interact directly with Rho1, RhoL, Rac2, Mtl, Rab4, Rab5, Rab7, and Rab11. In a similar study using the yeast 2-

hybrid system, it was also found that Rac1 also not physically interact with either GEFlong or βH33 (Seung-kyu Lee, personal communication). In a parallel experiment utilizing putative GEF domain of β_H-spectrin in pull down experiment, no Rac1 or any of the candidate Rho and Rab GTPases was pulled down (Graham Thomas, personal communication). Together, these observations suggest that β_H-spectrin does not have the ability to bind GTPase proteins, but our hypothesis cannot yet be rejected since there are many other GTPases that have not been tested. However, it is most likely at this time that β_H-spectrin modulates Rac1 signaling without involving a direct interaction with Rac1 or the most likely G-protein.

A previous member of our lab, Elizabeth Klipfell, had identified Eps15, Fax, and Nemy as positive interactors of βH33 in a yeast 2-hybrid library screen (Klipfell, 2006). These three proteins have became biologically relevent since Klipfell's original screen: Eps15 is affected by knockdown of Annexin B9 (a β_H partner; Tjota *et al*., 2011); Fax (*failed axon connection*) affects neuronal pathfinding, much was shown to be affected by spectrin mutation (Hülsmeier *et al*., 2007); and Nemy (*no extended memory*) is a cytochrome of a class with demonstrated roles in the endomembrane system (Iliadi *et al*., 2008). Nemy also interacts with Fax (flybase.org). Considering the possibility of false positive results in Y2H library screens, I decided to re-evaluate these three proteins using a better yeast 2-hybrid approach, namely the yeast spotting assay. Using cultures frozen away by Klipfell, my spotting assay did not detect any physical interaction between Fax and βH33 which contradicts the earlier Y2H result. At this point, we are not exactly sure about the cause of such inconsistency but we suspected that Fax could be a false positive. As for Eps15, only one out of three yeast clones expressing Eps15 and βH33 turned out to be positive for Y2H. Considering that the other two clones gave negative results, we suspected that the only positive Eps15 clone may be a result of cross-contamination from other samples

which might have occured when preparing the clones for the assay. Nonetheless, we could not reach a conclusion regarding Eps15 unless more tests are carried out. Nemy, on the other hand, tested positive in the spotting assay. This suggests that there could be an interaction between Nemy and βH33. To evaluate Nemy further, I decided to map its interaction with β_H using a set of more recently available βH33 deletion constructs.

Several efforts to isolate Nemy/pACT2 DNA directly from the yeast clones archived by Klipfell and revived by me, using the Smash and Grab method failed. A low copy number of Nemy DNA constructs in the clones could cause this problem. Subsequent efforts were therefore directed at amplifying the Nemy gene from a total RNA pool by using PCR to create a new clone. For reasons that are unclear, PCR of the Nemy gene proved very challenging, and in the end only one clone was successfully isolated. The resultant Nemy gene was cloned into pACT2 and verified by DNA sequencing. The sequence indicated a single nucleotide change (C-> T) resulting in an alanine to valine amino acid substitution at position 206. Obviously, such a change may well have a detrimental effect on the protein and will be fixed by site directed mutagenesis in the future. However, due to time constraints, I decided to proceed with the Y2H test using this clone. The Y2H assay failed to detect any physical interaction between the new Nemy clone and βH33. At this point, we are not sure whether the amino acid change causes the protein to fold incorrectly or whether the site where the amino acid change occurred are required for the interaction or whether Klipfell's frozen culture is mislabeled. Consequently, we do not have sufficient data to validate whether Nemy is truly a binding partner for βH33. Future efforts will be directed at correcting the amino acid sequence of the Nemy clone before conducting another Y2H assay.

Nonetheless, these Y2H results suggest that there is a possibility that Nemy could indeed interacts with β_H-spectrin. Like cytochrome b561, Nemy is predicted to function as a transmembrane electron transporter that shuttles electrons from the cytoplasm into the vesicles. The movement of electron by Nemy has been implicated as a mechanism in modulating various key vesicular processes including vesicular proteolytic processing, protein sorting and trafficking. Interestingly, β_H-spectrin has also been implicated in modulating endosomal protein sorting and trafficking. We considered the possibility that both Nemy and β_H-spectrin could be working in tandem in the same regulatory pathway. Understanding the interaction between these two proteins would be important in shedding some light on how cellular vesicles are regulated. Perhaps Nemy could have a role in recruiting β_H-spectrin into the vesicles *via* physical interaction.

In addition to this anchoring hypothesis, current research aimed at studying the molecular mechanism downstreams of Crumbs (an important apicobasal polarity determinant), reported that Crumbs represses a signaling pathway involving Rac1 and an unidentified NADPH oxidase (Chartier and Laprise, 2011). Considering that β_H-spectrin could provide a physical link between Crumbs and Nemy, we hypothesize that perhaps Nemy (also known as carbon monoxide oxygenase) could be part of this pathway. Presumably, the oxidase and Rac1 increases the activation of reactive oxygen species-dependent P13K/Akt activity while Crumbs inhibits P13K/Akt activity. The relationship between Crumbs, Rac1/NADPH oxidase, reactive oxygen species in the P13K/Akt pathway has been implicated to be important in maintaining epithelial organization (Chartier and Laprise, 2011). Since β_H inhibits Rac1 and is recruited by Crumbs (Lee and Thomas, 2011), it may well be part of this pathway.

In this thesis, we used the yeast 2-hybrid (Y2H) system as a molecular tool to test for protein-protein interaction. Despite the usefulness of this system, it does has several limitations that should be noted. This system is based on the co-expression of bait and prey fusion proteins and their ability activate certain reporter genes in yeasts (Fields and Sternglanz, 1994). Hence, the molecular basis of Y2H is restricted to the types of protein that can be used in this system.

First of all, the interaction between bait and prey proteins must occur in the nucleus to activate the reporter genes (Fields and Sternglanz, 1994). The nuclear translocation of these fusion proteins is mediated by the fused nuclear localization signal. Nevertheless, there are proteins that are not normally imported into the nucleus, thus, making the Y2H assay an abnormal compartment (Fields and Sternglanz, 1994). Integral membrane proteins are one of the types of proteins that are not normally imported into the nucleus. Similarly, the Y2H method may be unsuitable to assay for protein-protein interactions that occur outside the cell. These extracellular interactions such as the receptor-ligand binding cannot be truly reproduced in the nucleus, mostly because some of these interactions require the proteins to be glycosylated (Fields and Sternglanz, 1994).

Secondly, Y2H may not detect protein-protein interactions mediated by other post-translational modification such as phosphorylation (Fields and Sternglanz, 1994). Certain proteins need to be phosphorylated first before it can bind to its binding partners. As an example, the SH2 domain only binds to phosphorylated tyrosine residue but not to the unphosphorylated ones (Brown and Cooper, 1996). This poses a problem as yeasts may lack the ability to carry out specific post-translational modification on bait/prey proteins (Fields and Sternglanz, 1994). In addition to that, post-translational modification by yeast may be different than the one in the

species under test (Fields and Sternglanz, 1994). Consequently, this could affect protein-protein interaction, especially if the modification is needed for the interaction.

In addition, Y2H may be an unsuitable assay if the proteins cannot fold or orient properly: It is possible for a non-yeast protein to fold incorrectly when introduced into yeasts (Fields and Sternglanz, 1994). Sometimes, the orientation of the fusion protein can hinder protein-protein interaction. Usually in the construction of hybrid proteins, the DNA binding domain or activation domain is fused to N-terminus of protein of interest. The accessibility of the N-terminal region is then blocked by the transcription factor domains, preventing it from participating in protein-protein interaction (Fields and Sternglanz, 1994).

Last but not least, false positive results are common when Y2H is applied in library screens (Fields and Sternglanz, 1994). False positive are often caused by prey protein that can activate reporter genes without interacting with the bait protein (Fields and Sternglanz, 1994). This is likely to happen if prey itself is a transcription factor or is somehow involved in transcription activation (Fields and Sternglanz, 1994). To remedy this problem, the use of more than one auxotrophic selection markers can help eliminate false positives (Fang and Macool, 2002). Apart from that, positive results in Y2H do not necessarily reflect the actual interaction *in vivo* (Fields and Sternglanz, 1994). For example, two positive interactors in Y2H screens could be physically separated in different compartments within its native cell, indicating the likelihood of another type of false positive.

In light of its limitations, Y2H screen alone is insufficient to prove the interaction between proteins. There are alternative detection methods that can be used to supplement Y2H evaluation of protein-protein interaction. Other methods of detection include pull-down assay, co-immunoprecipitation, cross-linking assay, affinity electrophoresis, and label-transfer assay,

just to name a few (Phizicky and Fields, 1995). Thus, the negative results in this thesis may not be definitive.

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