Beach1 Functionally Antagonizes Rab11 During Development and in Regulating Synaptic Morphology

by

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ABSTRACT

BEACH proteins comprise an evolutionarily conserved family characterized by the presence of a BEACH (Beige and Chediak-Higashi) domain of unknown function. They have been shown to play a role in a number of important cellular processes, ranging from cytokinesis to synaptic transmission, and implicated in human diseases, such as Chediak-Higashi Syndrome and cancer. Analysis of several BEACH proteins suggests that they may be involved in membrane trafficking; however, little insight has been gained into their molecular mechanism of function. We identified *Drosophila* Beach1 in a gain-of-function screen: *beach1* overexpression in the photoreceptors drastically alters their growth cone morphology. In a subsequent genetic modifier screen, I identified *rab11* as a strong enhancer of the *beach1* eye overexpression phenotype. Rab11 is a small GTPase, which has been shown to regulate the delivery of vesicles and cargo to the plasma membrane via both the recycling and the biosynthetic pathways. Although *beach1* loss-of-function mutants exhibit no obvious phenotypes, a sensitized background of a *rab11* mutant revealed a requirement for *beach1* during development and in bristle extension. I also found that Beach1 functionally antagonizes Rab11 at the neuromuscular junction by suppressing the *rab11* synaptic overgrowth phenotype. Subcellular fractionation and double-labeling experiments suggest that these proteins may function in the same subcellular compartment; however, further experiments are needed to determine whether Beach1 and Rab11 interact directly, function in the same protein complex, or closely cooperate in the same molecular pathway. The interaction I found between Beach1 and Rab11 suggests a mechanism by which other BEACH proteins may be involved in vesicle trafficking.

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Chapter 1: Introduction
BEACH and Rab protein families

Motivation for studying Beach1

Drosophila Beach1 is a member of an evolutionarily conserved protein family characterized by the presence of a BEACH (Beige and Chediak-Higashi) domain. BEACH proteins are present in all eukaryotes and have been implicated in many diverse cellular processes ranging from cytokinesis to synaptic transmission. Mutations in several BEACH genes are also known to cause human disease. *Lyst* (lysosomal trafficking regulator), the first BEACH family gene to be discovered, is disrupted in Chediak-Higashi Syndrome (CHS) and in its mouse model *beige*. CHS is an often-fatal disease characterized by severe immunodeficiency, albinism, poor blood coagulation, and neurologic involvement (Introne et.al., 1999). Recently, another BEACH family member, *neurobeachin*, has been implicated as a candidate gene for autism (Castermans et.al, 2003). Furthermore, upregulation of *LRBA* (LPS-responsive and beige-like anchor) is seen in several types of cancer and appears to facilitate cancer growth (Wang, Gamsby et.al, 2004).

Since the identification of the *lyst* gene in 1996, some progress has been made towards the understanding of how BEACH proteins are involved in their respective cellular processes. Analyses of the loss-of-function phenotypes of several BEACH family genes suggest that they may play a role in membrane trafficking; however, little insight has been gained into their molecular mechanism of function.

BEACH proteins have been shown to be involved in a number of important cellular processes and implicated in human disease. Thus, the elucidation of their mechanism of function would be a significant advance in both biology and medicine. However, most BEACH proteins are very large, often close to, or over, 400kDa in size, making them extremely difficult to study.

In my graduate work I utilized the power of *Drosophila* genetics and molecular tools to try to shed some light onto the mystery of BEACH proteins.

This thesis will cover the identification of *beach1* in an overexpression screen in the *Drosophila* photoreceptors, the isolation and the characterization of the loss-of-function alleles in this gene, the identification of *rab11* as a modifier of the *beach1* overexpression phenotype, and the characterization of interactions between *rab11* and *beach1* throughout development and in regulating synaptic morphology. The main contribution of this work is in the discovery of a link between the poorly characterized BEACH proteins and the Rab GTPases--known regulators of membrane trafficking. This introduction will provide some background on these two families of proteins and the processes in which they are involved.

BEACH proteins

Beach1 belongs to a diverse family of large proteins named after a highly conserved BEACH (Beige and Chediak-Higashi) domain of unknown function. Proteins in this family share more than just the BEACH domain; in all of these proteins the BEACH domain is preceded by a novel pleckstrin-homology (PH) domain and followed by between four and six WD40 repeats. WD40 repeats fold into a beta-propeller structure that serves as a protein-protein interaction domain, a property that has been confirmed for one of the BEACH proteins--FAN (Adam-Klages et.al.,1996). The PH domain in these proteins is only similar to the canonical PH domains in structure, not in sequence, and its unique properties will be discussed in detail in the "Crystal Structure" section bellow. As a whole, the conserved PH-BEACH-WD40 module comprises only the C-terminal 25% of the total protein length, while the remaining 75% are mostly unique to each protein (Figure 1).

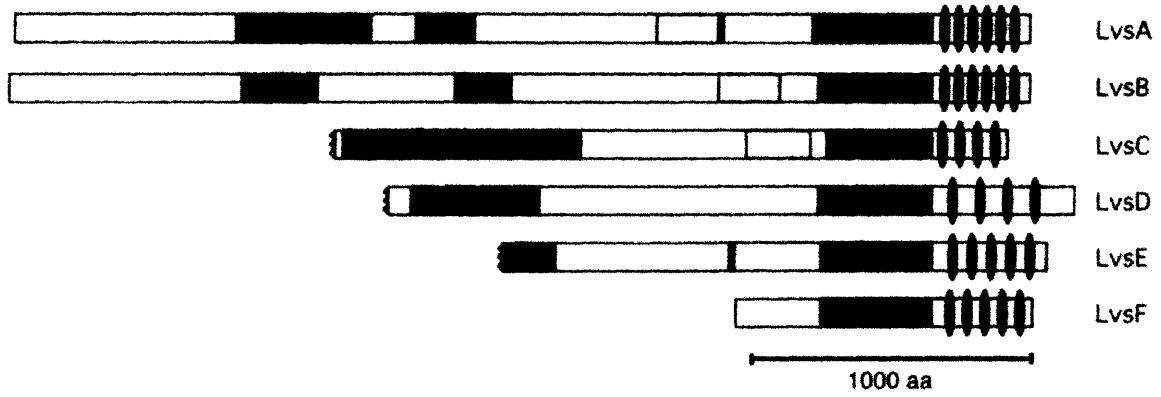

Figure 1 (Taken from De Lozanne, 2003): The domain organization of BEACH proteins. This diagram shows the domain organization of the six Dictyostelium BEACH proteins. The BEACH domain (green) is the most conserved portion of these proteins (50–60% identity). At the C-terminus of each protein there are multiple WD-40 repeats (ovals). Adjacent to the BEACH domain is a PH-like domain (purple). Other regions of homology (colored regions) are also shared among different Lvs proteins, but the similarity is low. The sequences of LvsC, LvsD and LvsE are truncated at the N-terminus. Accession numbers for these sequences are: LvsA, AAD52096; LvsB, AY159038; LvsC, AY159039; LvsD, AY159040; LvsE, AY159036; LvsF, AY159037.

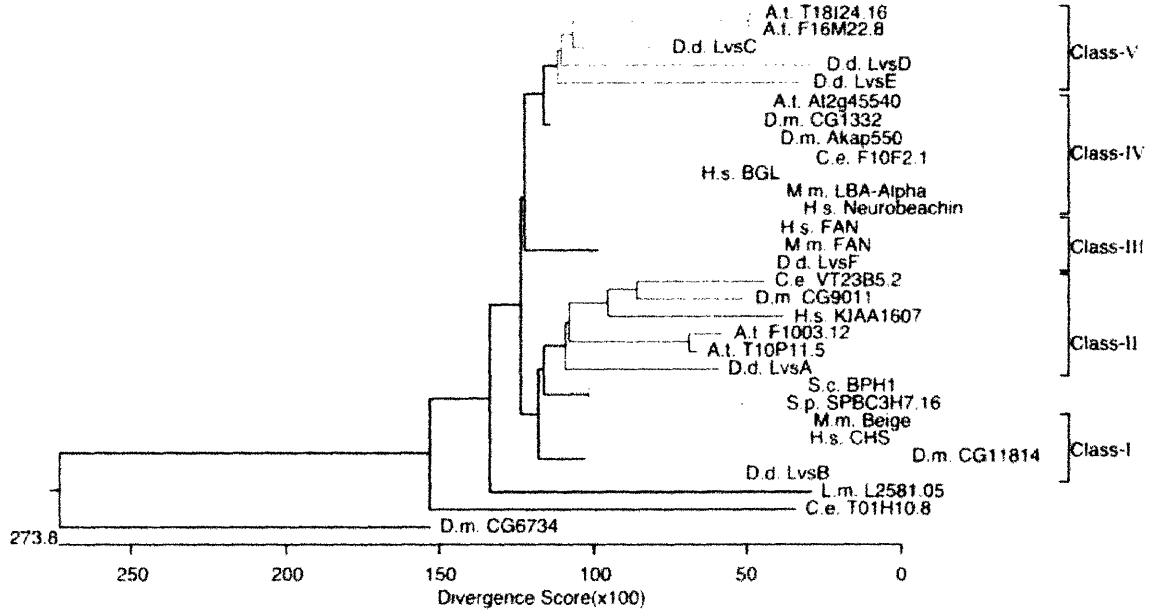

Figure 2 (Taken from De Lozanne, 2003): Phylogenetic tree of family of BEACH proteins. The BEACH and WD domains of the indicated sequences were aligned by the ClustalW algorithm and the alignment was used to construct this phylogenetic tree. The different classes of BEACH proteins are indicated by the brackets on the right.

*Correction: According to my sequence alignments this tree is not completely correct with respect to the human ortholog of Beach1. The protein most similar to Drosophila Beach1 in the human genome is Alfy (KIAA 0993). KIAA1607 is another homolog, which is less similar to Beach1 and does not have a FYVE domain.

Arturo De Lozanne's group used the BEACH-WD40 regions of BEACH proteins from different organisms to construct a phylogenetic tree of this protein family (Figure 2). Their analysis suggests that BEACH proteins are an ancient family that diversified early, before the separation of animals and plants. The genomes of *S. cerevisiae* and *S. pombe* have one BEACH protein each, *C.elegans* has three, *A. thaliana* and *D. melanogaster* have five, and *D. discodeum* and *H.sapiens* have six BEACH proteins each. Phylogenetic analysis grouped BEACH proteins into five classes; interestingly, proteins within each class possess additional regions of homology within their N-terminal regions, which are not shared with proteins in other classes (Figure 3). This finding raised the possibility that proteins within each class might represent a distinct functional group (Wang, Wu et.al., 2002). To test this hypothesis De Lozanne's group took advantage of the fact that *Dictyostelium* (an amoeba) has six BEACH proteins, Lvs (Large volume sphere) A-F, one belonging to each predicted class. By disrupting each *lvs* gene they found that, as expected, mutants in different genes had different loss-of-function phenotypes: *lvsA* nulls were unique in having defects in cytokinesis and osmoregulation, while only *lvsB* mutants showed defects in lysosomal traffic. The rest of the *lvs* single mutants did not have any obvious phenotypic defects and, thus, were not redundant (at least as single mutants) with either l*vsA* or *lvsB* (Wang, Wu et.al., 2002).

Given that BEACH proteins in *Dictyostelium* that belong to different classes have distinct cellular functions, the same is likely to hold true for BEACH proteins of other organisms.

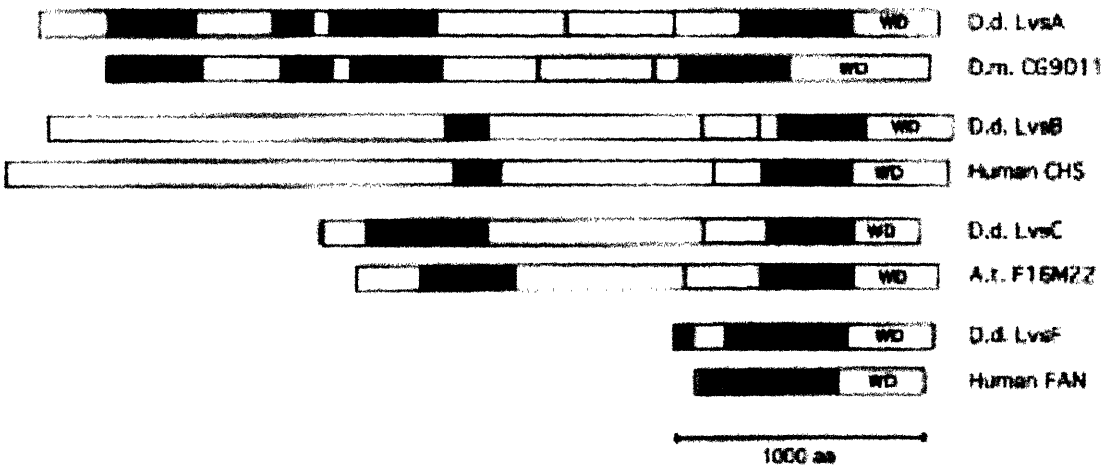

Figure 3 (taken from Wang, Wu et.al., 2002): Dictyostelium Lvs proteins share multiple domains with BEACH proteins of the same class. In addition to the BEACH domain (green) and the WD domain (WD), BEACH proteins within the same class have other domains in common. This diagram indicates in different colors the domains shared by BEACH proteins of the same class. These domains were identified as having a significant BlastP score in pairwise alignments.

Classification of BEACH proteins

According to the phylogenetic analysis, Beach1 falls into class II of BEACH proteins. However, in order to highlight the similarities and underscore the differences between different members of this protein family, it is useful to go over what is known about BEACH proteins in other predicted classes as well.

Class I

This class includes some of the more extensively studied BEACH proteins with putative roles in lysosomal membrane traffic, such as Lyst, mutated in Chediak-Higashi Syndrome (CHS) and its *Dictyostelium* ortholog LvsB. The hallmark of cells that lack the function of this gene, regardless of the organism, is the presence of large intracellular granules of lysosomal origin (Figure 4). For example, in patients with CHS, such large granules in

melanocytes, called melanosomes, are not properly transferred to keratinocytes, resulting in cutaneous albinism--the absence of pigment in the skin. In Cytotoxic T lymphocytes (CTLs) and natural killer (NK) cells these large lysosomes are similarly unable to undergo exocytosis, preventing the secretion of proteins used to kill infected cells (Ward, 2000). Several hypotheses about the origin of these giant lysosomes have been postulated: it has been suggested that Lyst and its homologs might regulate lysosome fusion, fission, or motility (Stinchcombe, Page et.al. 2000, Perou et.al., 1997, and Faigle et.al., 1998, respectively).

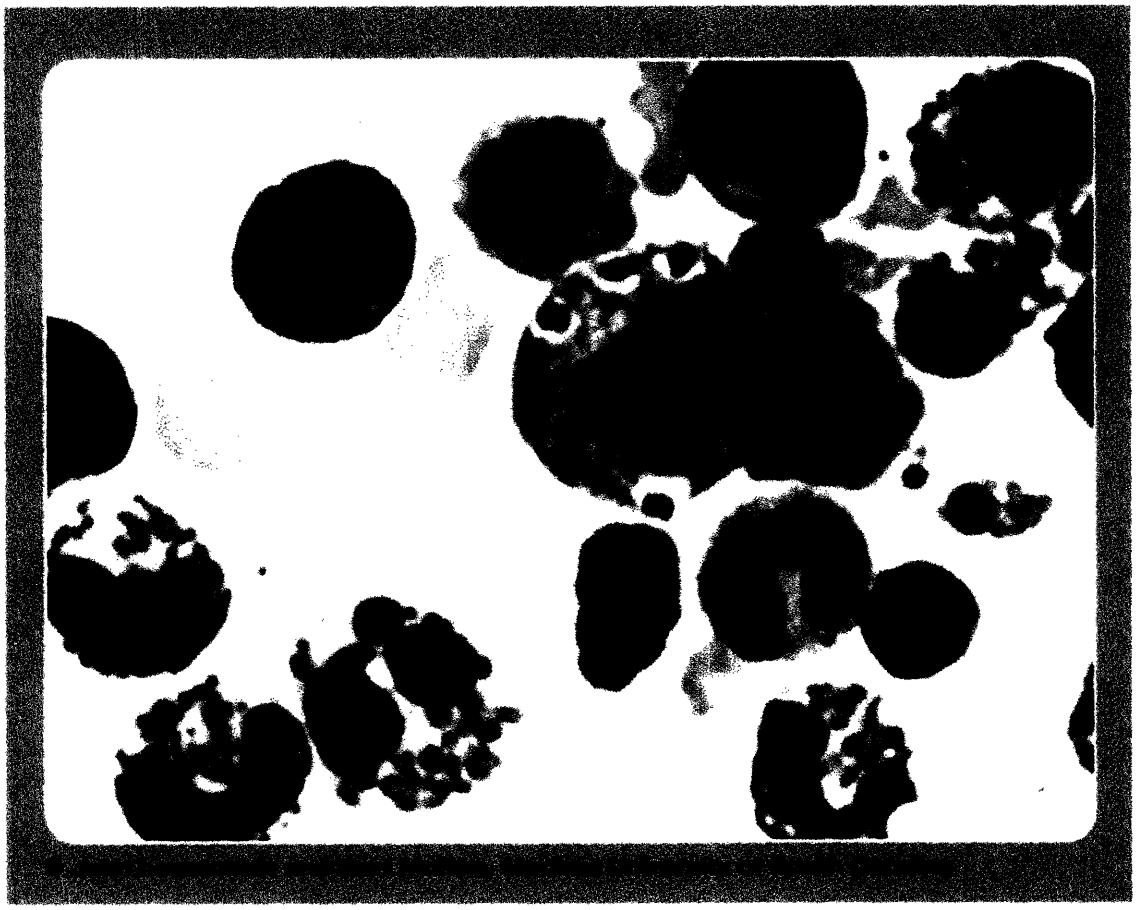

Figure 4 (Taken from Blood, 105(11), 2005): Bone marrow aspirate from a 17-year-old female with Chediak-Higashi syndrome is shown. Giant inclusions are present in the cytoplasm of the myeloid precursor cell (center of the image). Note also in both the granulocytes and eosinophils the multiple atypical large cytoplasmic granules that are characteristic of this disorder.

A simple pulse-chase experiment, in which populations of lysosomes were labeled with two different fluorescent dyes, helped to resolve this controversy: the oversized lysosomes in *Dictyostelium lvsB* mutants are likely to result from increased organelle fusion, not decreased fission (Figure 5) (Harris, Wang et.al., 2002). Although this observation clarified the way in which giant lysosomes formed, it failed to explain how the loss of LvsB function resulted in increased lysosomal fusion. There are many possible mechanisms that could result in such a phenotype. For instance, LvsB might regulate the rate of homotypic fusion between lysosomes, by acting as a negative regulator of SNAREs--the core executors of membrane fusion. Alternatively, LvsB could function as a regulator of lysosomal transport; its loss could allow lysosomes to remain in closer proximity with each other, thus increasing the probability of homotypic fusion.

Interestingly, a defect in lysosomal trafficking lies at the core of another human disorder, Griscelli Syndrome, which causes symptoms that are very similar to CHS. Griscelli Syndrome (GS), like CHS, affects immune cells and melanocytes; however, the subcellular defect in GS is different from that in CHS. Lysosomes in GS are normal in size, but are unable to dock and fuse with the plasma membrane. Griscelli Syndrome can be caused by mutations in Rab27a and in a number of other genes that have one thing in common—they all interact with this Rab GTPase. Rab27a is required for the release of lysosomal organelles from the microtubule cytoskeleton and their docking at the plasma membrane (Stinchcombe, Barral et.al., 2001), which explains the defects in GS. Since the subcellular phenotypes caused by mutations in *lyst* and *rab27a* are distinct, it is unlikely that Lyst interacts with this particular Rab. However, it has been suggested that Lyst, LvsB, and their homologs could interact with another member of the Rab GTPase family, one that, for example, regulates homotypic lysosome fusion (Harris, Wang et.al., 2002).

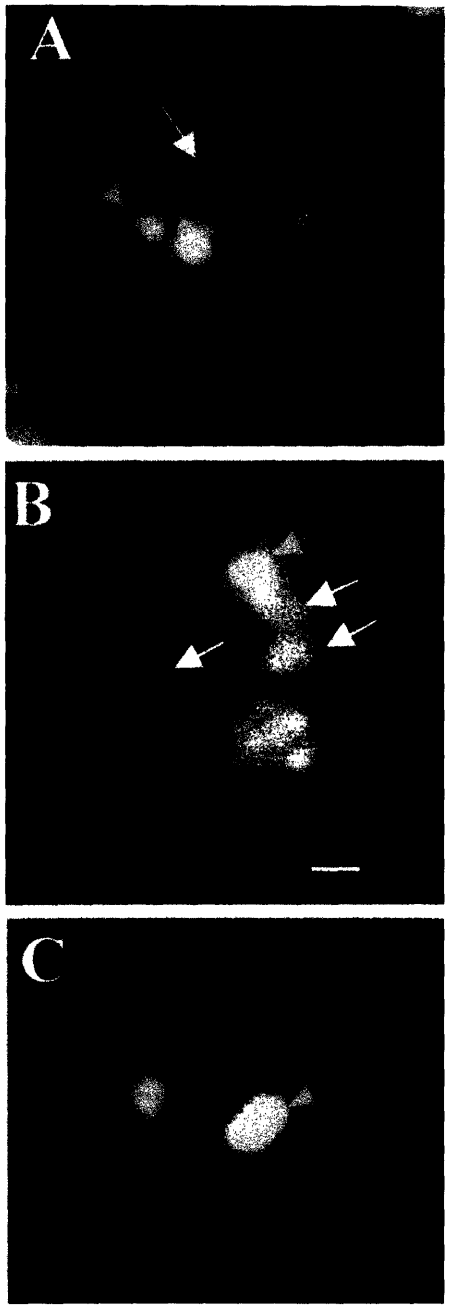

Figure 5 (Taken from Harris, Wang et.al., 2002):Large endolysosomes in the lvsB-null cells are a result of an increase in fusion. Control NC4A2, lvsA-null, and lvsB-null cells were pulsed with RITC-dextran, washed, pulsed with FITC-dextran, washed, chased for 5 min, fixed, and examined under a fluorescence microscope. Most of the red and green vesicles were distinct and separate from each other in the control (A), whereas, a higher percentage of vesicles in the lvsB-null (B) fused with each other. Examination of cells at the end of the double-pulse period revealed that little colocalization was observed in the mutant, suggesting that separately internalized vesicles fuse over time (C). Bar, 2 μm.

Class II

Class II BEACH proteins include the *Dictyostelium* LvsA, the *Drosophila* Beach1, and the human Alfy. Notably, each organism has at least one protein from this class, suggesting that Class II proteins might function in a basic process or processes common to all cells (De Lozanne, 2003). *LvsA* mutants in *Dictyostelium* have defects in cytokinesis: when grown in culture they are unable to divide and, as a result, form binucleate cells. LvsA seems to be required for the late step of cytokinesis: null mutants form the cleavage furrow normally, but are unable to complete its ingression and fail to separate (Kwak et.al., 1999). It is noteworthy that, similarly to LvsA, Rab11 has been shown to play a role in cytokinesis in *C.elegans* and in mammalian cells (Skop et.al., 2001, Wilson et.al., 2005, respectively). Furthermore, *Drosophila rab11* mutants are defective in the process of embryonic cellularization, which is mechanistically similar to cytokinesis (Pelissier et. al, 2003 and Riggs et.al. 2003). Like mutants in *lvsA*, *rab11* mutants in different organisms have a defect in the late stages of cytokinesis. It is possible that LvsA causes a defect in cytokinesis via its interaction with Rab11.

In addition to the cytokinesis defect, *lvsA* mutants are also defective in contractile vacuole (CV) function. The contractile vacuole is a set of membrane sacs and tubules that collect water and expel it by fusing with the plasma membrane, thus allowing amoebae to survive in a hypoosmotic environment. LvsA associates with the CV only during the expulsion phase, and is otherwise present in the cytosol in a punctate pattern. In *lvsA* mutants the CV network is disrupted and, although some vacuoles are able to swell, they are not able to discharge normally, making these mutants osmosensitive (Gerald et.al., 2002). Notably, Rab11 also localizes to the contractile vacuole in *Dictyostelium*. Moreover, the expression of a dominant-negative Rab11 GTPase causes a somewhat opposite defect in the CV morphology than the *lvsA* loss-of-function. Rab11 dominant-negative expression leads to a thicker-

appearing CV network, while the network of CV tubules in *lvsA* mutants is diminished (Harris, Yoshida et.al., 2001). Both mutants have a defect in osmoregulation; however, their subcellular phenotypes differ. Cells lacking LvsA function have small contractile vacuoles that fuse entirely with the plasma membrane and, unlike CVs of wildtype cells, those in *lvsA* mutants are not able to re-form after fusion (Gerald et.al., 2002; Wu et.al., 2004). In contrast, in *rab11* mutant cells contractile vacuoles appear to be unable to fuse with the plasma membrane and thus accumulate within the cell (Harris, Yoshida et.al., 2001). The involvement of Rab11 and LvsA in the regulation of contractile vacuole function in *Dictyostelium*, along with the data that implicates both Rab11 and LvsA in the highly conserved process of cytokinesis, suggests that their might be a functional link between class II BEACH proteins and the Rab11 GTPase.

Class III

Class III proteins, which only include members from *Dictyostelium* and mammals, are different from the rest in that they measure just one quarter of the length of the other BEACH proteins. Mammalian class III protein FAN has been shown to bind directly to the cytosolic tail of the tumor necrosis factor receptor,TNF-R55, via its WD40 repeats (Adam-Klages et.al., 1996). FAN is required for the activation of signaling downstream of the TNF receptor; however, the mechanism by which it does so is not understood. Furthermore, it is not clear whether, due to their much smaller size, FAN and its homologs might differ in their mechanism of function from the rest of the BEACH proteins.

Class IV

A unique feature shared by class IV BEACH proteins, which include the *Drosophila* AKAP550 (A-kinase anchor protein) and the mammalian Neurobeachin and LRBA, is a predicted binding site for the type II regulatory subunit of protein kinase A (PKA) in their N-terminal region. PKA is regulated

by cAMP and, in turn, controls many aspects of cellular function, including various membrane trafficking events (Muniz et.al., 1997; Ohashi and Huttner, 1994). AKAPs serve to target PKA function to various subcellular locations, thus, it is possible that some class IV proteins mediate their effects though PKA. The binding of DAKAP5550 and Neurobeachin to PKA RII has been confirmed in vitro; however, the biological significance of this interaction has not been addressed (Wang, Herberg et.al., 2000). Phenotypic analysis of mutants in several genes from this class revealed their involvement in different cellular processes. Neurobeachin appears to be involved in synaptic transmission, while DAKAP550 and LRBA are involved in signaling through the EGFR (Epidermal Growth Factor Receptor) pathway (Su et.al., 2004; Wech et.al., 2005; Wang, Gamsby et.al., 2004, respectively). Since members of this class of BEACH proteins associate with cellular membranes (Wang, Howson et.al., 2001 and Wang, Herberg et.al., 2000), it will be interesting to learn whether they mediate their respective effects by regulating some aspect of membrane trafficking.

Class V

Class V proteins, which only include members from *Dictyostelium* and *Arabidopsis*, have not yet been implicated in any cellular process (De Lozanne, 2003).

Structural Analysis

The BEACH domain

The BEACH domain--a 300 amino acid domain of unknown function, is highly conserved among BEACH family proteins and required for their function (Adam-Klages et.al., 1996; Karim et.al., 1997). Crystal structures of Neurobeachin and LRBA (Jogl et.al., 2002 and Gebauer et.al., 2004, respectively) revealed that the BEACH domain has an unusual fold, never before seen in any protein (Figure 6). The seven segments in its hydrophobic

core cannot be classified as either beta-strands or random coils. Furthermore, its secondary structure is held together by the hydrogen bonds between the main chain amides and carbonyls and the highly conserved side chains of the BEACH domain, rather than the hydrogen bonds between the main chain atoms (Jogl et.al., 2002). Since the BEACH domain fold is so unusual, the crystal structure of this domain did not provide much insight into its function. No obvious catalytic sites were detected in the structure, although the BEACH domain, being 35kDa in size, is certainly large enough to have an enzymatic function. Assigning a protein-protein interaction role to this domain is also problematic, since the outer surfaces of BEACH domains from different proteins are quite divergent, due to the fact that most of the conserved residues point towards the interior of the molecule, contributing to its fold (Jogl et.al., 2002). This could mean that BEACH domains of different family members have very different binding partners, or that the main function of the BEACH domain is to spatially organize the surrounding domains (De Lozanne, 2003).

The PH domain

An unexpected discovery from the crystal structure of Neurobeachin was a region with a similar backbone fold to a canonical plekstrin homology (PH) domain, found to lie directly upstream of the BEACH domain (Jogl et.al., 2002). Despite having the same structure, PH domains of BEACH proteins do not share any sequence homology with other PH domains. Moreover, unlike the canonical PH domains, those of BEACH proteins are not able to bind phospholipids (Gebauer et.al., 2004). This finding is consistent with the information obtained from the crystal structure, showing that the sites normally involved in phospholipid binding are occupied by portions of the BEACH domain (Jogl et.al., 2002). PH domains are also known to function in protein-protein interactions, and, in the case of BEACH proteins, such an interaction appears to be with the adjacent BEACH domain (Figure 7) (Jogl et.al., 2002). In fact, biochemical binding assays show that the binding

constant for the PH-BEACH domain interaction is indicative of a specific, fully reversible protein-protein interaction (Gebauer et.al., 2004).

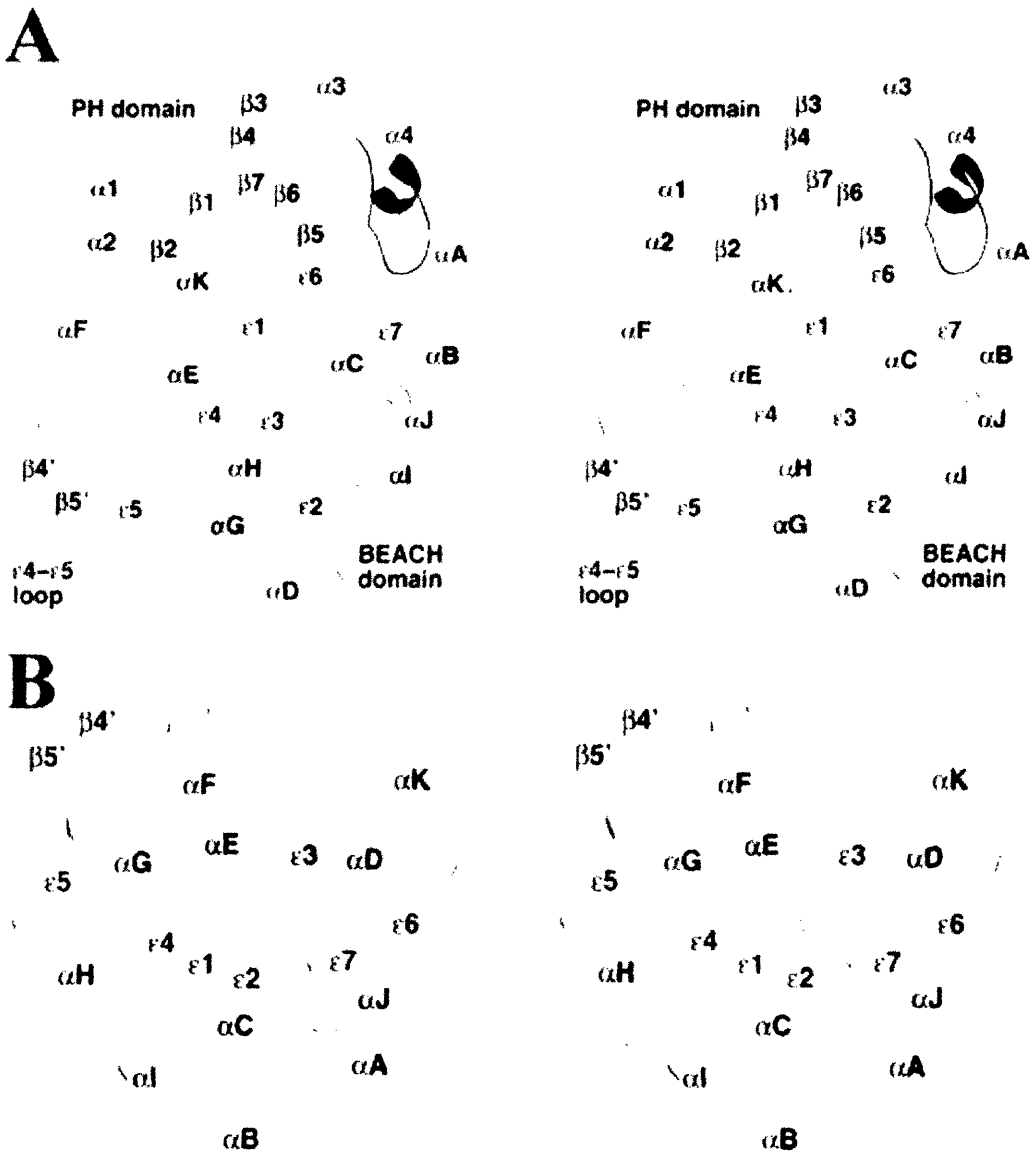

Figure 6 (Taken from Gebauer, 2004): Structure of the PH-BEACH domains of LRBA/BGL. (A) Schematic drawing of the structure of the PH-BEACH domains of human LRBA/BGL. The PH domain is shown in green, and the linker is shown in orange. For the BEACH domain, the extended segments are shown in cyan; the α-helices, in yellow; and the loops, in purple. (B) Schematic drawing of the structure of the BEACH domain of human LRBA/BGL. The view is related to that of A by roughly a 90 degree rotation around the horizontal axis, produced with ribbons.

Both the PH and the BEACH domains seem to be required for BEACH protein function, as has been demonstrated for the mammalian class III protein FAN. Only constructs containing both of these domains were capable of rescuing TNF signaling in *fan* mutant mouse fibroblasts. Moreover, mutations affecting the PH-BEACH interface were shown to reduce TNFR signaling, suggesting that a close interaction between these domains is required for proper function of FAN and, likely, other BEACH proteins (Jogl et.al., 2002). Interestingly, an allele of *beach1* that encodes a mutation in the predicted PH-BEACH interface has a strong effect on the function of this protein.

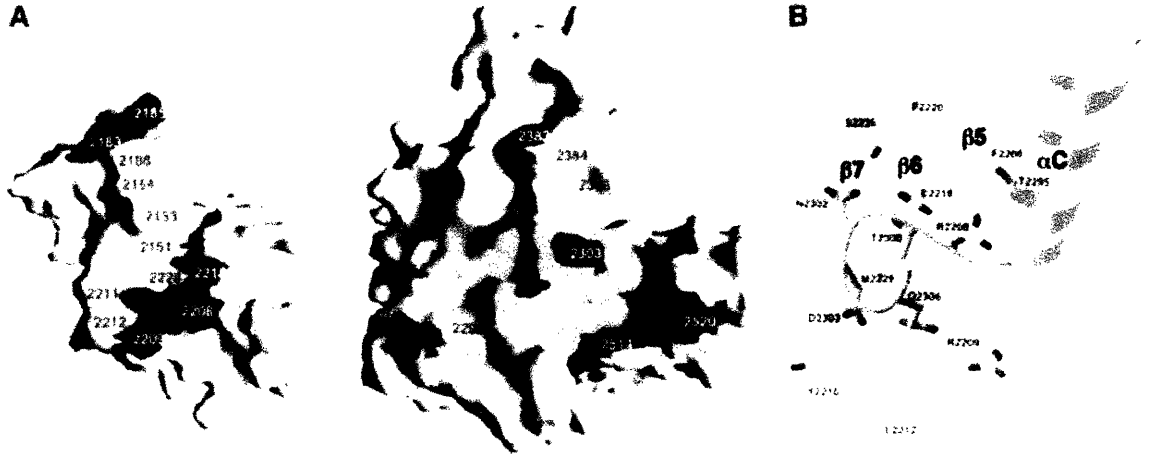

Figure 7 (Taken from Jogl, EMBO J, 2002): The interface between the PH and BEACH domains. (**A**) Molecular surface of the PH and BEACH domains. Residues in the PH–BEACH interface are shown in yellow for hydrophobic residues, green for polar residues, red for acidic residues, and blue for basic residues. (**B**) Schematic drawing of part of the interface between the PH and BEACH domains. The exposed residues of the back sheet (β5, β 6 and β 7) of the PH domain, shown in green, interact with the αC–ε1 linker of the BEACH domain (in purple). (A) was produced with Grasp (Nicholls *et al.*, 1991) and (B) was produced with Ribbons (Carson, 1987).

Structure-function analysis

Both the PH and the BEACH domains are vital for the function of BEACH proteins; however, structure-function analysis of the class II protein LvsA

revealed that almost the entire length of these proteins, including the unconserved N-terminus, may be required for normal function (Wu et.al., 2004). It was demonstrated that the LvsA protein missing just 689 N-terminal amino acids is only partially functional in osmoregulation, and the deletion of 1828 N-terminal amino acids results in a protein that is not functional in either cytokinesis or osmoregulation. Structure-function analysis of LvsA also suggested that it is the C-terminus of BEACH proteins that is responsible for membrane association: a truncated version of LvsA, missing all but the BEACH and WD40 domains, was still able to sediment with membranes (Wu et.al., 2004).

BEACH proteins are putative regulators of membrane trafficking

Localization of BEACH proteins to subcellular membranes, as well as class I and II mutant phenotypes in organelle morphology and function, suggest a role for this family of proteins in membrane trafficking. Unfortunately, phenotypic analysis of a number of BEACH mutants failed to uncover their molecular mechanism of action, nor did it reveal a common thread in how they regulate their diverse cellular processes. It seemed that biochemical and genetic studies into the nature of BEACH protein interactors were warranted to make further progress in this field. Unfortunately, biochemistry with BEACH proteins is difficult due to their enormous size and our continuing ignorance about the function of most of their protein domains. Therefore, in my research I took a genetic approach to studying Beach1, a *Drosophila* class II BEACH protein. I found that Beach1 functionally antagonizes a known regulator of membrane trafficking-- the Rab11 GTPase. The next half of the introduction will provide a brief overview of how Rab GTPases regulate vesicle trafficking, concluding with a summary of what is known about Rab11.

Membrane Trafficking--a Rab-centric view

Membrane organization and trafficking are extremely important in almost every aspect of cellular function; in particular, in eukaryotic cells, compartmentalization by membranes forms the basis of functional specialization. Research in the last several years has uncovered added levels of complexity in how subcellular membranes are organized: what we previously believed to be homogeneous organelles were found to be composed of many subdomains, each with different compositions of proteins and lipids, each representing a different functional specialization. For instance, the early endosome has at least four subdomians with different functionalities: one for the fast recycling of molecules directly to the surface, another for the slow recycling via the recycling endosome, another for downregulation by targeting to the lysosome, and yet another domain responsible for homotypic endosomal fusion (Pfeffer, 2003). Similarly, the late endosome has at least two different subdomains: one for the targeting of cargo to the lysosomes, and the other for targeting to the *trans*-Golgi Network (TGN) (Miaczynska and Zerial, 2002) (Figure 8).

Most of these membrane compartments and subdomains communicate with each other via different membrane trafficking pathways. For instance, in the biosynthetic pathway, vesicles carry newly made proteins from the trans side of the Golgi to their target destinations, including the plasma membrane. In the recycling pathway, molecules from the cell surface and the outside environment, such as transmembrane receptors and their ligands, are internalized and sorted for either recycling back to the surface or for lysosomal degradation. Thus, within a cell, cargo-carrying vesicles are continuously trafficked through multiple compartments, reaching their correct destinations with astounding accuracy. At the same time, the identities of organelles and the subdomains within them are carefully maintained in the face of this constant molecular flux. From recent research efforts, Rab GTPases have

begun to emerge as both the key regulators of membrane trafficking and the important determinants of organelle identity (Figure 9) (Zerial and McBride, 2001).

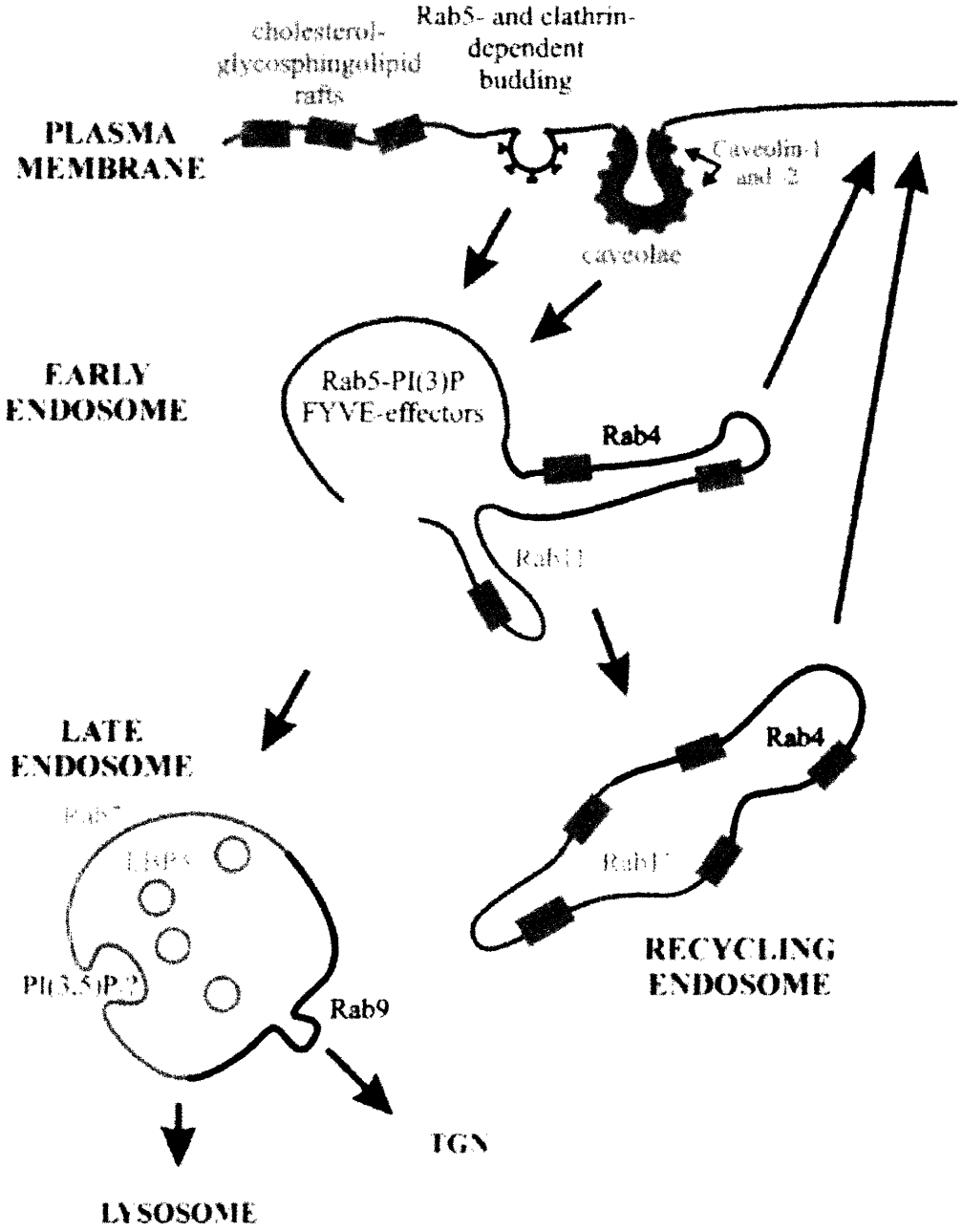

Figure 8 (Taken from Miaczynska and Zerial, 2002): Domain organization of the endocytic pathway. Abbreviations: ECV, endosomal carrier vesicles; LBPA, lysobisphosphatidic acid; TGN, trans-Golgi network; PI(3)P, phosphatidylinositol 3-phosphate; PI(3,5)P2, phosphatidylinositol 3,5-diphosphate.

Rab GTPases

Rabs are the largest family of small monomeric GTPases with 63 members in humans, 29 in each flies and worms, and 11 in yeast. Like all GTPases, Rabs function as molecular switches, they are active when GTP-bound and inactive when GDP-bound. In addition, the activity of Rabs is critically dependent on their membrane association: a Rab protein has to be both GTP-bound and associated with its correct membrane to be in a truly activated state. These two switches: the GTP/GDP switch and the membrane in/out switch, allow Rabs to act as both spatial and temporal regulators of membrane trafficking events (Seabra and Wasmeier, 2004).

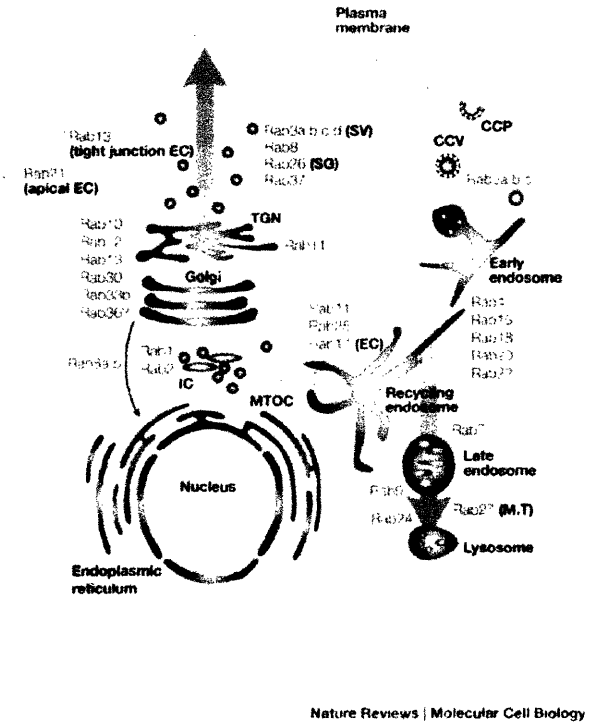

Figure 9 (Taken from Zerial and McBride, 2001): Map of intracellular localization of Rab proteins. Summarizes the intracellular localization of Rab proteins in mammalian cells. Some proteins are cell- (for example, Rab3a in neurons) or tissue-specific (for example, Rab17 in epithelia) or show cell-type-specific localization (for example, Rab13 in tight junctions). (CCV, clathrin-coated vesicle; CCP, clathrin-coated pit; EC, epithelial cells; IC, ER–Golgi intermediate compartment; M, melanosomes; MTOC, microtubule-organizing centre; SG, secretory granules; SV, synaptic vesicles; T, T-cell granules; TGN, trans-Golgi network.)

Rabs carry out their diverse functions in membrane trafficking via their many effectors--proteins that interact preferentially with the GTP-bound Rabs. Each Rab can have multiple effectors; for example, Rab5, a Rab that regulates many functions of early endosomes, has well over twenty known effectors. Since Rab GTPases are a highly conserved family, it was originally believed that their effectors would also fit nicely into a few protein families; however, this turned out not to be the case. Rab effectors identified so far interact specifically with one or a few closely related Rabs. Some effectors, however, do have features in common, such as the FYVE domain that is present in several Rab5 effectors. FYVE domains have been shown to bind specifically to phosphotidylinositol-3-phosphate (PtdIns(3)P), a phospholipid that is enriched in the membranes of early endosomes (Misra et.al., 2001), therefore, the presence of a FYVE domain might help a Rab5 effector to localize to early endosomes.

A possible explanation for the specificity of interaction between Rabs and their effectors was provided by the structural analysis of Rab3a bound to its effector, Rabphilin. The crystal structure revealed five regions of interaction between Rab3a-GTP and its effector. Two of these mapped onto the Switch I and II regions—the domains in a GTPase that change confirmation upon GDP/GTP exchange. However, the remaining three mapped onto the complementarity-determining regions (CDRs), which are divergent between different Rabs, but conserved within Rab protein subfamilies (Ostermeier and Brunger, 1999). Thus, it is thought that the CDRs impart specificity to the interaction between a Rab and its effector, while the Switch regions confer GTP dependence. In addition, it's becoming clear that, despite sequence similarity, subtle variations in shape can make different Rabs appear quite unique to their effectors. Nonconserved residues can influence the appearance of conserved regions by rendering certain amino acids sterically inaccessible or by changing the angle at which they are presented to the effector (Pfeffer, 2005).

Rabs regulate all steps of membrane trafficking

The process of vesicle trafficking can be broken down into a series of distinct steps: a vesicle must separate from its source, travel to its destination, find the correct target, and, finally, it must fuse with the target membrane. Much has been learned about the core executors of each of these steps in membrane trafficking (Figure 10). It is relatively well understood how coat proteins, such as Clathrin, mediate vesicle budding and cargo selection and how actin- and microtubule-based motors deliver vesicles to their targets (Bonifacino and Lippincott-Schwartz, 2003; Schliwa and Woehlke, 2003). We have also begun to understand how tethering complexes ensure proper vesicle docking, and have made substantial advances in the area of SNARE-mediated membrane fusion (Scales et.al., 2000). However, we still do not have a good grasp on how the specificity of membrane trafficking is achieved.

Recently, Rabs and their effectors have been identified as regulators of each step in the vesicle trafficking cycle. During vesicle formation Rabs can help collect cargo for the inclusion in the transport vesicle. For example, Rab9 has been shown to bind to both an adaptor protein and to the Mannose-6-Phosphate Receptor during the formation of vesicles destined to travel from the late endosome to the Golgi (Pfeffer, 2003). Rabs can also link vesicles or organelles to motor proteins: Rab27a connects mature melanosomes with Myosin Va for transport to the plasma membrane (Stinchcombe, Barral et.al., 2001). Rabs have also been shown to interact directly with tethering factors, such as the exocyst complex, which targets vesicles to sites of membrane addition. For instance, Sec4p, a yeast Rab, binds directly to one of the exocyst complex components, Sec15p and thus recruits Sec4p positive vesicles to the site of exocytosis at the plasma membrane. Finally, Rab effectors have been shown to interact directly with SNAREs, the core mediators of vesicle fusion. For instance, the binding of EEA1, a Rab5 effector, to a target SNARE Syntaxin 13 is required for early endosome fusion (Zerial and McBride, 2001).

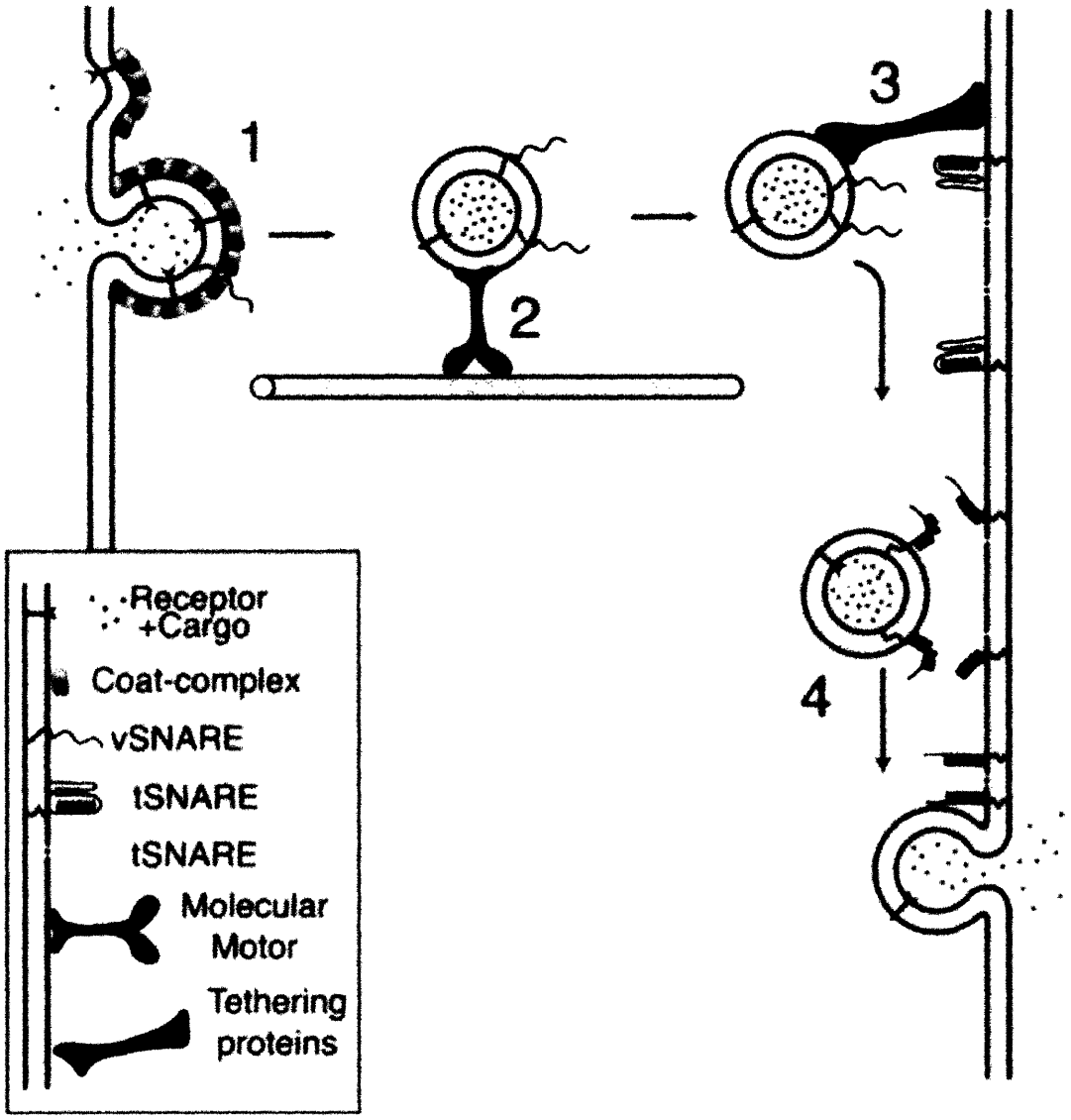

Figure 10 (Taken from Prekeris, 2003): Transport vesicle formation and fusion model. Coat proteins mediate vesicle budding from the donor compartment as well as cargo selection (step 1). Actin- and microtubule-based molecular motors are responsible for delivering the transport vesicle to its final destination (step 2). Tethering proteins dock transport vesicles to target membranes (step 3). Finally, SNARE proteins, namely syntaxins and VAMPs, mediate membrane fusion and delivery of cargo to the acceptor compartment (step 4). VAMPs are usually present on transport vesicles (vSNAREs), while syntaxins are present on target membranes (tSNAREs).

In addition to regulating vesicle trafficking, Rabs serve as domain organizers within organelles. This has been best described for Rab5, the localization of which to the early endosome is the key step in the assembly of the Rab5 microdomain, rich in Rab5 effectors and other molecules essential for early endosome function (Figure 11) (Zerial and McBride, 2001).

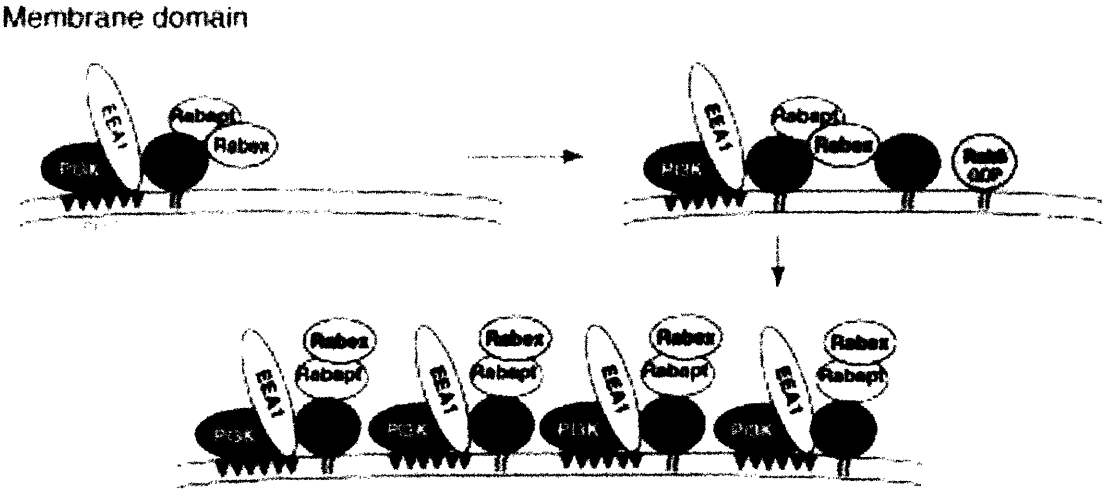

Figure 11 (Taken from Seabra and Wasmeier, 2004): (a) Model depicting the recruitment of several Rab5 effectors that may act in concert to generate a membrane domain. Activated Rab5 recruits PtdIns(3)K, leading to the generation of PtdIns(3)P in the endosomal membrane. The presence of the lipid then allows the binding of EEA1. GTP–Rab5 also interacts with the Rabaptin/Rabex complex, which, through the GEF activity of Rabex, results in the activation of additional Rab5 molecules. This in turn is followed by recruitment of further effectors, leading to the formation of a functionally distinct membrane subdomain.

The Rab cycle

In order to regulate all the various aspects of membrane trafficking, Rabs themselves must be carefully regulated: they must localize to the correct membrane and their activity (GTP/GDP state) must be carefully controlled. We do not yet understand exactly how each Rab finds its target membrane; however, some pieces of the puzzle have been put into place.

Most Rabs are post-translationally modified on the C-terminus by two hydrophobic geranylgeranyl groups. This double prenyl lipid group not only

anchors Rabs to the membrane, but also is necessary for their correct localization. Mono-prenylated Rabs are unable to localize properly and are, therefore, nonfunctional (Gomes et.al., 2003). Rabs are delivered to their target membranes in a GDP-bound form by a GDI—GDP dissociation inhibitor. Once associated with the right membrane, Rabs undergo a GDP to GTP exchange with the help of GEFs—Guanine nucleotide exchange factors. Upon activation, Rabs recruit downstream effectors through which they carry out their diverse functions. After inactivation by GAPs—GTPase activating proteins, Rabs are extracted from the membrane by a GDI and recycled. They can exist in the cytosol in the GDP-bound, GDI-associated state (Figure 12) (Seabra and Wasmeier, 2004).

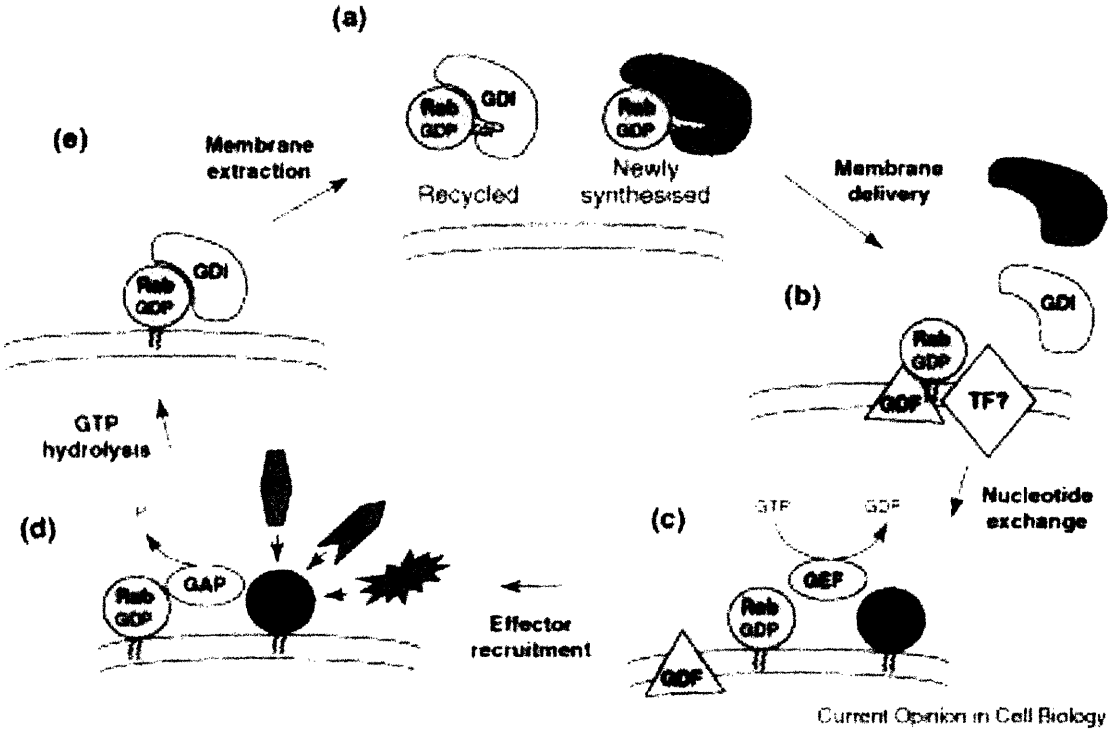

Figure 12 (Taken from Seabra and Wasmeier, 2004): Schematic representation of the Rab cycle showing membrane recruitment and activation. (a) GDP-bound Rab proteins form a cytosolic complex with RabGDI. (b) Membrane delivery and RabGDI displacement are mediated by a GDF, probably aided by unidentified targeting factors (TF), followed by (c) Rab activation through GEF-catalysed nucleotide exchange. (d) GTP-bound Rab recruits effector molecules to the membrane. (e) GAP-mediated GTP hydrolysis returns the Rab to its inactive state, resulting in re-extraction from the membrane by GDI.

There are only two GDI proteins in the human genome and they bind indiscriminately to all Rabs. Thus, while a GDI can regulate the membrane association/dissociation cycle of Rabs, it cannot by itself specify membrane localization for different Rabs. Recently, a molecule called Yip3 (Ypt-interacting protein 3) was shown, in vitro, to catalyze the dissociation of Rab9 from GDI and its transfer to the membrane (Sivars et.al., 2003). However, this has yet to be confirmed in vivo. Yip3 is a member of a family of small integral membrane proteins that bind promiscuously to prenylated Rabs in vitro, and are present on both endosomal and Golgi membranes in vivo (Seabra and Wasmeier, 2004). Furthermore, the Yip family of proteins has 16 members in humans, 12 of which are ubiquitously expressed, for the 63 human Rabs, making it apparent that they cannot be the sole determinants of Rab localization (Pfeffer and Aivazian, 2004).

While it was once thought that the C-terminal hypervariable regions, which are the most divergent among Rabs, determine their membrane localization, domain-swapping experiments disproved this hypothesis. It is now clear that different regions in different Rabs are required for targeting, suggesting that diverse mechanisms might be responsible for the recruitment of different Rabs to their membranes (Seabra and Wasmeier, 2004). Thus, the understanding of how Rabs are localized will likely require more than the identification of a family of "targeting" proteins.

We have a better grip on how the temporal activity of Rabs is regulated: GEF-- Guanine nucleotide exchange factors, turn them on and GAPs--GTPase activating proteins, turn then off. However, there are many Rabs for which these regulators have not been identified. Moreover, it is not well understood how GEFs and GAPs are involved in the switching of a Rab between its numerous effectors, or what signals lie upstream of these regulators.

Rab11

The Rab11 GTPase is interesting and complex in that, unlike most other Rabs, it regulates a variety of different membrane trafficking pathways. In mammals, the Rab11 family has grown to include three closely related proteins: Rab11a, Rab11b, Rab25a, while in flies and worms there is only one Rab11 GTPase.

The recycling pathway

When Rab11 was originally identified, it was found to localize to the pericentriolar recycling endosome and to regulate both the morphology and the function of this compartment. For instance, the expression of a dominant-negative form of Rab11 in mammalian cells inhibits the recycling of internalized transferrin, which instead accumulates in the recycling endosome (Ren et.al., 1998 and Ullrich et.al., 1996). In *Drosophila*, *rab11* mutant oocytes also show a defect in transferrin recycling (Dollar et.al., 2002).

The biosynthetic pathway

In addition to the recycling endosome, Rab11 has also been localized to the *trans*-Golgi Network (TGN) and the post-Golgi vesicles, suggesting that it plays a role in the biosynthetic exocytic trafficking pathway. Indeed, dominant-negative Rab11 expression blocks the transport of vesicular stomatitis virus (VSV)-G protein to the basolateral cell surface and causes it to accumulate in the TGN (Chen et.al., 1998). Moreover, recently the Ready lab beautifully demonstrated that Rab11 is required for the apical delivery of newly synthesized Rhodopsin from the TGN to the developing rhabdomere in the *Drosophila* photoreceptors (Satoh et.al., 2005).

Polarized membrane trafficking

As described above, Rab11 plays a role in polarized membrane transport and, interestingly, in different processes it is required for vesicle trafficking to either the apical or the basolateral cell surfaces. Examples of trafficking to the

apical surface include the transport of Rhodopsin in the *Drosophila* photoreceptors (Satoh et.al., 2005) and the trafficking of endosomally-sequestered H^+-K^+ ATPase to the luminal surface of acid secreting parietal cells in the stomach (Duman et.al., 1999). Examples of basolateral transport include the trafficking of VSV-G in kidney cells (Chen et.al., 1998) and the recycling of membrane to the expanding lateral membrane surface during *Drosophila* embryonic cellularization (Pellisier et.al., 2003 and Riggs et.al., 2003). Thus, Rab11 functions in both the apical and the basolateral polarized membrane trafficking.

Specialized trafficking

Rab11 also plays a role in the recycling of G protein-coupled receptors (GPRCs) such as the CXCR2 chemokine and the M4 muscarinic acetylcholine receptors, which seems to proceed through a specialized recycling compartment (Fan et.al., 2004 and Volpicelli et.al., 2002, respectively). It might also play a role in the important process of translocating the Glucose transporter 4 (GLUT4) from intracellular stores to the plasma membrane in response to insulin (Muller et.al., 2002).

How is the regulator regulated?

It is clear that Rab11 is a key regulator of multiple vesicle trafficking pathways; however, little is known about the mechanisms by which it regulates these events. Recently, a family of Rab11 interacting proteins, known as FIPS (Rab11 family of interacting proteins), has been described. FIPs share a conserved 20 amino acid Rab11/25 binding protein (RBD) domain, but otherwise, different classes of FIPs are quite dissimilar (Figure 13). Unlike the canonical Rab GTPase effectors, FIPs do not require Rab11 for their membrane localization, and, conversely, FIPs do not appear to be sufficient to recruit Rab11 to the appropriate membranes (Prekeris, 2003). However, like other Rab effectors, FIPs appear to recruit proteins required for membrane trafficking to Rab11-postive vesicles and organelles. For example,

FIP2 acts as an adaptor between Rab11 and the actin-based motor protein Myosin Vb, which is required for the transport of vesicles out of the pericentriolar recycling endosome compartment (Hales et.al., 2002). There is also evidence that class I FIPs might target Rab11-positive vesicles to docking sites on the plasma membrane via their C2 domains (Lindsay and McCaffrey, 2004). Finally, class II FIPs have been implicated as dual ARF/Rab11 interacting proteins (Hickson et.al., 2003). Some ARF GTPases are known regulators of cytoskeletal rearrangements, thus, class II FIPs might serve to couple the function of Rabs and ARFs in the processes, such as cytokinesis, where both membrane addition and cytoskeletal rearrangements must be closely coordinated (Wilson et.al., 2005). Interestingly, Nuf, a *Drosophila* homolog of an ARF effector, physically associates with Rab11 and, like Rab11, is required for embryonic cellularization, a process in many ways similar to cytokinesis (Rigss et.al., 2003).

In addition to FIPS, recent studies suggest that the exocyst--a tethering complex that targets vesicles to sites of membrane addition, is a Rab11 effector. In mammalian cells, Sec15, a member of the exocyst, binds to Rab11 in a GTP-dependent manner (Zhang et.al., 2004). Moreover, *Drosophila* photoreceptors mutant in *sec6*, an exocyst component, have an identical Rhodopsin trafficking defect to *rab11* mutant photoreceptors. In addition, Rab11 physically interacts with Sec5, another exocyst component. These results suggest that Rab11-positive vesicles might be targeted to the plasma membrane via their interaction with the exocyst complex (Beronja et.al., 2005).

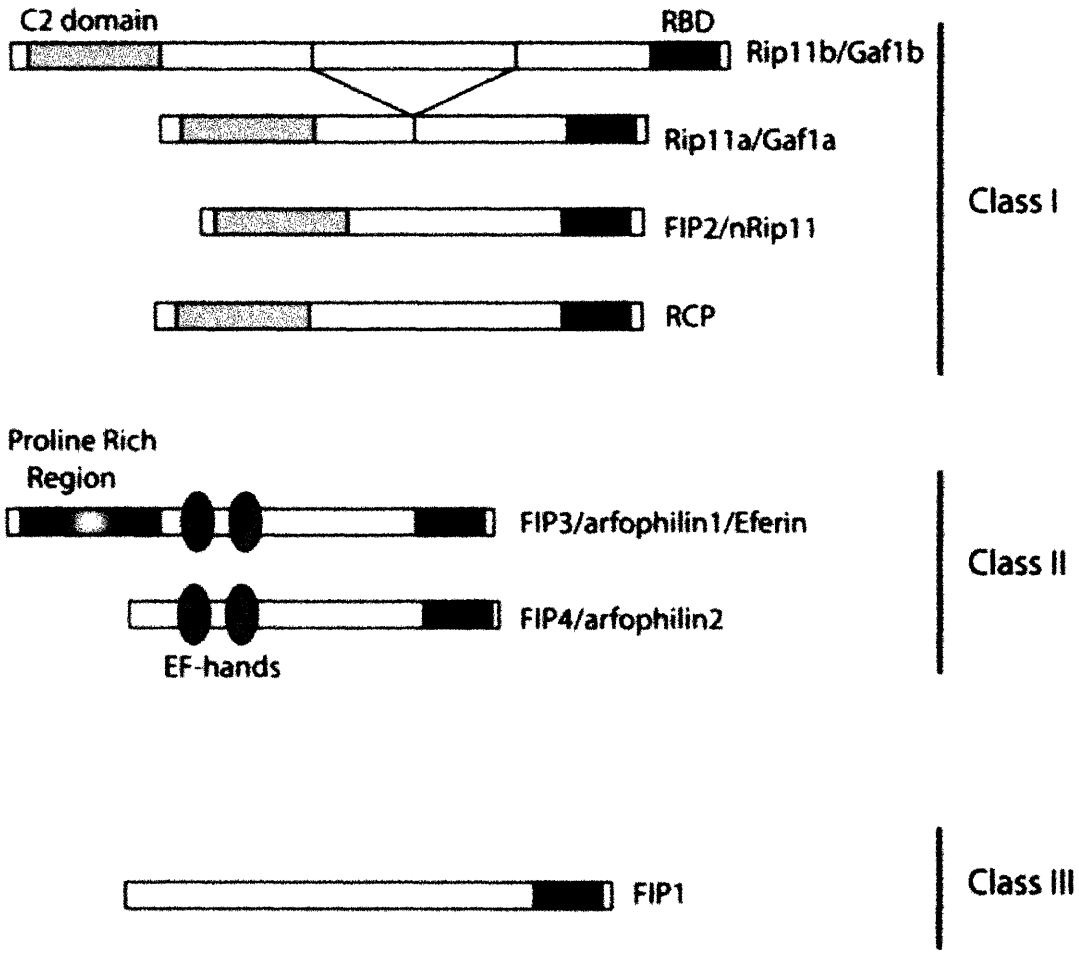

Figure 13 (Taken form Prekeris, 2003): The structure of Rab11 family interacting proteins (FIPs). Based on their domain structure, all FIPs can be divided into three main classes. Class I FIPs (Rip11a, Rip11b, RCP, and FIP2) contain a C2 domain at the N-terminus end of the protein. Class II FIPs (FIP3 and FIP4) contain two EF-hands and a proline rich region. Class III includes only one member, FIP1, which exhibits no homology to known protein domains. RBD stands for Rab11 binding domain.

Remaining questions about Rab11

Although we are beginning to learn about how Rab11 executes its diverse functions, many questions remain. It will be important to learn how Rab11 is localized to its target membranes, and furthermore, what factors are involved in its differential targeting to the TGN versus the recycling endosome. No Rab11 GEFs or GAPs have been identified to date; therefore, it will be interesting to find out what they are and how they play a role in the regulation of Rab11 localization and function. Rab5 is estimated to have close to forty effectors, as might Rab11. So far, only a handful of Rab11 effectors have been identified; it will be interesting to find other Rab11 interactors and learn how they function in various Rab11-dependent processes.

Concluding Remarks

BEACH proteins comprise an evolutionarily conserved protein family with roles in important cellular processes and some members associated with human disease. BEACH proteins have been implicated in vesicle trafficking; however, their molecular mechanism of function is not known. In my work I took a genetic approach to studying Beach1, a previously uncharacterized Drosophila BEACH protein. Although we identified Beach1 in an overexpression screen due to a strong gain-of-function phenotype in the photoreceptor growth cones, we found that loss-of-function *beach1* mutants had no obvious defects. However, through a genetic modifier screen, I found the *beach1* overexpression phenotype to be strongly enhanced by a reduction in *rab11* dosage. Rab11 is a small GTPase required in several membrane trafficking pathways that culminate in the delivery of recycled or newly synthesized proteins to the plasma membrane. Analysis of *beach1, rab11* double mutants revealed that *beach1* suppresses the previously described viability and bristle defects of *rab11*. Furthermore, guided by the synaptic localization of Beach1 and Rab11, I found that *rab11* mutants also have synaptic morphology defects at the neuromuscular junction (NMJ), which are suppressed by the loss of *beach1*. This confirmed that *beach1* is indeed involved in the regulation of the morphology of nerve terminal regions, as was originally suggested by its overexpression phenotype. Thus, my work showed that Beach1 functionally antagonizes Rab11 during Drosophila development and in regulating synaptic morphology. Furthermore, since the neuromuscular junction is highly amenable to genetic, functional, and structural analysis, future studies at the NMJ are likely to not only contribute to our understanding of how Rab11 and Beach1 function in membrane trafficking, but to also broaden our knowledge about the mechanisms regulating synaptic growth.

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Chapter 2

Identification and characterization of Beach1, a *Drosophila* BEACH protein

This chapter includes joint work with Adela Augsburger, who was a postdoc in the Garrity lab at the time. We worked together on the overexpression screen and the EMS screen for the isolation of *beach1* mutants.

Some of the work described in this chapter, specifically, localization experiments at the neuromuscular junction, was done in the Schwarz Laboratory at Harvard Medical School (Children's Hospital) and funded, in part, by the Harvard MD/PhD program.

Introduction

Mutational analysis of the genomes of model organisms is a powerful way to gain insight into biological processes. Mutagenesis screens are advantageous in that one needs no prior knowledge to implicate a gene in a particular process. To create lesions in the DNA, mutagens, such as chemicals, ionizing radiation or transposable elements, are used. Most mutations generated in this fashion are loss-of-function mutations—they reduce or eliminate the function of a given gene. However, gain-of-function mutations, ones that increase the level or broaden the area of expression, have also proven useful in the understanding of how a given gene is involved in various biological processes. For example, upregulation of oncogenes leads to cancer, consistent with the normal roles of these genes in cell growth. One of the best examples of informative missexpression is probably the missexpression of homeotic genes in the *Drosophila* embryo that results in stunning segmental transformations, helping to elucidate the normal functions of homeotic genes in the establishment of the *Drosophila* body plan (Lewis, 1978 and Schneuwly et.al., 1987).

At the start of my graduate work, I was interested in the mechanisms behind axon guidance, the process by which neurons navigate to their targets using growth cones, motile structures at the end of axons that steer them along their path. At that time, several loss-of-function screens for regulators of axon guidance and growth cone morphology had already been done (Martin et.al., 1995 and Garrity et.al., 1996); therefore, we decided to use a gain-of-function approach. We hoped that, by doing an overexpression screen, we would increase our chances of identifying new genes involved in these processes.

For our screen we chose to use the compound eye of *Drosophila melanogaster*, which had already been proven to be a powerful system for the identification of axon guidance regulators (Martin et.al., 1995 and Garrity

et.al., 1996). The fly eye is made of approximately 750 ommatidia, each containing eight photoreceptor or R-cells, which can be divided into three subtypes: R1-6, R7 and R8. R-cell axons from each ommatidium project as a bundle from the developing eye into the brain's optic lobe; however, R1-6 stop in the lamina, while R7 and R8 continue to the deeper medulla layer. The precise and repetitive nature of R-cell projections makes even subtle defects in targeting or morphology easily observable. In addition, the fly eye is not required for viability of the animal, making it an ideal tissue for studying molecules that are otherwise required for proper nervous system development and function.

To conduct our screen we used an EP modular misexpression system designed by Pernille Rorth and a collection of molecularly mapped EP insertion lines (Rorth, 1996). We screened through over 1300 EP lines and found that several genes with known loss-of-function defects in axon guidance and growth cone morphology had overexpression phenotypes in our assay. Moreover, we identified genes that were not previously implicated in the regulation of these processes. We focused our analysis on Beach1, a previously uncharacterized member of the conserved BEACH family of proteins with putative roles in vesicle trafficking. Overexression of *beach1* caused a dramatic alteration in growth cone morphology; however, when we generated strong loss-of-function mutants in this gene we found that they had no obvious phenotypes. Therefore, further analysis is needed to understand how Beach1, a synaptically enriched protein, is involved in the regulation of the morphology of nerve terminals.

Results

Overexpression Screen

Screen design and goals

To conduct our screen we systematically crossed a collection of molecularly mapped EP elements, each containing fourteen UAS elements (Gal4 binding sites) to a GMR-Gal4 pattern line. GMR is a photoreceptor specific promoter that drives high levels of the yeast transcription factor, Gal4, in the fly eye. This, in turn, initiates photoreceptor specific transcription of genes located directly downstream of the EP insertion site. We analyzed photoreceptor targeting and growth cone morphology of animals carrying both the GMR driver and the target EP during the third instar larval stage of development, after many photoreceptor neurons have reached their targets in the brain.

We were looking for mutants with defects in axon targeting, manifesting in phenotypes of "shoot through", caused by a failure of R1-R6 axons to stop in the lamina, as well as "short stop", caused by a failure of axons to reach the lamina target layer. Weaker phenotypes, described as "uneven lamina", "holes in lamina", and "thick bundles", were also recorded. Such phenotypes could also result from a failure of a fraction of axons to stop in the lamina; however, such phenotypes are more difficult to interpret than the unambiguous short stopping and shoot through defects. The rest of the phenotypes, where the projection was not wild type, but the nature of the defect was difficult to qualify, were termed "abnormal". We tried to screen against defects in eye development, which manifest themselves in the uneven patterning of the eye disk. However, it is possible that subtle defects in eye patterning were missed, thus, some of the lines with defects in the axon projection might also have defects in eye development.

Summary of screen results

We screened through 1372 lines and identified 97 EP insertions that caused defects in photoreceptor projections. Tables summarizing the results of our screen are provided in the Supplementary Materials section at the end of this chapter (Supplementary Tables S1-S9). For each EP line the following information is provided:

- gene of insertion,
- orientation of EP with respect to the open reading frame of the linked gene,
- position on the chromosome,
- brief summary of function, and
- other overexpression screens in which it was identified.

The lines that showed high penetrance—in which a close to expected proportion of the animals exhibited the phenotype, are indicated in the tables by an asterics. Those lines that had high expressivity of the defect, for example, where a high percentage of the axons was short stopping or shooting through, were qualified as "strong".

Overall, we identified:

- 8 known regulators of axon guidance and growth cone morphology,
- 17 genes with roles in protein modification (such as phosphorylation and ubiquitination),
- 10 genes involved in vesicle trafficking, cytoskeletal organization, and cell adhesion,
- 11 genes involved in signaling (such as ligands, receptors, and other players in the pathway),
- 17 genes involved in transcriptional regulation,
- 14 involved in basic metabolism and homeostasis,
- 5 genes involved in RNA processing and localization,
- 1 gene involved in DNA replication,
- 11 inserts in genes whose function is unknown, and

- 10 EPs for which the gene of insertion is unclear.

Some of the genes are included in more than one category (Supplementary Tables S1-S9).

Known regulators of axon guidance and new candidate gene were identified in the screen

We identified eight EP lines that mapped to known regulators of axon guidance and growth cone morphology in the eye, the embryo, or the larva. Among these were three transmembrane molecules, two transcription factors, a kinase, an activator of a Rho-family GTPase, and an actin-binding protein. Only half of the eight had highly penetrant defects, and of these, three had the EP inserted in the orientation predicted to cause overexpression (Tables S1-S9 and Figure 1).

Two of the eight EPs were inserted in genes with known loss-of-function phenotypes in the photoreceptors: *misshapen* (*msn*), a serine/threonine kinase, causes shoot through and growth cone defects when mutated (Su et al., 2000). Loss-of-function in *scribbler* (*sbb*), a transcription factor, also causes shoot though phenotypes (Rao et.al., 2000). The short stopping phenotype caused by a gain-of-function in *msn* is clearly opposite to its loss-of-function phenotype (Figure 1). However, the same clearly opposite phenotype is not seen for *scribbler*; its overexpression causes a weak, more ambiguous defect, best characterized as "uneven lamina".

In our screen we identified five EPs that caused strong and penetrant axon guidance defects. Two of these are in the known regulators of axon guidance: *msn*, which is described above, and *longitudinals lacking* (*lola*)—a transcription factor. The others include a transcription factor, *escargot*, *v(2)k05816*--an acetyltransferase, putatively involved in fatty acid biosynthesis, and an insertion in a novel gene CG33523, which has no conserved domains.

Figure 1. Examples of Photoreceptor Axon Guidance and Growth Cone Morphology Defects Identified in the Gain-of-Function Screen

Optic lobes of 3rd instar larvae labeled with antibody that recognizes photoreceptors and their projections are shown

(A) A control animal, showing wild type projection pattern and morphology. Photoreceptors(R-Cells) from the eye disk (not shown) project through the optic stock (os) into the brain. Some photoreceptors (R1-R6) stop in the lamina (la), while others (R7 and R8) pass through the lamina and terminate in the medulla (me).

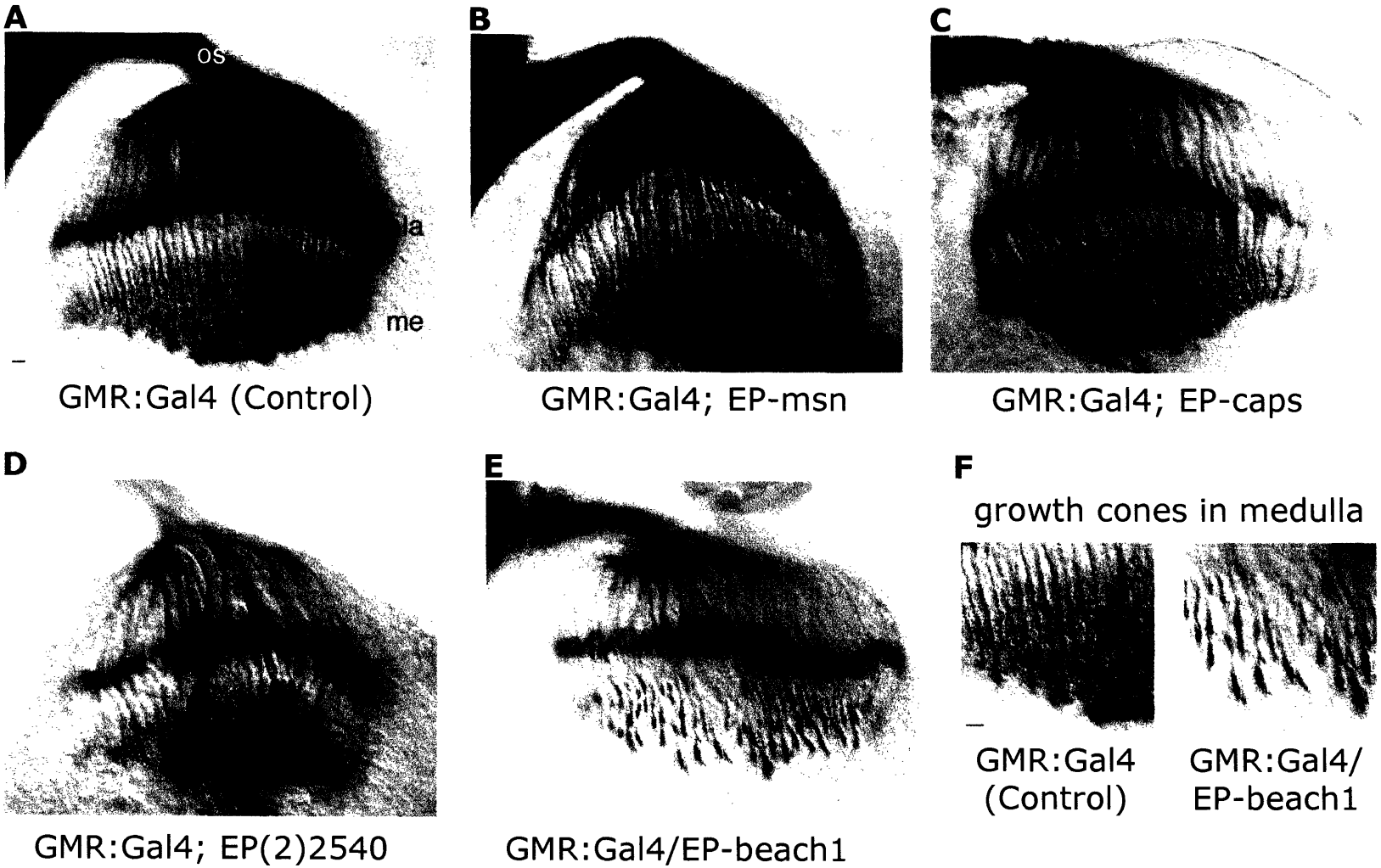
(B) Overexpression of a known axon guidance regulator *misshapen*, which encodes a serine/threonine kinase, causes most R1-R6 photoreceptors to stop before reaching the lamina. This is an example of a strong short stopping defect.

(C) Overexpression of a known regulator or axon guidance and synapse formation *capricious* (*caps*), which encodes a transmembrane cell adhesion molecule results in projection with uneven lamina, holes in the lamina, and few thick bundles that project into the medulla.

(D) Phenotype resulting from putative overexpression of EP(2)2540, which is not linked to a gene: lamina is uneven, has holes, and abnormal axon crossing is observed.

(E) Overexpression of *beach1*, a putative regulator of vesicle trafficking with conserved PH, BEACH, WD40, and FYVE domains, leads to an alteration of growth cone morphology, but not to targeting defects.

(F) A magnified view of growth cones in control versus *beach1* overexpressing animals.


A
os
la
me
GMR:Gal4 (Control)
B
GMR:Gal4; EP-msn
C
GMR:Gal4; EP-caps
D
GMR:Gal4; EP(2)2540
E
GMR:Gal4/EP-beach1
F
growth cones in medulla
GMR:Gal4
(Control)
GMR:Gal4/
EP-beach1

Beach1, a candidate regulator of growth cone morphology, was identified in the screen

We identified one line, EP(2)2299 that showed normal axon targeting, but had a strong and highly penetrant defect in growth cone morphology. EP(2)2299 is inserted in the first intron of *beach1,* an evolutionarily conserved gene with a putative role in vesicle trafficking. When this EP line is crossed to a photoreceptor-specific driver, it leads to high levels of *beach1* mRNA expression in the photoreceptors (Figure 3B). Beach1 overexpression leads to a striking alteration in growth cone morphology: growth cones have a large, blebby core or central area and are less well expanded compared to controls, giving the appearance of increased spacing between growth cones in the medulla (2A', A'' and B', B''). The eye disk in *beach1* overexpressing animals is normally patterned, suggesting that eye development is normal; however, the adult eye is reduced in size and glazed in appearance (Figure 2A and B). Since *beach1* had one of the strongest phenotypes observed in the screen, we decided to focus our efforts on the characterization of this gene.

Figure 2. Overexpression of *beach1* in the photoreceptors leads to defects in larval growth cone and adult eye morphology

(A) Control (GMR-Gal4) adult eye has highly organized rows of ommatidia (individual facets). Scale bar 100um.

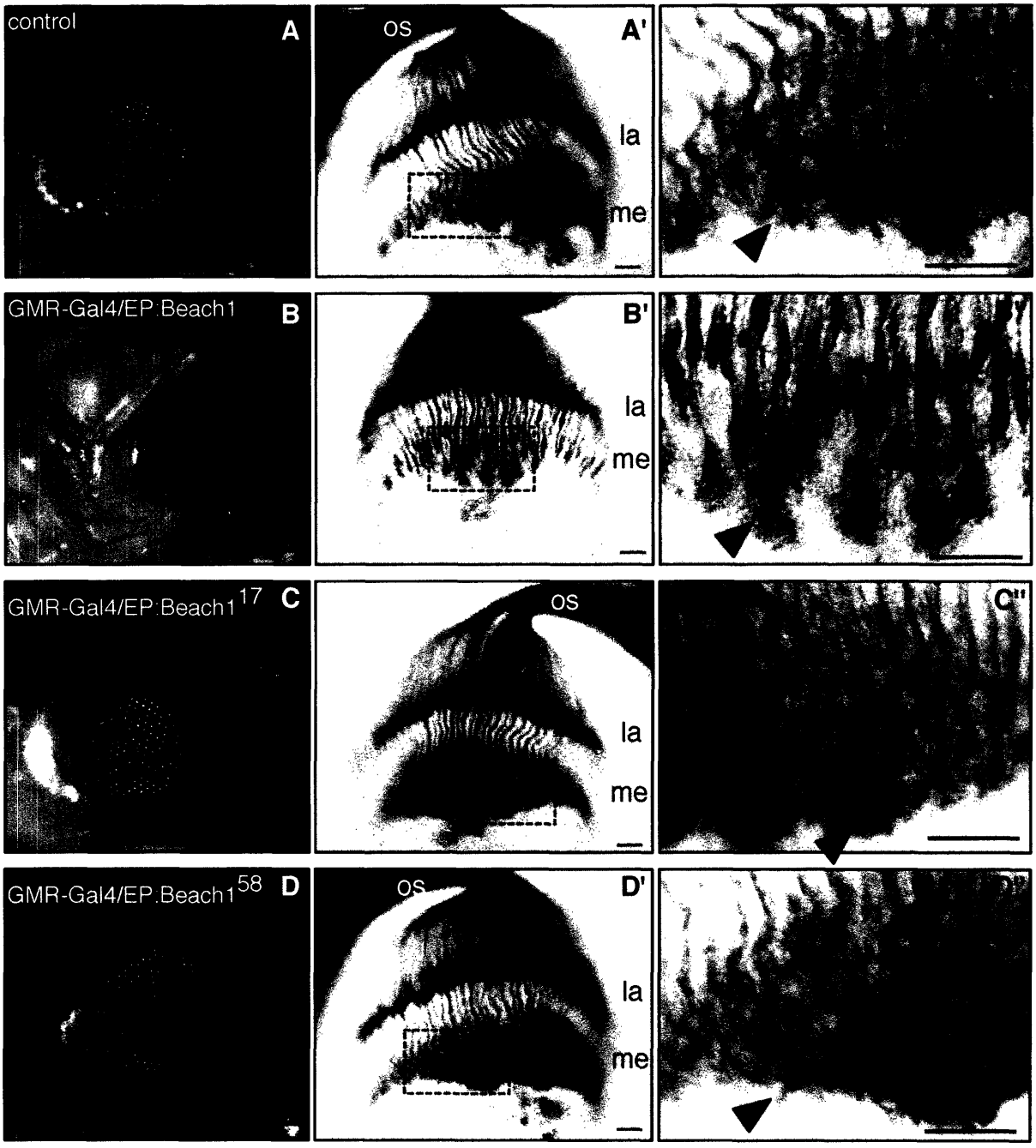
(B) Beach1 overexpression leads to a reduced, glazed eye, without defined ommatidia.

(C), (D) Alleles $beach1^{17}$ and $beach1^{58}$, both of which encode premature Stop codons, completely suppresses the *beach1* eye overexpression phenotype when driven in the photoreceptors.

(A') Control photoreceptor projection pattern. Axons project from the eye disk (not shown) through the optic stalk (os), into the brain. R1-R6 terminate in the lamina (la), R7 and R8 terminate in the medulla (me).(A'') Zoomed in view of the medulla from the region outlined in A' by a dotted line shows the normal morphology of growth cones. Scale bar 10um.

(B') In *beach1* overexpressor, axons project normally and are correctly targeted to lamina and medulla layers, but growth cone morphology is abnormal. (B'') Growth cones have a large, blebby central area and are not as well expanded as the control, giving the impression of increased spacing between them. Scale bar 10um.

(C'),(C'') and (D'),(D'') Alleles $beach1^{17}$ and $beach1^{58}$ completely suppress the *beach1* growth cone overexpression phenotype. Scale bar 10um. Arrowheads in A'', B'', C'' and D'' point to individual growth cones in the medulla.


control
A
os
A'
la
me
GMR-Gal4/EP:Beach1
B
B'
la
me
GMR-Gal4/EP:Beach1^{17}
C
os
C''
la
me
GMR-Gal4/EP:Beach1^{58}
D
os
D'
la
me

Confirmation of *beach1* genomic and mRNA sequence

When we began studying *beach1*, no published data about this gene existed, and only the predicted genomic and cDNA sequences were available. I sequenced genomic DNA from the EP(2)2299 (EP:Beach1) strain and found that my results matched the predicted sequence. We also sequenced the *beach1* cDNA from S2 cell RT PCR and from an available partial cDNA. Our results confirmed that the *beach1* gene is spliced into a 9473 bp mRNA. Aside from several silent polymorphisms, no differences were found between the predicted and the experimentally determined cDNA sequences. I also determined the location of the START site using 5′ RACE and confirmed that the EP element is inserted in the 1st intron of *beach1*, 822 base pairs upstream of the START codon (Figure 3A). This suggests that when the EP Beach1 line is crossed to a Gal4 driver, the full-length Beach1 protein is overexpressed.

beach1 mRNA is highly expressed in the nervous system

To determine the endogenous expression pattern of the *beach1*, we carried out an RNA in situ hybridization experiment, which showed that *beach1* mRNA is enriched in, but not restricted to, the embryonic central nervous system (Figure 4A). Since *beach1* is an evolutionarily conserved gene, I also did northern blot analysis of the mouse *beach1* homolog expression. Similarly to *Drosophila beach1*, the mouse homolog is highly expressed in, but not restricted to, the CNS; it is also highly expressed in the mouse embryo and ovary, and present in other tissues (Figure 4B). This suggests that *beach1* and its homologs might function in many cell types and tissues.

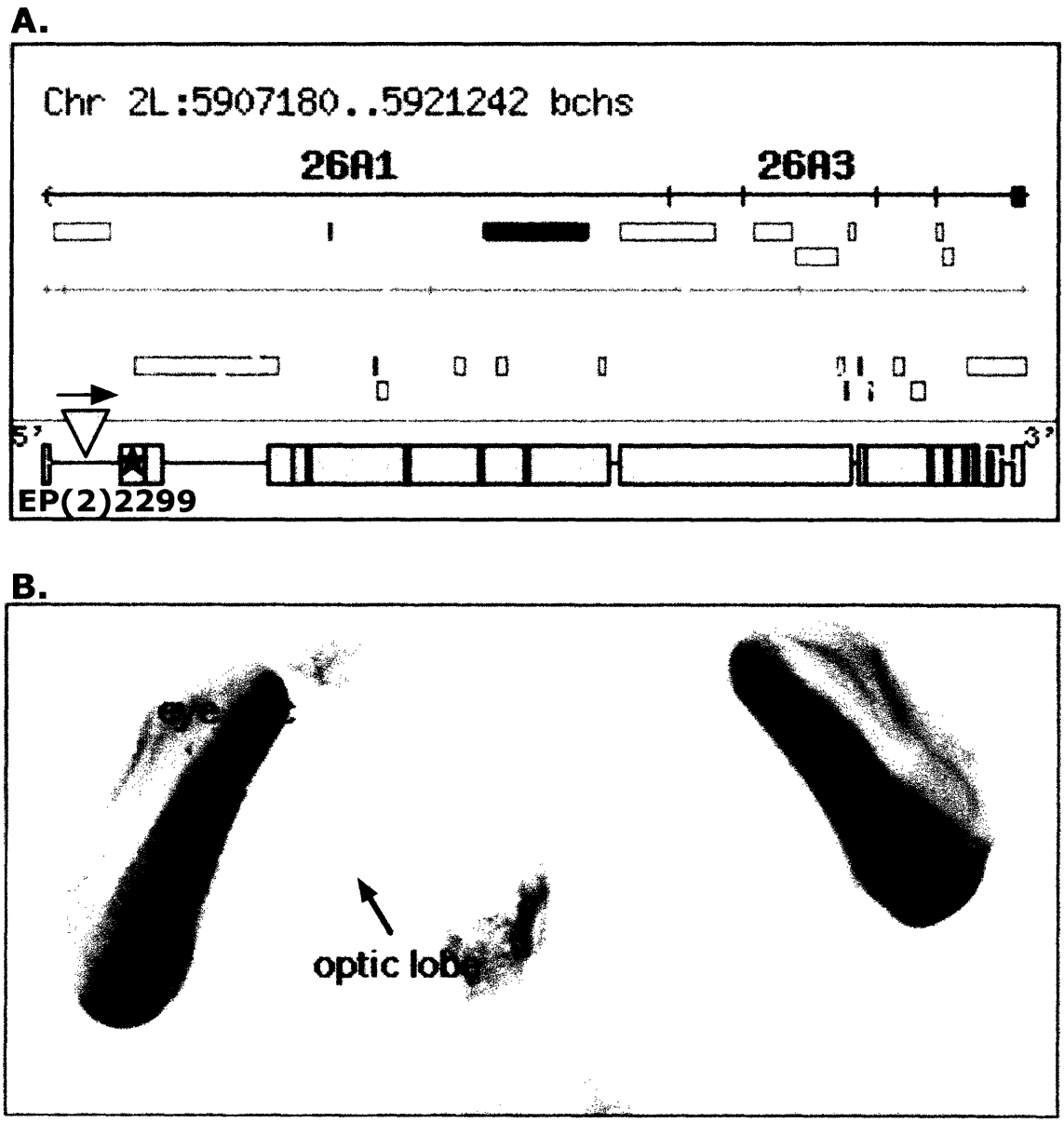


Figure 3. EP(2)2299 Causes Overexpression of *beach1* in the Photoreceptors
(A) Image modified from FlyBase. EP(2)2299 (indicated by a green triangle) is inserted in the 1st intron of beach1 (also known as bchs), 822 bps upstream of the START codon (indicated by a blue star). EP is in the proper orientation to drive expression of a full length beach1 message.
(B) When EP(2)2299 or EP:Beach1 is crossed to a photoreceptor-specific driver GMR-Gal4, it leads to high levels of *beach1* mRNA expression in the photoreceptors (eye disk)

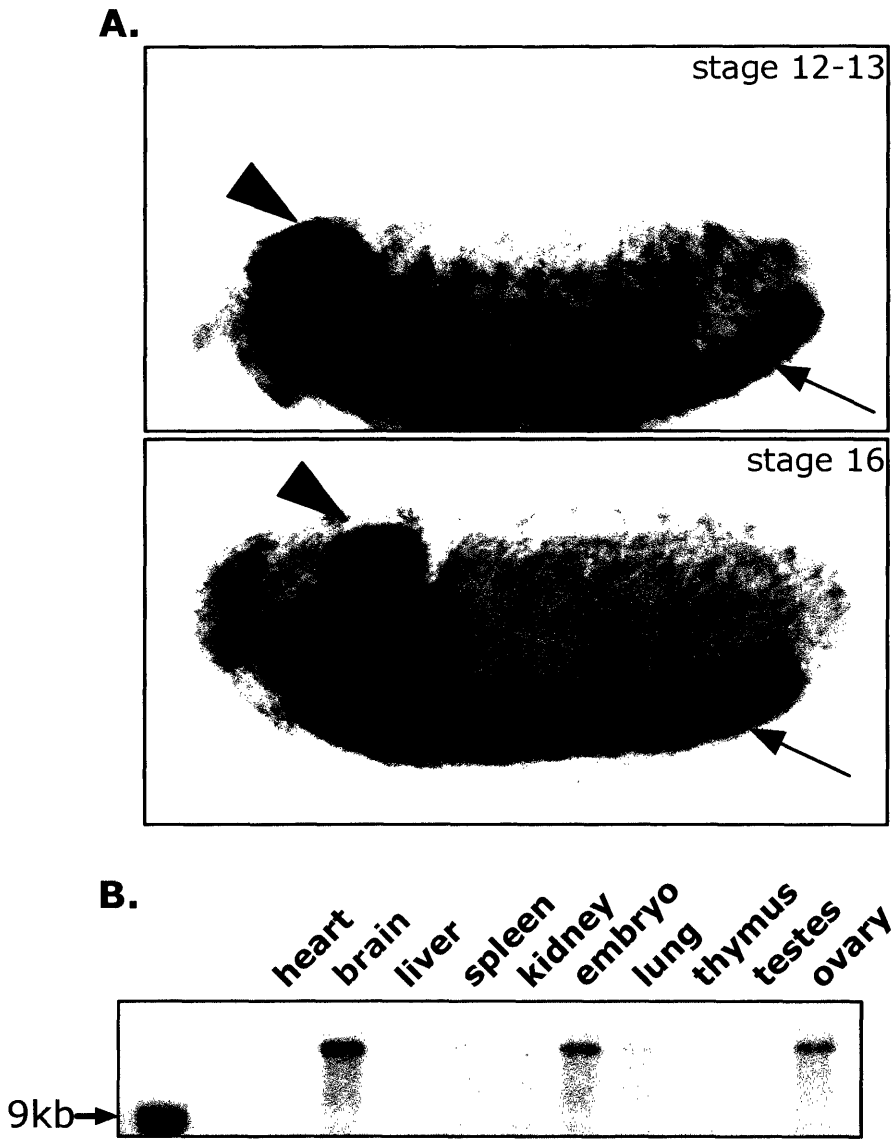


Figure 4. *beach1* and its mouse homolog are highly expressed in the CNS

(A) *beach1* mRNA is highly expressed in the brain (arrowhead) and in the ventral nerve cord (arrow) of the embryonic CNS. It is also highly expressed in the salivary glands (star). Embryos in developmental stages 12-13 and 16 are shown.

(B) Murine *beach1* homolog is highly expressed in the brain. It is also expressed in other tissues, such as the ovary, and during embryogenesis.

Beach1 Is evolutionarily conserved

Beach1 is a 3491 amino acid protein with a predicted size of 390 kDa. As discussed in the introduction, it belongs to Class II of the BEACH protein family, and has a homolog in every organism, from *Dictyostelium* to humans. We did an alignment between the *Drosophila* Beach1 and its human homolog Alfy, and found that there is a high degree of homology between these two proteins (Figure 5A). Like most other BEACH proteins, Beach1 has a PH-BEACH-WD40 domain module in its C-terminus, which is shared among all BEACH proteins. The remaining N-terminus, covering about 70% of the total protein length, is not conserved between different BEACH classes, but shares some regions of homology with Alfy and the other Class II proteins. From here on I will refer to this region as CRAB—conserved region in Alfy and Beach1. In addition to the PH-BEACH-WD40 module, Beach1 also has a FYVE (Fab1p, YOTB, Vac1p, and EEA1) domain in its very C-terminus. FYVE domains have been shown to bind to phosphotidylinositol-3-phosphate (PtdIns(3)P) and are thought to be involved in the localization of proteins to membranes rich in PtdIns(3)P, such as those of early endosomes (Misra et.al., 2001). Interestingly, Beach1 homologs in *Dictyostelium* (LvsA) and in *Arabidopsis* lack this C-terminal FYVE domain.

Mutations in *beach1* were isolated in the EMS screen for the suppressors of the eye overexpression phenotype

Based on the EP orientation and on the in situ data showing increased *beach1* mRNA levels in photoreceptors of the GMR-Gal4/EP:Beach1 animals (Figure 3), we inferred that the observed defects in the growth cone morphology and the adult eye were caused by the overexpression of Beach1 protein. We reasoned that if we created a mutation in the *beach1* locus downstream of the EP element, which resulted in the production of a nonfunctional (either truncated or unstable) protein, we would restore the growth cone and adult eye morphology back to wildtype. Therefore, to isolate

loss-of-function mutations in the *beach1* gene, we decided to take advantage of the adult eye phenotype caused by *beach1* overexpression. To this end, we carried out an EMS mutagenesis screen for suppressors of the adult eye phenotype.

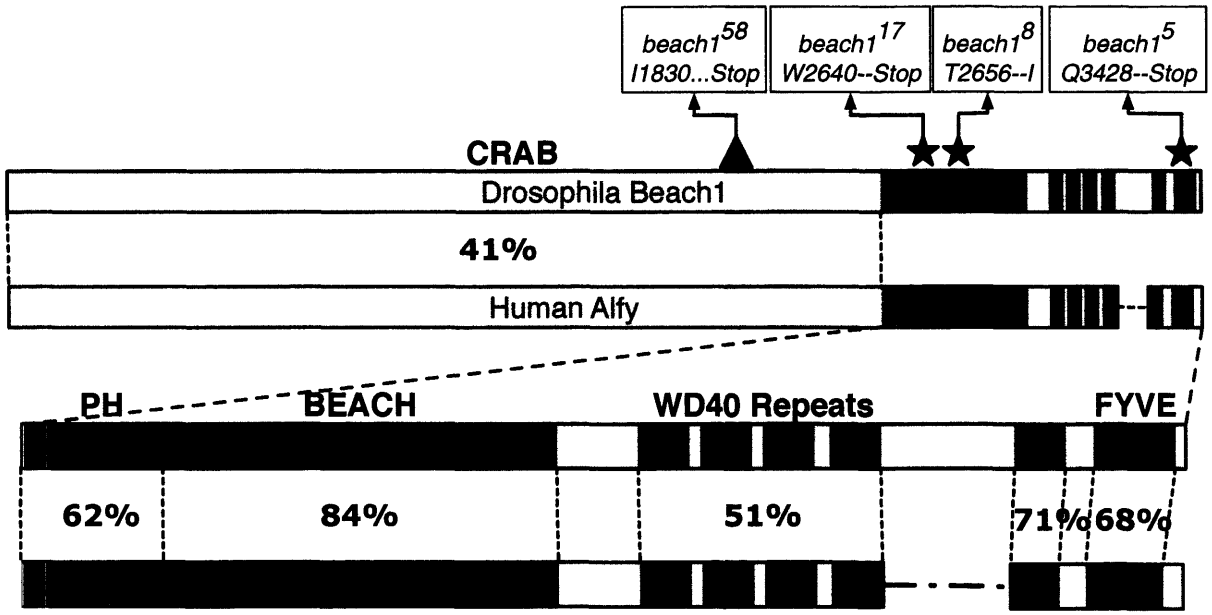
We identified 17 independent lines that completely or partially suppressed the adult eye phenotype (Figure 2C and D, and Table 1). The degree of eye phenotype suppression showed a precise correlation with the degree of growth cone phenotype suppression; confirming our suspicion that the growth cone and the eye phenotypes are linked (Figure 2C',C'' and 2D', D''). I sequenced genomic DNA from 11 suppressor lines that were homozygosed for the EP:Beach1 carrying chromosome, and found that 8 of them had mutations in the *beach1* gene (Figure 5A and Table 1). I did not identify a mutation in $beach1^{12}$, which is a strong suppressor of both the growth cone and the adult eye phenotypes. However, during my attempts to PCR from the *beach1* locus in this stock for sequencing, I found that a PCR reaction that consistently worked in every other line completely failed in the $beach1^{12}$ line. This suggests that the lesion in this line might be a DNA rearrangement. Sequencing from lines $beach1^{29}$ and $beach1^{37}$, both of which are weak suppressors, did not reveal mutations in the *beach1* locus, suggesting that the eye phenotype suppression in these lines might be due to a second site mutation.

Figure 5. Alleles of *beach1*, an evolutionarily conserved gene, were generated by EMS mutagenesis

(A) Protein alignment between *Drosophila* Beach1 and human Alfy. Conserved domains are shown in different colors: PH in orange, BEACH in green, WD40 in purple, FYVE in blue, and CRAB (Conserved Region in Beach1 and Alfy) in grey. % refers to percent amino acid identity between the indicated domains. The broken line in Alfy indicates a region that is not conserved with Beach1. Positions of alleles that encode point mutations are indicated by a star, and the one that encodes a small deletion is indicated by a triangle.

(B) Western blot probed with antibody against Beach1 (3777). Beach1 protein level is increased when expression of EP:Beach1 is driven in the photoreceptors with GMR-Gal4. This overexpression in suppressed when a mutation encoding a premature Stop condon is introduced downstream of the EP (EP:$beach1^{17}$), as shown for allele $beach1^{17}$. The original EP:beach1 chromosome has Beach1 protein level similar to control. Alleles $beach1^{12}$, $beach1^{17}$, $beach1^{17}$ have no detectable protein. Allele $beach1^{8}$ has about half of the control amount of Beach1 protein. Antibody against Elav, a pan-neuronal protein is used to show equal loading. A band of invariant intensity across the lanes, seen under the Beach1 band, is not specific for Beach1, since it is present in allele $beach1^{58}$, which is truncated prior to the region detected by this antibody.

A.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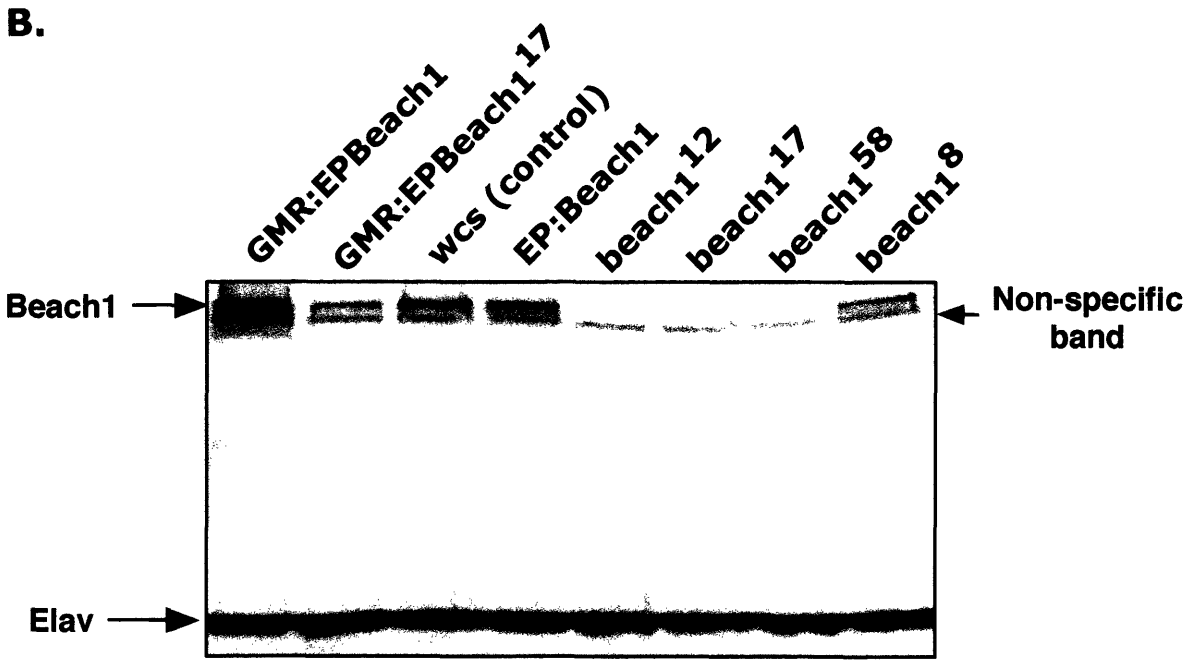
beach1^17
W2640--Stop
beach1^8
T2656--I
beach1^5
Q3428--Stop
CRAB
Drosophila Beach1
41%
Human Alfy
PH
BEACH
WD40 Repeats
FYVE
62%
84%
51%
71%
68%

B.


GMR:EPBeach1
GMR:EPBeach1^17
wcs (control)
EP:Beach1
beach1^12
beach1^17
beach1^58
beach1^8
Beach1
Non-specific band
Elav

Protein null alleles of *beach1* were isolated in the EMS screen

I analyzed protein levels in the *beach1* EMS-induced alleles with antiserum raised against the 2283-2636 amino acid portion of the Beach1 protein. On a Western blot this antiserum recognizes a high molecular weight band, running at the very top of the gel and well above the 250kDa marker, which is consistent with the 390kDa predicted size of the Beach1 protein (Figure 5B). Furthermore, the intensity of this band is increased in the animals that are overexpressing the original EP:Beach1 transgene in the photoreceptors compared to control. This band is absent in lanes with homogenate from animals that have mutations in the *beach1* gene, confirming the specificity of the antiserum. Moreover, the intensity of this band is decreased even when an attempt is made to overexpress allele $beach1^{17}$ in the photoreceptors, suggesting that this line is incapable of making stable Beach1 protein even when its expression is boosted by a powerful driver, such as GMR-Gal4.

It is also noteworthy to mention that allele $beach1^{8}$, which is a complete suppressor of both the eye and the growth cone phenotypes, shows only about a 50% reduction in the Beach1 protein on a Western blot (Figure 5B). Furthermore, when overexpressed, this allele is capable of generating very high levels of protein. Allele $beach1^{8}$ encodes a protein in which a conserved Threonine residue in the interface between the PH and the BEACH domains is changed to an Isoleucine (Figure 5A and Table 1).

A lower molecular weight band of unchanging intensity across the lanes is also seen on the western blot. It represents a non-specific band, since it is present in allele $beach1^{58}$, which encodes a protein truncated prior to the region that would be recognized by the antibody.

Table 1: Loss-of-Function Alleles in *beach1*

Allele number	Molecular lesion	Type of mutation and domains affected	Beach1 protein level	Suppression of adult eye
$beach1^{4}$	?	?	low	weak
$beach1^{5}$	Q_{3428}-Stop	Truncation: removes most of the FYVE domain	low	weak
$beach1^{8}$	T_{2656}-I	Missense mutation in BEACH domain: affects predicted PH-BEACH interface	50% of wildtype	strong
$beach1^{10}$	W_{3406}-Stop	Truncation: removes the FYVE domain	very low	strong
$beach1^{12}$	?	?	undetectable	strong
$beach1^{13}$	W_{2217}-Stop	Truncation: removes PH, BEACH, WD40, FYVE domains	very low	strong
$beach1^{14}$	?	?	wildtype	weak
$beach1^{17}$	W_{2640}-Stop	Truncation: removes most of BEACH, all of PH, WD40, FYVE domains	undetectable	strong
$beach1^{26}$	?	?	undetectable over df	strong
$beach1^{29}$	?	?	increased	weak
$beach1^{32}$	?	?	wildtype	weak
$beach1^{37}$	?	?	wildtype	weak
$beach1^{38}$	?	?	wildtype	weak
$beach1^{40}$	G_{1224}-D	Missense mutation in CRAB	very low	strong
$beach1^{43}$	?	?	undetectable over df	strong
$beach1^{52}$	Q_{410}-Stop	Early Stop, appears to have some read-through the Stop codon	very low	strong
$beach1^{58}$	I_{1830}...Stop	Truncation: removes PH, BEACH, WD40, FYVE domains	undetectable	strong

Beach1 is enriched in synaptic regions of the CNS and localizes to presynaptic terminals at the neuromuscular junction

I examined the localization of the Beach1 protein in the 3rd instar larval whole mount and found that it is enriched in the synaptic neuropil region, where its expression pattern overlaps with that of a known synaptic protein, Synaptotagmin1 (Figure 6A). This neuropil staining pattern is specific for Beach1, since no similar signal can be detected in *beach1* mutants. To determine whether Beach1 accumulates pre or postsynaptically I drove the expression of an HA-tagged Beach1 in the ellipsoid body—a central complex structure with spatially separated presynaptic and postsynaptic regions (Zhu et.al., 2003). I found that while a membrane-associated murine CD8 protein tagged with GFP was evenly distributed along the ellipsoid body neurons, Beach1 accumulated in the presynaptic regions (Figure 6B).

I also examined the localization of Beach1 at the 3rd instar neuromuscular junction (NMJ). I found that Beach1 is enriched in the terminals of motor neurons, but is also present in the muscles (Figure 6C and D). Most of the Beach1-positive puncta are contained within the HRP staining, which specifically labels the plasma membrane of a neuron, suggesting that Beach1 is enriched presynaptically.

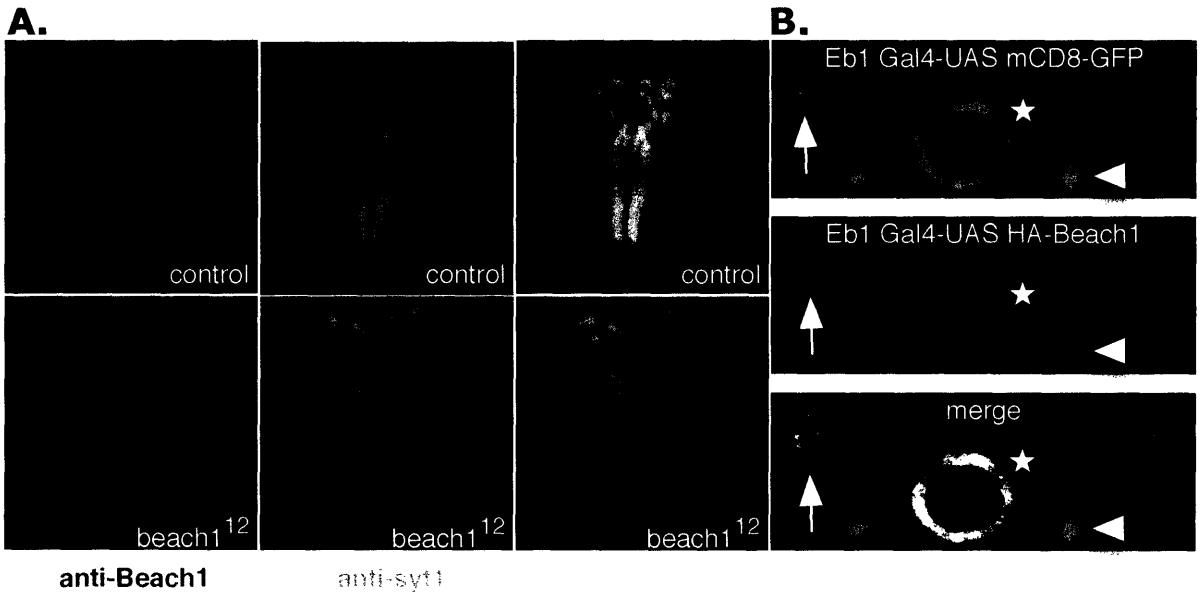
Figure 6. Beach1 protein is enriched in synaptic regions

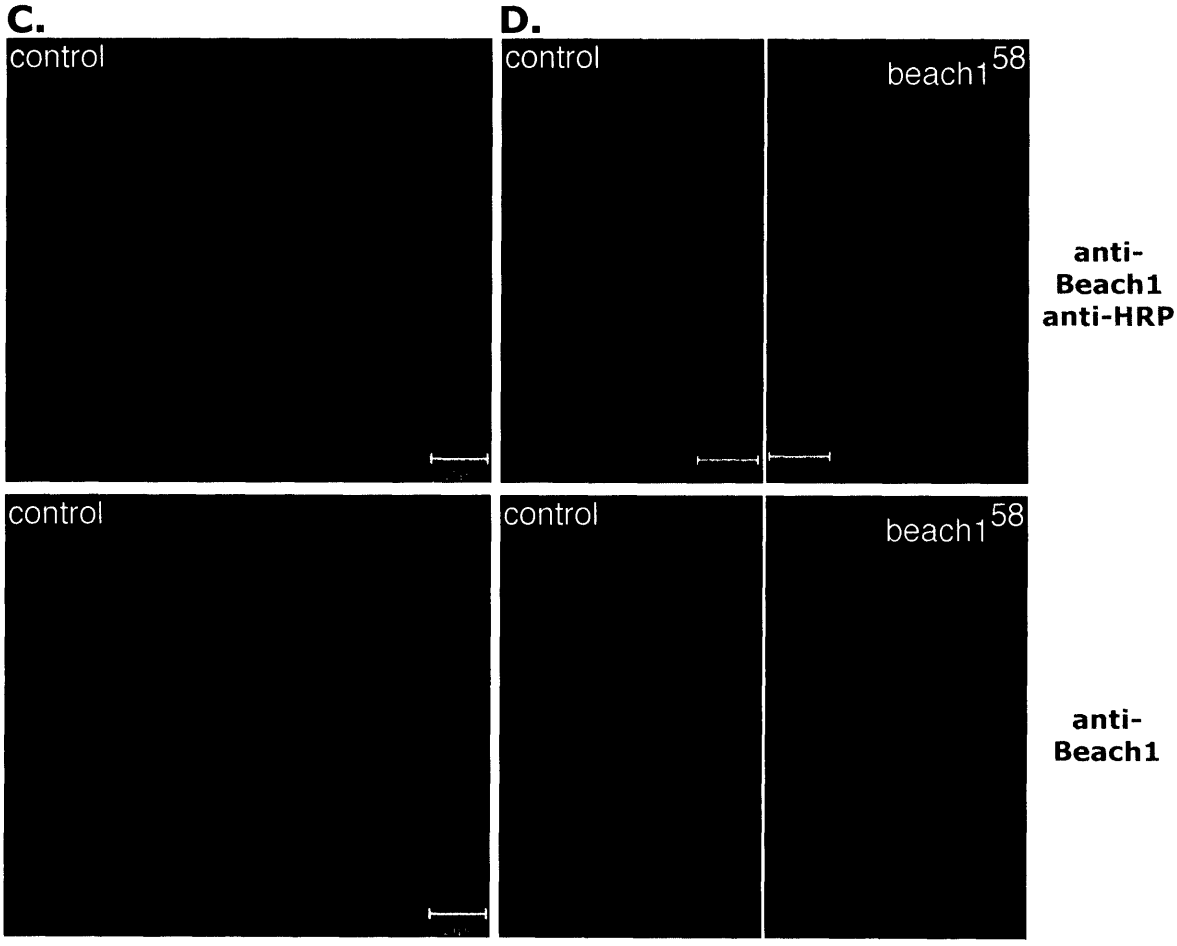
(A) Beach1 protein (red) is enriched in the synaptic neuropil of the 3rd instar larval brain and nerve cord, where its expression pattern overlaps with that of a known synaptic protein Synaptotagmin1 (green). Beach1 is undetectable in allele *beach1*^{12}.

(B) When expressed in the adult ellipsoid body, a central complex structure, HA-tagged Beach1 protein accumulates in the presynaptic regions (red), while a murine transmembrane CD8-GFP (green) distributes evenly throughout these neurons. Star indicates the presynaptic region, arrowhead points to the postsynaptic region, arrow points to the cell bodies.

(C) At the 3rd instar larval neuromuscular junction (muscle 6-7), Beach1 protein (red) is enriched in synaptic boutons. The neuronal membrane is outlined by ant-HRP staining (green). A confocal stack through the NMJ is shown. Scale bar is 20um.

(D) Beach1 puncta (red) are enriched within the HRP-labeled (green) neuronal membrane, suggesting that Beach1 localizes presynaptically at the NMJ. Beach1 is undetectable in allele *beach1*^{58}. A single confocal slice through an NMJ at muscle 6-7 is shown. Scale bar is 5 um.


A.
B.
control
control
control
beach1^12
beach1^12
beach1^12
anti-Beach1
anti-syt1
Eb1 Gal4-UAS mCD8-GFP
Eb1 Gal4-UAS HA-Beach1
merge


C.
D.
control
control
beach1^58
anti-Beach1 anti-HRP
control
control
beach1^58
anti-Beach1

beach1 mutants do not have an obvious loss-of-function phenotype

Sequencing and western blot analysis confirmed that strong loss-of-function alleles in *beach1* were isolated in the mutagenesis screen. Furthermore, to make sure I was working with the strongest allelic combination possible, I obtained two deficiencies that were predicted to remove the entire *beach1* locus, which I confirmed by PCR analysis (Figure 7). However, analysis of multiple allelic combinations, including protein null alleles over the deficiency, failed to uncover a loss-of-function phenotype: *beach1* mutants are viable, fertile, and have no overt defects. Examination of the 3rd instar larval eye brain complex revealed that eye disk patterning, photoreceptor axon guidance, and growth cone morphology were completely normal. The embryonic nervous system was also examined for subtle defects in axon guidance morphology, but none were found.

beach1 mutants do not have a shortened lifespan or exhibit age-dependent neurodegeneration

Around the time when we isolated mutants in *beach1*, it was reported that mutants in this gene had a shortened life span and exhibited neurodegeneration (Finley et.al., 2003). Although our mutant stocks showed no signs of premature death, I carried out careful experiments to assess the lifespan of three of our protein null alleles: *beach1*^{12}, *beach1*^{17},*beach1*^{58}. In repeated independent experiments we found that all of our *beach1* mutants lived longer than the wild type controls (Figure 8A). I also examined plastic brain sections from aged *beach1* mutant strains, *beach1*^{12} and *beach1*^{17}, for telltale signs of neurodegeneration, such as vacuolization, and found none (Figure 8B). Thus, our results concerning the lifespan and neurodegenaration in *beach1* mutants do not agree with the published data.

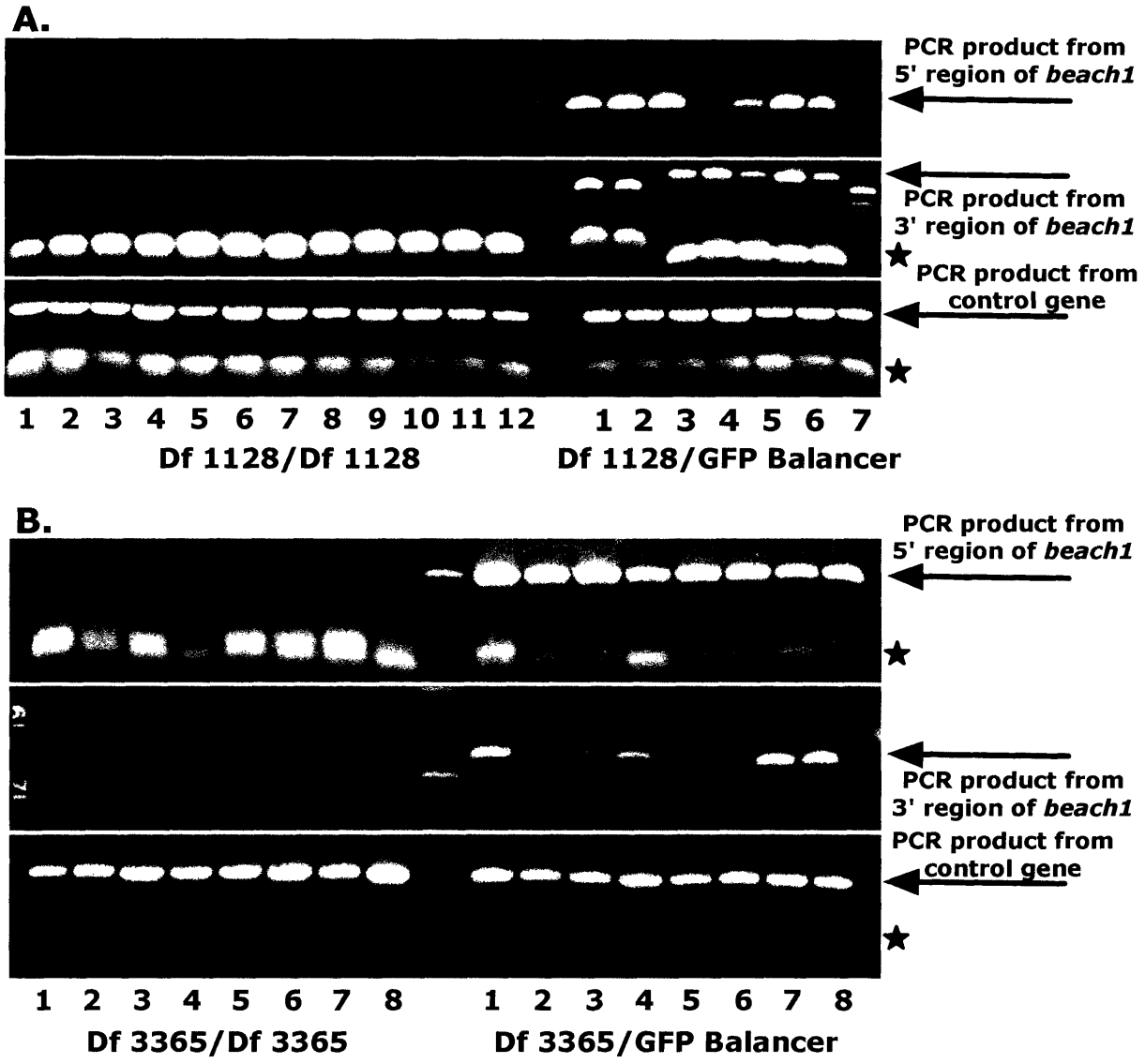


Figure 7. Deficiencies 1128 and 3365 remove the *beach1* locus

(A) For each panel, DNA from the same single embryo (1-12 for Df1128/Df1128 and 1-7 for Df1128/GFP Balancer) was used. Top panel shows a DNA gel from single embryo PCR reactions with primers against a 5' region of the *beach1* gene. Middle panel shows PCR against a 3' region of the beach1 gene. Bottom panel shows PCR against an unrelated gene gp160-Dtrk that verified the presence of DNA in each single embryo extraction. Arrow points to the positions of PCR products. Positions where primers from PCR reactions are seen are marked with a star.
(B) The results for the same set of experiments is shown for Deficiency 3365.

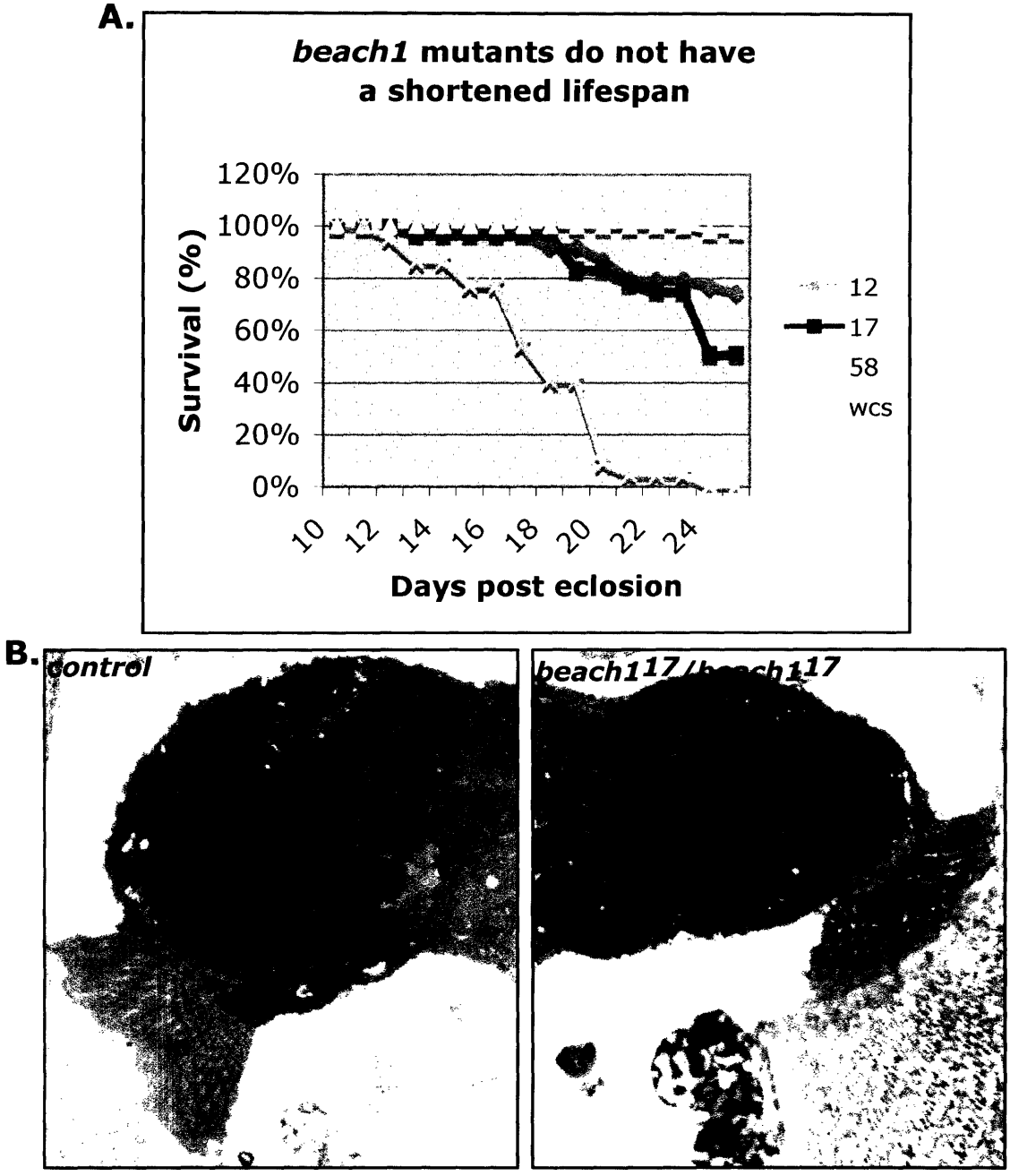


Figure 8. *beach1* mutants do not exhibit a shortened life span or neurodegeneration (A) Results of one of the life span experiments, showing that strong loss-of-function mutants *beach1*^{12}, *beach1*^{17} and *beach1*^{58} live longer than the control strain (wcs). (B) Brains of aged b*beach1*^{17} mutants do not reveal signs of neurodegeneration, such as vacuoles. An animal heterozygous for *beach1* was used as a control. Plastic 1um thick toluidine blue-stained sections are shown. White areas seen in a few places in both control and mutants brains are not vacuoles, but paths of giant fiber tracks. la-lamina, lo-lobula, me-medulla, lp-lobula plate areas of the brain are indicated.

Could *beach1* have a role in olfactory learning and memory

Since Beach1 protein is enriched in presynaptic regions, we wondered if, despite the absence of overt phenotypes, *beach1* mutants might have a subtle defect in synaptic plasticity. To test this hypothesis we collaborated with the lab of Scott Waddell (Umass, Worcester) to examine *beach1* flies for defects in olfactory learning. The olfactory learning assay is an example of classical associative conditioning in which flies are taught to associate a particular odor with a negative stimulus. In this assay, flies are exposed to one odor in conjunction with an electric shock, and then exposed to another odor in the absence of an electrical stimulus. These "trained" flies are then placed at a choice point where two air currents, each carrying one of the odors, are converging. Wildtype flies preferentially avoid the shock-associated odor, while learning and memory mutants distribute more randomly with respect to odor (Figure 9A). Mutant strains can be tested in this assay for defects in olfactory learning—performance within three minutes after training, or memory—performance after a longer time interval following training (Waddell and Quinn, 2001). Our collaborators found that *beach1*^{17} and *beach1*^{58} had lower learning scores than the controls and, when *beach1* function was restored in neurons using a transgene, learning scores improved (Figure 9B). However, not all the learning data were consistent. When a transheterozygote for two truncation alleles was tested, its learning score was not statistically different from that of the control (Figure 9B). Thus, it is unclear whether the olfactory learning defect in *beach1* mutant strains is due to a lack of *beach1* function or to a background effect.

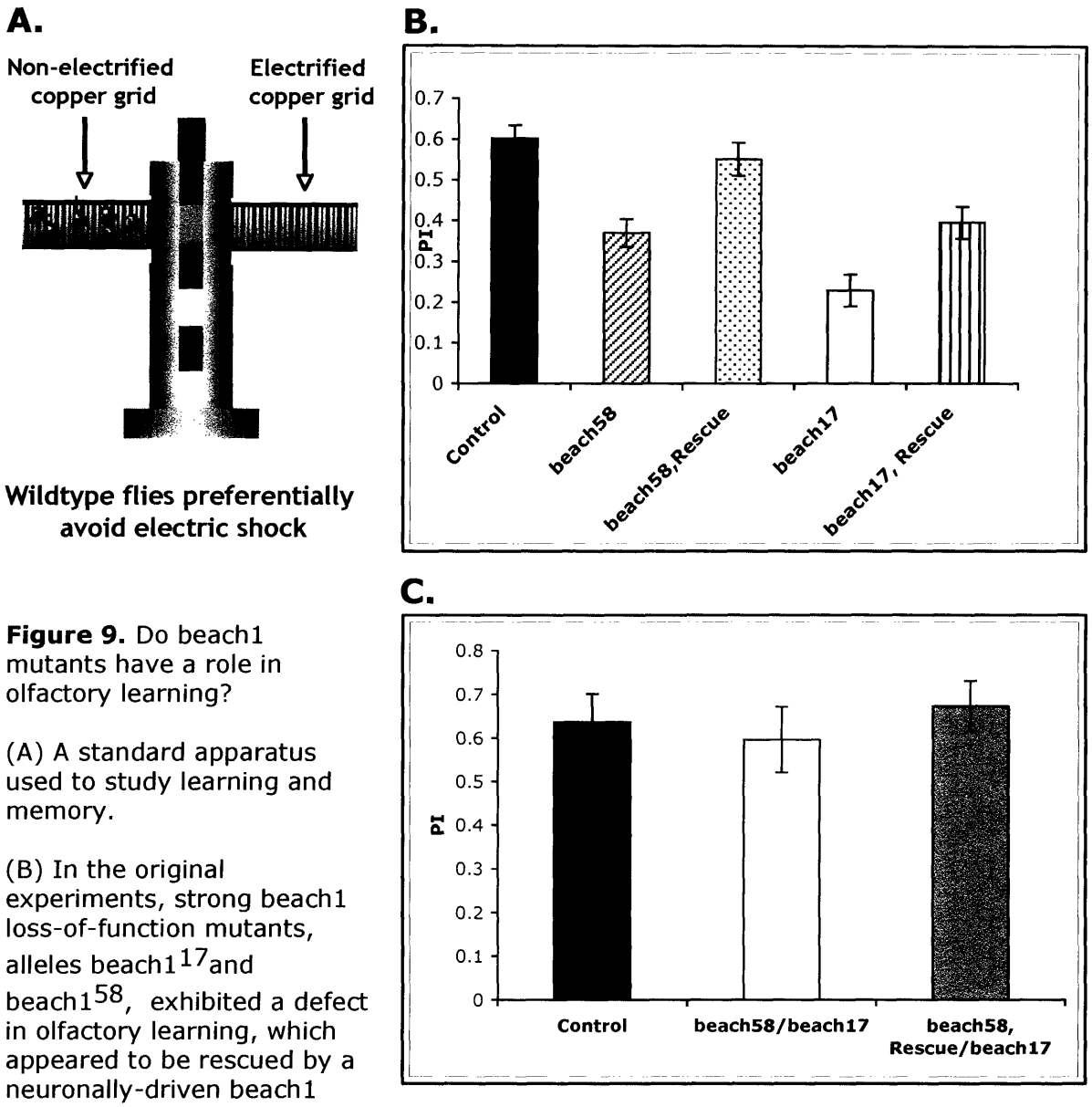


Figure 9. Do beach1 mutants have a role in olfactory learning?

(A) A standard apparatus used to study learning and memory.

(B) In the original experiments, strong beach1 loss-of-function mutants, alleles beach1^{17}and beach1^{58}, exhibited a defect in olfactory learning, which appeared to be rescued by a neuronally-driven beach1 rescue construct.

(C) A transheterozygote for strong loss-of-function alleles beach1^{17}and beach1^{58}, as well as the same mutant in combination with a neuronally-driven rescue construct, have learning scores statistically similar to those of the control strain.

$$\mathbf{PI}(\text{Performance Index}) = \frac{\#\text{ of flies avoiding shock} - \#\text{ of flies not avoiding shock}}{\text{total }\#\text{ of flies}} \times 100\%$$

Discussion

Subcellular mechanism behind the *beach1* overexpression phenotype

Photoreceptors in the animals that overexpress Beach1 protein have a strong defect in growth cone morphology: growth cones have a larger core or central area and are less well "spread out" compared to controls. Other *Drosophila* mutants have been reported to have defects in the photoreceptor growth cone morphology, such as mutants in *dreadlocks* (*dock*), which encodes an SH2-SH3 adaptor protein (Garrity et.al., 1996 and Rao and Zipurski, 1998) as well as mutants in the Ste20-like kinase *misshapen* (*msn*) (Su et al., 2000). Dock and Msn are believed to be part of a signaling pathway that translates extacellular cues, encountered by the growth cone, into changes in the cytoskeletal organization. In turn, these cytoskeletal rearrangements enable the axon to correctly navigate toward its target. Both the actin and the microtubule components of the cytoskeleton have been implicated in growth cone morphology and dynamics (Dent and Gertler, 2003). Thus, the *beach1* overexpression phenotype could be due to a misregulation of the actin or the microtubule cytoskeleton. However, the collapsed growth cone phenotype seen in *dock* mutants, as well as the phenotype seen in *msn* mutants, is quite different from the morphology seen in *beach1* overexpressors (Garrity et.al., 1996 and Rao and Zipurski, 1997). Specifically, *dock* and *msn* mutants do not exhibit what appear to be membrane bulges in the central areas of their growth cones, as are seen in *beach1* overexpressors. Therefore, it is unlikely that these phenotypes would be caused by very similar underlying defects in the cytoskeleton.

Membrane dynamics also play an important role in growth cone morphology and function. While cytoskeletal rearrangements regulate the direction and the rate of growth cone guided neurite extension, the availability

of membrane in the right place in the growth cone is necessary to make this extension and growth possible. Such neurite extension requires exocytosis of membrane precursors to the growth cone area (Pfenninger et.al., 2004 and Craig et al., 1995). Transmembrane receptors, such as Dscam and Frazzled, are thought to regulate growth cone dynamics by their effects on the downstream cytoskeletal regulators (Li and Guan, 2004 and Forsthoefel et.al., 2005). However, it is membrane recycling and the sorting of receptors to endosomes that determines the type and the number of receptors present on the growth cone plasma membrane (Myat et.al. 2002 and Keleman et.al., 2002). It has also been shown that endocytic recycling is involved in the formation of ruffles and lamellipodia in migrating cells (Bretscher and Aguado-Velasco, 1996 and 1998). Furthermore, endocytosis of extra membrane is important for growth cone collapse that occurs in response to negative guidance cues (Jurney et.al. 2002). Therefore, it is possible that the growth cone morphology defect seen in *beach1* overexpressors could be caused by misregulation of membrane dynamics, rather than misregulation of the cytoskeleton. Genetic interactions between *beach1* and the known vesicle trafficking regulator *rab11*, which will be discussed in the next chapter, suggest that this might, indeed, be the case.

Structure-function clues from *beach1* alleles

Assuming that the *beach1* overexpression phenotype is a consequence of its normal subcellular function, and not simply a neomorphic effect, some insight about the relationship between its structure and function can be gained from the characteristics of certain mutant alleles. However, most alleles that were isolated in the EMS mutagenesis screen are not informative for such structure-function analysis, because they are protein null. For example, early truncations encoded by $beach1^{17}$ and $beach1^{58}$ do not result in an overexpression of truncated protein, likely due to nonsense-mediated decay. On the other hand, allele $beach1^{5}$, which encodes a protein without a FYVE

domain, as well as allele *beach1*^{8}, which results in a Threonine to Isoleucine change in the interface between the PH and the BEACH domains, produce large amounts of overexpressed protein (Table 1). Allele *beach1*^{5} is a partial suppressor of the eye and growth cone defects, which suggests that the FYVE domain is not required for the generation of the overexpression phenotype. However, it is difficult to gauge whether the partial suppression is due to a lower level of overexpression of this truncated protein or because the FYVE domain contributes to the overexpression phenotype. Notably, the *Dictyostelium* and the *Arabidopsis* homologs of *beach1* do not have a FYVE domain. Furthermore, there is another predicted human homolog of Beach1, with a lower degree of similarity to Beach1 than Alfy, which does not have a FYVE domain. FYVE domains have been shown to bind to PtdIns(3)P--a phospholipid that is highly enriched in the membranes of early endosomes; however, one FYVE domain by itself is not sufficient to recruit a protein to such membranes (Misra et.al., 2001). Perhaps the FYVE domain of Beach1 facilitates, but is not required for its localization and, thus, its removal does not completely abrogate its function. Interestingly, protein encoded by allele *beach1*^{5} is still transported to the synaptic terminals at the neuromuscular junction, suggesting that the FYVE domain is not required for synaptic localization.

Protein encoded by allele *beach1*^{8} can be overexperessed at high levels; however, this allele suppresses the growth cone and the adult eye phenotypes completely. This allele carries a missense mutation, which changes a conserved Threonine to an Isolucene in the PH-BEACH domain interface. Threonine has a polar side chain, which is smaller than the hydrophobic side chain of Isoleucine; therefore, this substitution might disrupt the PH-BEACH interface. It has been previously demonstrated that the function of a BEACH family protein FAN is disrupted by single-site mutations in its PH-BEACH interface (Jogle et.al., 2002), suggesting that the interaction between these domains is required for the function of BEACH family proteins. My data,

demonstrating the suppression of the *beach1* gain-of-function phenotype by a mutation in the PH-BEACH interface, supports this hypothesis.

The learning phenotype--real or background?

Although the initial results of the olfactory learning experiments were promising, the data showing normal learning scores for the transheterozygote *beach1* allelic combination puts the earlier data into question. Although it is theoretically possible that a transheterozygous allelic combination would give a weaker phenotype than the individual homozygous mutants, this is usually the case when a protein in question works as a dimer or a multimer, and each allele has partial functionality that complements the other allele. In the case of $beach1^{17}$ and $beach1^{58}$ this explanation does not fit, since both alleles encode truncated proteins that are missing the C-terminal conserved domains. And, more importantly, neither of these alleles makes a detectable amount of protein (Figure 5B). Therefore, the transheterozygote combination would be expected to be as strong a loss-of-function mutant as the individual alleles. Thus, if the learning data for this allelic combination holds up, it would rule out *beach1* as the cause of the learning defect in *beach1* mutant strains. This defect might therefore be due to a background mutation or mutations in our stocks.

Shortened lifespan and neurodegeneration—Why the discrepancy?

According to published data, *beach1* mutants exhibit neurodegeneration and have a shortened lifespan (Finley et.al, 2003). However, I do not observe these phenotypes in our protein null alleles. It is difficult to explain the discrepancy between our findings and the published data. Most alleles described in the Finley paper are large deletions that remove not only the *beach1* locus, but also the adjacent *dissatisfaction* gene. Other alleles used in that paper are transposable element insertions on the CyO balancer chromosome, which, like all balancer chromosomes, has many inversions to

prevent recombination. These are less precise disruptions of the *beach1* gene than the point mutation alleles we generated. Perhaps decreased lifespan and neurodegeneration, seen by Finley et.al., result from mutation or mutations that lie outside of the *beach1* locus and are caused by a combination of a large deletion and inversions associated with a balancer chromosome. I examined animals from two of our protein null *beach1* alleles, which have been raised at 29°C for 26 days, a time point when almost all of the mutants in the Finley paper are dead, and still did not observe neurodegeneration. Therefore, I do not believe that neurodegeneration and decreased lifespan observed by Finley et.al. are due to a loss-of-function in the *beach1* gene.

Suggested involvement of Beach1 and Alfy in autophagy

Autophagy is the vacuolation of a portion of the cell's cytoplasm within a membrane and its subsequent digestion after fusion with a lysosome. It can be used by the cell to eliminate entire organelles in order to maintain the balance between organelle biogenesis and degradation. Autophagy is sometimes used for elimination of protein aggregates that are inefficiently degraded by the proteosome. It has been suggested that the human homolog of Beach1, autophagy-linked FYVE protein (Alfy), might target cytosolic protein aggregates for autophagic degradation (Simonsen et.al., 2004). However, all these claims are based on the staining pattern in HeLa cells, which the authors attribute to Alfy. Unfortunately, the specificity of this anti-Alfy antibody on tissue is never demonstrated, putting all the conclusions of this paper into question. The authors find that the anti-Alfy antibody detects an epitope in the nucleus, on the nuclear envelope and on autophagic membranes (Simonsen et.al., 2004). However, when I transfect a construct that expresses a full length YFP-tagged Beach1 protein into human HeLa cells, I observe a very different pattern of localization. Beach1 is excluded from the nucleus and does not localize to the nuclear membrane. Instead, it shows a punctate localization throughout the cytoplasm and is enriched at what

appears to be the leading edge of cell. This enrichment at the leading edge is consistent with the enrichment of Beach1 at the synapse, and with the growth cone overexpression phenotype in *Drosophila* photoreceptors. Of course, it is possible that the *Drosophila* protein is mislocalized in a heterologous cell, or that the fly and the human proteins have diverged enough to have very different localization patterns.

Strong gain-of-function in the absence of a loss-of-function phenotype

Beach1 has one of the strongest gain-of-function phenotypes we observed in the overexpression screen; however, we were unable to detect a loss-of-function phenotype in the strong *beach1* mutants that we generated. There could be several explanations for this result, for instance, Beach1 could be redundant with another protein in the fly genome. Although there are four other BEACH-WD40 proteins in the genome, none of them are significantly similar to Beach1 outside of the PH-BEACH-WD40 module. Beach1 is the only class II BEACH protein in the fly, and, according to the evidence from *Dictyostelium*, BEACH proteins in different classes are likely to have different cellular functions.

Another possibility is that the gain-of-function phenotype is due to a neomorphic effect caused by misexpression of *beach1* in a tissue where it is not normally present. This would explain why a loss-of-function in *beach1* shows no phenotype in the photoreceptor growth cones.

Yet another explanation could be that Beach1 is a negative regulator in a pathway that affects growth cone morphology. Such a pathway is likely to be subject to many levels of regulation, both positive and negative. Consequently, a removal of just one negative regulator, such as Beach1, might increase the activity of the pathway, but not enough to have an observable effect on the animal. On the contrary, its overexpression might override the influence of positive regulators, effectively shutting down the

pathway and causing a strong phenotype. Data that will be presented in the next chapter suggests that this may be the best explanation.

The overexpression screen

Our goal in doing the overexpression screen was to quickly identify candidate genes involved in the regulation of axon guidance and growth cone morphology. We hoped to use both the observed phenotypes and the knowledge about the identity of the candidate genes when deciding which of them to pursue. However, only six EP lines in our screen gave rise to strong phenotypes, and two of them had been previously characterized in our process of interest. The remaining three with strong axon guidance defects included a transcription factor, an acetyltransferase, and a gene that encoded a protein with no obvious domains: not exactly the type of molecules we were hoping to identify.

Among the rest of the candidates, which had weaker and less penetrant phenotypes, many belonged to classes of molecules that have been shown to play roles in axon guidance and growth cone morphology, such as protein kinases, transmembrane receptors, cytoskeletal and vesicle trafficking regulators, and molecules with roles in RNA localization. However, it was difficult to know, based on their phenotypes, which of these might be worth following up.

We chose to focus on *beach1*, a gene with a strong overexpression phenotype in the growth cones, which had interesting domains that pointed to a role in vesicle trafficking, a process known to be important for growth cone function. Since *beach1* is highly evolutionarily conserved and does not have a close homolog in flies, we reasoned that it would be likely to have a loss-of-function phenotype that we would be able to study. This did not turn out to be the case.

The pitfalls of overexpression screens

Since the time we had completed our gain-of-function screen in the photoreceptors, many similar screens were carried out in *Drosophila*, using the same EP collection created by Pernille Rorth. They were done in various cell types and aimed at identifying new candidate genes involved in a variety of processes. For example, one such screen was done in the adult external sensory organs to examine the processes of lateral inhibition, asymmetric cell division and cell cycle progression (Abdelilah-Seyfreid et.al., 2000). Another looked at axon guidance and synaptogenesis in the larval motor neurons, (Kraut et.al., 2001), another examined growth and cell cycle progression using the developing eye (Tseng and Hariharan, 2002), and yet another looked at stem cell development and maintenance in the *Drosophila* male germ-line cells (Schulz et.al., 2004). Each of these screens identified numerous candidate genes; however, almost no follow-up papers, describing the loss-of-function analysis of the candidates from any of these screens, have been published. Among the few candidates for which loss-of-function analysis was carried out by either RNAi or traditional mutant analysis, none had a phenotype in the process of interest (DiAntonio et.al., 2001; Tseng and Hariharan, 2002). From reviewing the outcomes of these screens, and from conversations with some of the scientists who have done them, I learned that the experiences of others have been similar to ours. Here I would like to discuss the pros and cons of these screens as compared to traditional loss-of-function screens.

An ode to loss-of-function screens

Traditional loss-of-function screens are not easy to do: they are time-consuming and often require complicated genetics. However, if designed and executed well, one can trust that most of the genes identified in such screens are involved in the process of interest and have loss-of-function phenotypes that are informative with respect to their roles in the process. Overexpression screens are much more simple to execute, but their results can be difficult to

interpret. Below is a discussion of some advantages that loss-of-functions screens have over their gain-of-function counterparts.

Things you don't know can't hurt you

When doing a loss-of-function screen, one need not have any prior knowledge about the genes that are involved in the process of interest. A screen allows one to get an answer to this question from the organism itself, without any biases or preconceived notions. In overexpression screens carried out using the Rorth method, the sites of EP insertions and, thus, the identities of candidate genes are known. Although this is usually cited as an advantage, I don't believe that it necessarily is. Such knowledge gives one the opportunity to use one's own bias when choosing a candidate gene to pursue, instead of focusing solely on the phenotype. When conducting a screen, it is important to be selective and to discard mutants with less than optimal phenotypes. This is much easier to do when one doesn't know that the line being discarded is a mutant in their favorite gene.

Go for the obvious

There is never enough time to study everything that comes out of a screen, so one must choose candidates using a very simple criterion: a good phenotype. One must choose those candidates with the strong, unambiguous, penetrant phenotypes and leave the rest behind. At the start of our screen we thought that we would be able to rely on the same principles when sorting through candidate genes, but this turned out to be more difficult than we thought. The majority of EP lines we identified had weak phenotypes and showed a low degree of penetrance. This was especially difficult to interpret, since even those genes with known loss-of-function phenotypes in the process had weak overexpression phenotypes. For example, in our screen overexpresson of *scribbler* (also known as *brakeless*), caused a weak, poorly penetrant phenotype; however, the loss-of-function in this gene is known to have a strong shoot-through phenotype (Rao et.al., 2000). Conversely,

overexpression of *beach1* causes a very strong defect in growth cone morphology, while no defect is observed in the null *beach1* mutants. Similarly, DiAntonio et.al. identified a deubiquitinating protease *fat facets* (*faf*) in an overexpression screen at the neuromuscular junction as having a strong synaptic overgrowth defect; however, loss-of-function mutants in *faf* did not exhibit defects in synaptic growth (DiAntonio et.al., 2001). Thus, it seems that it is often not possible to use the strength of the overexpression phenotype as a basis for candidate selection in gain-of-function screens.

Too much is not the opposite of too little

The beauty of loss-of-function screens is that one not only learns that a gene has a role in a certain process, but also gains some insight from the phenotype about what this role might be. When we began our screen, we hoped that we would similarly be able to interpret the overexpression phenotype, that too much expression of a gene would give a phenotype opposite of too little expression. However, in our screen and in other gain-of-function screens this was hardly ever the case. For instance, in the screen for genes involved in axon guidance and synaptogenesis in the motor neurons, the overexpression of *amnesiac*, a predicted neuropeptide, lead to the thinning of nerve branches, while loss-of-function in *amnesiac* is known to cause a reduction in the size of synapses. This was not the exception, but rather the rule, since the authors concluded that, overall, the overexpression phenotypes were not opposite of the known loss-of-function phenotypes (Kraut et.al.,2001). Furterhmore, in the screen for genes with roles in the development of the external sensory organ (the mechanosenory bristle), genes that are known cell cycle regulators, such as *string*, caused overexpression phenotypes that would have been predicted to result from a defect in the process of asymmetric cell division (Abdelilah-Seyfried et.al., 2000). Thus, it is difficult to infer from the overexpression phenotype what role a given gene might normally play in the process of interest.

You are at the right place, at the right time

When one finds that a given mutant has a strong loss-of-function phenotype in a cell type or a tissue, it usually means that this gene is likely to function in the affected cell. Of course, there are exceptions, since some defects in the surrounding tissues can cause non-cell-autonomous phenotypes. However, in overexpression screens, phenotypes caused by misexpression of genes in the wrong place or at the wrong time can be a serious caveat. Knowing that overexpression phenotypes can be non-physiological, it is worrisome that over 38 percent of inserts identified in our screen where also identified in other overexpression screens. While it would make sense that some of the genes we identified should also be picked up in the screen for axon guidance regulators in the motor neurons, the fact that more than one third of all genes that caused phenotypes in our screen also did so in screens looking at seemingly unrelated processes is cause for concern. Based on this finding, it appears that overexpression screens are likely to pick up genes that can non-specifically disrupt many different processes.

I don't want to imply that overexpression phenotypes are useless. They can be used to create a sensitized background, which can serve as a basis for an interactor screen. They can also be useful, as I will describe in the next chapter, to learn something about a gene of unknown function through a modifier screen. Overexpression phenotypes can sometimes be informative for understanding a function of a gene; however, it is usually best to know the loss-of-function phenotype before turning to overexpression. However, based on my experience, and on the results published (or not published) by others, it seems that gain-of-function screens are not a good approach to identifying new candidate genes involved in a process of interest. Sure, these screens are quicker to do than the traditional loss-of-function screens; however, at the end, you are not likely to find what you are looking for. As for the claim that one can identify genes with weak or no loss-of-function phenotype, well, you will get what you wished for, but you might later regret it...

Materials and Methods

Fly Stocks

EP lines, originally characterized by P. Rorth (Rorth, 1996), were obtained from the Rebay lab (Whitehead Institute, MIT). Df1128 (BL1128-Df(2L)GpdhA/CyO breakpoints 25D7-E1;26A8-9 and Df3365 (BL3365-Df(2L)cl7, pr[1] cn[1]/CyO, breakpoints 25E1-2;26A7) were obtained from the Bloomington stock center. W-cs strain, originally obtained from the Quinn Lab (MIT), was used to outcross all the EMS *beach1* alleles and subsequently used as a control in the immunohistochemistry, lifespan, and learning and memory experiments.

In situ hybridization

Template for the fly in situ probe was made by PCR from genomic fly DNA using primers

5'GAATTAATACGACTCACTATAGGGAGAGCACACAAAGTTCGATCTTGAC5' and 5'AATTAACCCTCACTAAAGGGAGA GTTCGCCTACAAGCACATCG3'. RNA probes were made using the DIG RNA labeling kit (Roche). Embryo and 3rd instar in situ hybridization were done according to standard protocol.

Mouse northern blot

Template for the mouse RNA probe corresponding to region 4737-5413 base pairs of the cDNA for Wdfy3 (BWF1) was made by PCR from total mouse cDNA (gift from the Gertler lab, MIT) (Primers used 5'T3CCTAAGCCTGTCGCCACTACTTTAC3' and 5'T7CCAAACTTCTTCTT CTGCTCCCG3'. The probe was synthesized using the Strip-EZ RNA (T7) kit from Ambion and a-P^{32}UTP 800ci/mMole from Amersham Biosciences, following the recommended protocol. The hybridization procedure was carried out as suggested in the ULTRAhyb manual from Ambion, using the Ultrasensitive hybridization buffer and NorthernMax High and Low Stringency

Wash Buffers. Millennium Marker Probe Template (Ambion) was labeled and used to detect RNA size markers according to suggested protocol.

EMS mutagenesis screen

Homozygous EP Beach1 3-day-old males were starved on water for 6 hours and then allowed to feed on a 30mM EMS, 1% sucrose solution overnight. These males were then crossed to virgins homozygous for the GMR Gal4 transgene. We screened through the resulting progeny for the suppression of the rough eye phenotype and crossed them to GMR Gal4 flies to check for transmission through the germline. Those lines that were found to transmit were balanced over a second chromosome balancer CyO and subsequently homozygosed. From 13,500 progeny of the original EMS-fed males, 17 independent suppressor of the adult eye phenotype were recovered.

Sequencing of the *beach1* locus

The *beach1* gene was amplified in 3 parts using the following sets of primers:

- 5'CAAACCCCACGGACATGC3' and 5'GCTGGTGTGGACTGACGCC3',
- 5'GCACGCTCCCTCCGTTCG3' and 5'CAAACTTGGAGCACTGCCTGAG3',
- 5'CAACCAGTTACAGGGTCGGAATC3' and 5'GCGCTGACCACTTTTGTAGTCTG3'.

Sequencing was done by the MGH DNA Sequencing Core Facility.

PCR to check deficiencies

Df1128(BL1128-Df(2L)GpdhA/CyO breakpoints (25D7-E1;26A8-9) and Df3365(BL3365-Df(2L)cl7, pr[1] cn[1]/CyO, breakpoints25E1-2;26A7) were put over the CyOKrGFP balancer. Embryos of stage 16 and 17 were sorted under the fluorescence microscope to separate those GFP negative (Df/Df) from GFP positive (Df/CyOKrGFP). PCR from individual embryos was done using two different primer sets: one covering the 1st intron and exon, the other in the middle of the region that encodes the PH domain: 5'CAACCAGTTACAGGGTCGG AATC3' and 5'GGTAGAAGACATCATTACGCATCG3'.

The control primers for the presence of DNA were directed against gp160-Dtrk:
5'GAATTAATACGACTCACTATAGGGAGAAGAAACTTAGCACCTGGAAGAAGG3' and 5'GAATTAATACGACTCACTATAGGGAGACGTGGGCTGAGGAA CAAACTTTAG3'.

Antibody production

DNA region corresponding to base pairs 6849-7908 of the *beach1* cDNA was amplified by PCR and cloned into the pET28a(+) bacterial expression vector behind a T7 promoter and a 6XHis tag. This construct was transfected into the Rosetta pLysS *E.Coli* strain for protein expression. The resulting protein was purified under denaturing conditions over a Nickel column (Ni Sepharose 6 Fast Flow from Amhersham Biosciences). The purified protein was sent to Covance Inc. for polyclonal antibody production in rats. Three animals were injected, numbered 3775, 3776, 3777. Animals 3775 and 3777 had higher antibody titers; therefore, crude antisera from these animals were used for western blotting and immunohistochemistry.

Immunohistochemistry

Fixation and staining of 3rd instar whole mounts was performed as previously described by Garrity et.al. 1999. For photoreceptor labeling anti-Chaoptin antibody 24B10 was used at 1:200 (Hybrydoma bank) and goat anti-mouse HRP-conjugated secondary (Jackson Laboratories) was used at 1:200. For the endogenous protein visualization, Beach1 antiserum (3777) was preabsorbed against *beach1* null animals and used at 1:500. Goat anti-rat Cy3 secondary (Jackson Laboratories) was used at 1:500. Polyclonal rabbit Syt1 antibody (generously provided by T. Littleton) was used at 1:500 and goat anti-rabbit FITC-conjugated secondary (Jackson Laboratories) was used at 1:200. Adult brains for the Eb Gal4 experiment were fixed and stained in the same manner as the 3rd instar whole mounts. Mouse Anti-HA (Covance) was used at 1:1,000 and goat anti-mouse Cy3 secondary (Jackson Laboratories)

was used at 1:1,000. Third instar and adult whole mount samples were visualized using a Nikon PCM2000 confocal microscope.

Third instar body wall dissections were done in PBS and fixed in 4% PFA in PBS. Beach1 antiserum (3775) was preabsorbed against *beach1* null animals and used at 1:500. Goat anti-rat Cy3 secondary (Jackson Laboratories) was used at 1:500. Cy5-conjugated anti-HRP was used at 1:100. Confocal data was acquired as single images or image stacks of multi-tracked, separate channels with a Zeiss LSM 510 microscope. Three-dimensional projections of image stacks were made with the 3D Zeiss software package.

Western blotting

Each lane of a 6% SDS-polyacrylamide gel was loaded with a homogenate of 9 adult heads ground in 1X Laemmli buffer in PBS (130 mM NaCl, 175mM Na2HPO4, 60 mM NaH2PO4). The gel was run, then transferred to Hybond-P membrane (Amersham Pharmacia). Membranes were blocked in 5% nonfat milk and probed with anti-Beach1 antisera diluted 1:1,000 and a rat monoclonal antibody against Elav diluted at 1:1,000, followed by an HRP-conjugated goat anti-rat secondary antibody (Jackson Laboratories) diluted at 1:5,000. The anti-Elav antibody served as a control for equal amounts of material loaded into each lane.

cDNA cloning and transgene constructs

A full-length cDNA was assembled using PCR (Expand High Fidelity PCR System, Roche) from the existing partial cDNA (clone LD02084) and from S2 cell RT PCR reaction (S2 cell RNA was generously provided by the Pardue Lab (MIT) and RT reaction was done using the RETROscript First Strand Synthesis Kit for RT-PCR, Ambion). The following primers were used:

- 5'CGGGATCCATGAATGTAATGCGTAAGCTGCG3',
- 5'CGGAATTCGCCACCAAGGACTTGATGATTTCG3',
- 5'CGGAATTCTGCTTCGCACCACGCAGGTC3',
- 5'CGGGATCCCGAGCGGACAACAAAAGCATTG3',
- 5'ACGCGTCGACCAGATTCCGACCCTGTAACTGG3',

- 5'GCAACCACGAGTTGGAATTCATTGGC3', and
- 5'ATTTGCGGCCGCCCTAATTGTCCAACGAGTTCGTGC3'.

All segments, generated by PCR, were sequenced prior to being assembled together in the pcDNA6/V5-His vector modified with a 5' HA or a FLAG tag (Invitrogen). Sequencing was done by the MGH DNA Sequencing Core Facility.

Full-length *beach1* cDNA, tagged with HA, was cloned into the pUASt vector (Brand and Perrimon, 1993) for the ellipsoid body localization experiment and the FLAG-tagged full-length cDNA was cloned into pElav vector for rescue in the olfactory learning experiment.

Lifespan experiment and Plastic head sections

For lifespan experiments flies were monitored for survival and transferred to fresh food every 1-2 days.

Flies used for head sectioning were raised at 29C for 26 days. In preparation for plastic section, adult heads, with the mouthparts removed, were fixed in 4% Paraformaldehyde and 2.5% Glutaraldehyde in 0.1M Cacodylate buffer, pH7.4 for 2 hrs and postfixed in 2% OsO_4 in 0.1M Cacodylate buffer at 4C overnight. Heads were washed twice in 0.1M Cacodylate buffer and dehydrated in a graded alcohol series. Heads were then transferred to Propylene Oxide, then, in a progressive series transferred to the resin mixture (50% Epon 812, 29% NMA, 19% DDSA and 2% DMP-30, all reagents from Ted Pella) for embedding. Heads were oriented prior to polymerization, which was done at 65C for 48 hrs. Semithick sections (1.0 μm) were collected using slotted grids and placed onto glass slides on a hotplate. Sections were stained with 1% toluidine blue and 1%borax on a hotplate, then rinsed with distilled water.

Olfactory learning assays

Olfactory learning assays were done by a graduate student A. Keene in the Waddell Lab (UMass, Worcester) as previously described (Waddell et.al., 2000).

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Supplementary Materials to Chapter 2

Table S1: Known regulators of axon guidance and growth cone morphology

EP number (chrom)	Phenotype	Gene of Insertion & Orientation	Map Position	Gene Description	Other OE screens
365(2)	breaks in lamina	in frazzled (fra)	49B2-3	Netrin receptor involved in axon guidance	
2137(2), 2396(2)	shoot through (strong)	in intron of longitudinals lacking (lola)	47A11-13	Transcription factor involved in axon guidance in the embryo	Abdelilah-Seyfried et al., 2000
2225(2)*	uneven lamina	in 5'UTR of enabled (ena), +/-	56B5--C1	Actin binding cytoskeletal regulator, involved in axon guidance	
2461(2)	uneven lamina	in scribbler (sbb), +/+	55C2	Transcription factor involved in photoreceptor axon guidance	Kraut et.al., 2001
609(3)*	short stop (strong)	1st intron/5'UTR of misshapen (msn),+/+	62E6-7	Ste-20 serine/threonine kinase, involved in photoreceptor axon guidance and growth cone morphology	Huang and Rubin, 2002, Kraut et.al., 2001
3415(3)*	abnormal	in pebble (pbl)	66A18-19	RhoGEF involved in actin reorganization. Axon guidance phenotypes and eye phenotypes	Abdelilah-Seyfried et.al., 2000, Kraut et.al., 2001, Tseng and Hariharan, 2000
3420(3)*	uneven lamina w/holes, few thick bundles	5' UTR of capricious (caps),+/+	70A3-4	Transmembrane cell adhesion molecule involved axon guidance and synapse formation	
3508(3)	abnormal	1st intron of gliolectin (glec),+/+	93F14	Intergral membrane carbohydrate binding molecule involved in axon guidance in the embryo	Abdelilah-Seyfried et.al., 2000, Pena-Rangel et.al., 2002

Table S2A: Protein Modification - Kinases and Their Regulators

EP number (chrom)	Phenotype	Gene of Insertion & Orientation	Map Position	Gene Description	Other OE screens
921(2)	abnormal	in 1st intron/5'UTR of grapes (grp), +/+	36A10	Serine/threonine kinase, involved in DNA damage checkpoint	Abdelilah-Seyfried et.al., 2000
2081(2)	abnormal angle of projection	upstream of CG30492,+/-	43E4	Protein of unknown function with Protein kinase-like, FYVE/PHD zinc finger domains	
2254(2)	abnormal	upstream of A kinase anchor protein 200 (Akap200), +/+	29C3-4	Protein kinase A binding, involved in negative regulation of Ras protein signal transduction	Huang and Rubin, 2000
2445(2)	abnormal	downstream of CG7097, +/-, antisense?	56C4	Serine/threonine kinase	
609(3)*	short stop (strong)	1st intron/5'UTR of misshapen (msn),+/+	62E6-7	Ste-20 serine/threonine kinase, involved in photoreceptor axon guidance and growth cone morphology	Huang and Rubin, 2000, Kraut et.al., 2001
3656(3)	abnormal	in Ecdysone-induced protein 63E (Eip63E) , +/-	63E3--5	Serine/threonine kinase	
3657(3)	abnormal	in 5'UTR of JIL-1, +/+	68A5--6	Serine/threonine kinase	Kraut et al., 2001

Table S2B: Protein Modification - Ubiquitination

EP number (chrom)	Phenotype	Gene of Insertion & Orientation	Map Position	Gene Description	Other OE screens
1184(2)	abnormal	in 3' of morgue, +/+	24F2	Ubiquitin conjugating enzyme involved in apoptosis	
2063(2)*	abnormal	upstream of Nedd8, +/+	37B7	Involved in ubiquitin-dependent protein catabolism	
2077(2)	abnormal	upstream of lemming (lmg), +/+	29D4-5	Ubiquitin-protein ligase	Kraut et.al., 2001
381(3)*	uneven lamina	1st intron/5'UTR of fat facets (faf),+/+	100D2	Ubiquitin protease, rough eye (lof) and nmj defects (gof)	Abdelilah-Seyfried et.al., 2000, Kraut et.al., 2001,Pena-Rangel et.al., 2002, Tseng and Hariharan, 2002
414(3)	thick bundle in the middle	upstream of CG5555,+/+	91F10-11	Ubiquitin-protein ligase	
662(3)	abnormal	in 1st exon/5'UTR of supernumerary limbs(slmb),+/+	93B13-C1	Ubiquitin-protein ligase involved in regulation of frizzled signaling pathway, eye clones don't differentiate	

Table S2C: Protein Modification - Other

EP number (chrom)	Phenotype	Gene of Insertion & Orientation	Map Position	Gene Description	Other OE screens
2019(2)*	messy lamina	in 5'UTR of Fkbp13,+/+	57E6-8	FK506 binding putatively involved in protein folding (ER)	
2393(2)	uneven lamina	Upstream of Ribosomal protein L18A (RpL18A),+/+	54C3-7	Structural constituent of ribosome, involved in protein biosynthesis	
2421(2)	abnormal	in Tim10	57F8	Protein translocase involved in protein-mitochondrial targeting	
1141(3)*	abnormal	upsteam of slamdance (sda),+/+	97D7-9	Membrane alanyl aminopeptidase, putatively involved in proteolysis and peptidolysis (bang sensitive)	

Table S3: Trafficking and Cytoskeleton

EP number (chrom)	Phenotype	Gene of Insertion & Orientation	Map Position	Gene Description	Other OE screens
1357(X)	uneven lamina	in 1st intron/5'UTR of RhoGAPp190, +/+	16B10	RhoGAP involved in mushroom body development	
2043(2)	uneven lamina	in lissencephaly-1 (lis-1)	52F4-5	Dynein binding, putatively a component of the dynein complex, required for axonal transport	
2080(2)	abnormal lamina	upstream of GDP dissociation inhibitor (GDI),+/+	30B8	GDP-dissociation inhibitor, involved in vesicle-mediated trafficking via Rabs	
2225(2)*	uneven lamina	in 5'UTR of enabled (ena), +/-	56B5--C1	Actin binding cytoskeletal regulator, involved in axon guidance	
2299(2)*	abnormal growth cones (strong)	in 1st intron/5'UTR of Beach1,+/+	26A1	BEACH, WD40,FYVE protein, putatively involved in vesicle trafficking	Abdelilah-Seyfried et.al., 2000, Kraut et.al., 2001
2363(2)*	uneven lamina with holes	in 1st exon/5'UTR of spinster,+/+	52E6-9	Late endosomal membrane protein implicated in TGF-β-mediated synaptic growth regulation	
2425(2)	abnormal	upstream of Tetraspanin 42Ej (Tsp42Ej), +/-	42E5	Lysosomal transmembrane protein tetraspanin, putatively involved in the light-induced degradation of rhodopsin	
877(3)	abnormal	in 1st inron/5'UTR of Syndapin (synd), +/+	93A1-2	Structural constituent of cytoskeleton involved in synaptic vesicle endocytosis	
3104(3)*	abnormal	Upstream of klarsicht (klar), +/+	61C3-4	Atpase, component of the dyenin complex, involved in nuclear migration (lof rough eye)	Abdelilah-Seyfried et.al., 2000
3415(3)*	abnormal	in pebble (pbl)	66A18-19	RhoGEF involved in actin reorganization. Axon guidance phenotypes and eye phenotypes	Abdelilah-Seyfried et.al., 2000, Kraut et.al., 2001, Tseng and Hariharan, 2002

Table S4: Receptors and Other Molecules Involved in Signaling

EP number (chrom)	Phenotype	Gene of Insertion & Orientation	Map Position	Gene Description	Other OE screens
365(2)	breaks in lamina	in frazzled (fra)	49B2-3	Netrin receptor involved in axon guidance	
2023(2)	shoot through & uneven lamina	upstream of rhomboid-6 (rho-6),+/+	33D2	Receptor binding putatively involved in signaling	
2125(2)	abnormal	1st exon/5'UTR of Misexpression suppressor of KSR 2 (MESK2), +/-	57E8-9	Interacts genetically with Ras85D , ksr and peb	Huang and Rubin, 2000
2201(2)	uneven lamina	in 1st intron/ 5'UTR of spitz(spi), +/-	37F2	Transmembrane ligand of EGFR, lof mutants have photoreceptor phenotypes	Kraut et.al., 2001
2232(2)*	uneven and abnormal angle of projection	in decapentaplegic (dpp), likely +/+	22F1--3	TGFb ligand (extracellular)	Tseng and Hariharan, 2002, Schulz et.al., 2004, Rorth et.al., 1998
2254(2)	abnormal	upstream of A kinase anchor protein 200 (Akap200), +/+	29C3-4	Protein kinase A binding, involved in negative regulation of Ras protein signal transduction	Huang and Rubin, 2000
2363(2)*	uneven lamina with holes	in 1st exon/5'UTR of spinster,+/+	52E6-9	Late endosomal membrane protein implicated in TGF-β-mediated synaptic growth regulation	
2378(2)	crossed axons and holes	middle of spitz (spi),+/-	37F2	Binds to and activates signaling from the EGFR	Kraut et.al., 2001

EP number (chrom)	Phenotype	Gene of Insertion & Orientation	Map Position	Gene Description	Other OE screens
2395(2)	abnormal	upstream of crossveinless 2 (cv-2),+/+	57D12-13	Putatively involved in the BMP-like signaling pathway, genetically interacts with dpp	
2425(2)	abnormal	upstream of Tetraspanin 42Ej (Tsp42Ej), +/-	42E5	Lysosomal transmembrane protein tetraspanin, putatively involved in the light-induced degradation of rhodopsin	
496(3)	shoot through & uneven lamina	upstream of trapped in endoderm-1 (tre1),+/+	5A11-12	Taste receptor (G protein coupled R), involved in perception of sweet taste	

Table S5A: Transcriptional Regulation (Transcription Factors)

EP number (chrom)	Phenotype	Gene of Insertion & Orientation	Map Position	Gene Description	Other OE screens
1335(X)*, 1390(X)*	uneven lamina	in 1st intron of NFAT, +/+	12A9-B2	Transcription factor	Kraut et.al., 2001, Tseng and Hariharan, 2002, Abdelilah-Seyfried et.al., 2000, Pena-Rangel et.al., 2002
1342(X)*	slightly uneven	in 1st intron/5'UTR of CHES-1-like, +/+	7B6	Transcription factor, Checkpoint suppressor homologue	
1425(X)	abnormal	Upstream of CrebB-17A,+/+	17A	Cyclic-AMP response element binding protein B at 17A, overexpression might block long term memory	
488(2)	clumps in lamina	in bunched (bun),+/+	33E5-9	Transcription factor, R cells misproject due to incomplete medulla rotation, R cells missing	
633(2)*	abnormal	upstream of escargot (esg), +/-	35D2	Transcription factor	
2137(2)	shoot through	in intron of longitudinals lacking (lola)	47A11-13	Transcription factor involved in axon guidance in the embryo	Abdelilah-Seyfried et al., 2000
2169(2)*	uneven lamina and rough eye	1st intron/5'UTR of lola like (lolal) +/-	55B9	Transcription factor, bristle phenotypes	Abdelilah-Seyfried et.al., 2000, Kraut et.al., 2001, Tseng and Hariharan, 2002
2173(2)*	uneven lamina	upstream of no ocelli (noc), +/-	35B2	Transcription factor activity (ocelli and bristle defects)	
2361(2)	abnormal	near CG6751	47C5	Transcription factor	

EP number (chrom)	Phenotype	Gene of Insertion & Orientation	Map Position	Gene Description	Other OE screens
2396(2)	abnormal	in 1st intron/5'UTR of lola, +/-	47A11-13	Transcription factor involved in axon guidance in the embryo	Abdelilah-Seyfried et al., 2000
2408(2)*	shoot through (strong)	in escargot (esg), likely+/+	35D2	Transcription factor	Abdelilah-Seyfried et.al., 2000, Pena-Rangel et.al., 2002, Tseng and Hariharan, 2002
2461(2)	uneven lamina	in scribbler (sbb), +/+	55C2	Transcription factor involved in photoreceptor axon guidance	Kraut et.al., 2001
3098(3)	abnormal	in Ecdysone-induced protein 78C, +/-	78C2-3	Transcription factor involved in larval and pupal development	Tseng and Hariharan, 2002

Table S5B: Transcriptional Regulation (Chromatin Structure)

EP number (chrom)	Phenotype	Gene of Insertion & Orientation	Map Position	Gene Description	Other OE screens
2371(2)	abnormal	Upstream of domino(dom),+/+	57D11-12	DNA-dependent ATPase, a component of the chromatin remodeling complex, involved in gene silencing	
2374(2)	abnormal	Upsteram of pipsqueak (psq), +/+	47A11-13	DNA binding protein involved in gene silencing	
3541(3)	uneven lamina and rough eye	in 5'UTR of trithorax (trx), +/+	88B1	Contributes_to histone lysine N-methyltransferase activity, lof mutants have defects in photoreceptor differentiation	Pena-Rangel et al., 2002
3641(3)	uneven lamina	upstream of Histone H2A variant (His2Av), +/+	97D3	Histone (overexpression causes rough eye)	

Table S6: Basic Metabolism and Cell Homeostasis

EP number (chrom)	Phenotype	Gene of Insertion & Orientation	Map Position	Gene Description	Other OE screens
1235(X)*	abnormal	upstream of raspberry (ras), +/+	9E1-2	IMP dehydrogenase involved in GMP biosynthesis	Pena-Rangel et.al., 2002
1300(X)*	uneven lamina	upstream of CG8062,+/+	18C2	Monocarboxylic acid transporter, integral membrane protein	
600(2)*	shoot through (strong)	near v(2)k05816	23C5	S-acetyltransferase, putatively involved in fatty acid biosynthesis	Abdelilah-Seyfried et.al., 2000
670(2)	abnormal	in Glutathione S transferase S1 (GstS1)	53F7-8	Glutathione transferase activity, involved in response to oxidative stress	Pena-Rangel et.al., 2002
757(2)	abnormal	in 2nd exon/5'UTR of CG8446,+/+	52F7-8	Lipoyltransferase, putatively involved in coenzyme metabolism	
1061(2)	messy	Upstream of SRY interacting protein 1 (Sip1), +/-	54B6-7	Sodium:hydrogen antiporter regulator, putatively involved in cell homeostasis	
2021(2)*	uneven lamina	in Eip55E, +/+	55E2	Cystathionine gamma-lyase involved in sulfur amino acid metabolism	
2172(2)	uneven	near NTPase	23C1	Nucleoside-diphosphatase, involved in nucleotide metabolism	
2177(2)*	abnormal	in1st intron/5'UTR of l(2)k01209, +/-	54B10-11	Uridine kinase,putatively involved in pyrimidine base metabolism	

EP number (chrom)	Phenotype	Gene of Insertion & Orientation	Map Position	Gene Description	Other OE screens
2360(2)	abnormal	Upstream of retinal degeneration B (rdgB), +/+	54D6	Phosphatidylinositol transporter, involved in phosphoinositide metabolism and polyphosphoinositide synthesis (R cell degeneration)	
2482(2)*	messy lamina and bolwig's defect	near CG17646, likely +/+	22B1-2	Transporter activity, putatively involved in anion transport	Schulz et.al., 2004
720(3)*	abnormal	in CG4825	77C6-7	CDP-diacylglycerol-serine O-phosphatidyltransferase, putatively involved in lipid metabolism	
857(3)*	abnormal	upstream of alpha-Man-IIb,+/+	89A5	Alpha-mannosidase activity, putatively involved in carbohydrate metabolism	
1066(3)	abnormal	near CG8036	85A5	Transketolase, putatively involved in pentose-phosphate shunt	

Table S7: RNA Localization & Processing, DNA Replication

EP number (chrom)	Phenotype	Gene of Insertion & Orientation	Map Position	Gene Description	Other OE screens
1526(X)	abnormal	downstream of pac-man (pcm),+/-	18C7	5'-3' exoribonuclease, involved in nonsense-mediated mRNA decay	
372(2)	abnormal	upstream of csl4, +/+	32D4	3'-5'-exoribonuclease involved in mRNA processing	
2132(2)	abnormal	1st exon(or intron)/ 5'UTR of Nup44A +/+	44A2	Structural constituent of the nuclear pore, putatively involved in RNA local-ization	
2493(2)*	uneven lam-ina	in quaking related 58E-3 (qkr58E-3)	58D7-8	RNA binding, involved in apoptosis	
3109(3)	abnormal	1st intron/5'UTR of CG11486,+/+	62E3-4	Protein of unknown function, with pu-tative exoribonuclease activity puta-tively involved in RNA elongation	Kraut et.al., 2001
2060(2)	abnormal lamina and eye disk	in double parked (dup)	51F11	DNA binding involved, in DNA repli-cation	

Table S8: Proteins of Unknown Function

EP number (chrom)	Phenotype	Gene of Insertion & Orientation	Map Position	Gene Description	Other OE screens
437(2)	abnormal direction of projection	upstream of CG30332, +/+	55E1-2	Unknown function	
618(2)	abnormal	in 1st intron/ 5'UTR of CG9047,+/+	60E5	Protein of unknown function	
755(2)	abnormal	in CG30424, +/+	60E11	Unknown function	
2081(2)	abnormal direction of projection	upstream of CG30492,+/-	43E4	Protein of unknown function with Protein kinase-like, FYVE/PHD zinc finger domains	
2419(2)	abnormal	upstream of CG13790, +/+	28B1-2	Unknown function	Pena-Rangel et.al., 2002, Tseng and Hariharan, 2002
3109(3)	abnormal	1st intron/5'UTR of CG11486,+/+	62E3-4	Protein of unknown function, with putative exoribonuclease activity putatively involved in RNA elongation	Kraut et.al., 2001
3523(3)	uneven lamina	l(3)L0539	67E1-E2	Unknown function	Pena-Rangel et al., 2002
3648(3)*	shoot through (strong)	in CG33523, +/+	64F5--65A1	Unknown function	
413(3)	messy lamina and thick bundles	in CG9986,+/+	98D6	Unknown function	
3690(3)	uneven lamina & thick bundles	in 5'UTR of CG8121, +/+	85D11	Unknown function	Pena-Rangel et.al., 2002 and Schulz et al., 2004

Table S9: No Linked Gene

EP number (chrom)	Phenotype	Gene of Insertion & Orientation	Map Position	Gene Description	Other OE screens
1523(X)	abnormal direction of projection	no linked gene	7B1-2		Abdelilah-Seyfried et.al., 2000
566(2)	abnormal	no linked gene	22F1-4		Pena-Rangel et.al., 2002, Schulz et.al., 2004
2414(2)	abnormal	no linked gene	?		
2496(2)	uneven lamina and abnormal eye disk	no linked gene	?	Unknown function	
2520(2)	abnormal	no linked gene	37F2		
2540(2)	holes in lamina, axon crossing	no linked gene	37A1		
417(3)	shoot through	no linked gene	?		Pena-Rangel et al., 2002
3005(3)	abnormal	no linked gene	100D2		
3064(3)	abnormal	no linked gene	89A2		
3088(3)	abnormal direction of projection	no linked gene	98B2		Abdelilah-Seyfried et.al., 2000

Chapter 3

Beach1 Functionally Antagonizes Rab11

Some of the work described in this chapter, specifically, all the experiments at the neuromuscular junction and the majority of viability and bristle studies, were done in the Schwarz Laboratory at Harvard Medical School (Children's Hospital) and funded, in part, by the Harvard MD/PhD program.

Introduction

Beach1, a member of an evolutionarily conserved BEACH family of proteins with putative roles in vesicle trafficking, was identified in a gain-of-function screen in the *Drosophila* eye. When overexpressed in the photoreceptors, Beach1 causes an alteration in their growth cone morphology: growth cones have a large, blebby central area and are less well expanded than their wildtype counterparts. The nature of these defects suggests that Beach1 might be involved either in the organization of the cytoskeleton or in membrane trafficking in nerve terminals. However, our attempts at addressing this hypothesis by examining *beach1* loss-of-function mutants were not informative, since they have no obvious phenotype. Therefore, to try to gain insight into its function, we carried out a screen for modifiers of the *beach1* overexpression phenotype in the adult eye, in which we identified *rab11*. Rab11 is a member of a large family of monomeric GTPases that are known to regulate all aspects of membrane trafficking.

Rab11 has been studied in several experimental systems, including *C.elegans*, *Drosophila*, and mammalian tissue culture. It has been found to play a role in many different cellular processes, from cytokinesis in *C.elegans* to the insulin-mediated glucose uptake in mammalian cardiac muscle cells (Skop et.al., 2001; Muller et.al., 2002, respectively). Furthermore, Rab11 has been shown to function in multiple membrane trafficking pathways, such as the exocytic biosynthetic pathway and the recycling pathway (Satoh et.al., 2005; Ren et.al., 1998). In *Drosophila*, a requirement for *rab11* has been demonstrated during embryonic cellularization, polarization of the oocyte, and the morphogenesis of the rhabdomere--a photosensing organelle of photoreceptors (Riggs, et.al., 2003; Dollar et.al., 2001; Satoh et.al., 2005). *Rab11* is an essential gene in *Drosophila*; however, some hypomorphic allelic combinations survive and exhibit easily observable external phenotypes, such as bristle loss. I was able to take advantage of these phenotypes to show that

loss-of-function in *beach1* suppresses *rab11* lethality and bristle defects. This discovery of an interaction between Beach1 and Rab11 is the first clue to a mechanism by which BEACH proteins could be involved in membrane trafficking.

Although it was clear from these studies that *rab11* and *beach1* interacted during *Drosophila* development, I was still interested in finding out whether *beach1* was involved in the regulation of nerve terminal morphology, as the overexpression phenotype suggested. Thus, guided by the enrichment of Beach1 at the neuromuscular junction, I employed this experimental system to see whether its overexpression phenotype could also be tied to its interaction with Rab11.

The *Drosophila* neuromuscular junction (NMJ) is a convenient system for studying multiple aspects of nervous system development and function, because it is amenable to powerful genetic analysis and offers anatomical and electrophysiological accessibility. The NMJ has particular advantages for studying synaptic growth and plasticity, a process by which connections between a neuron and its target are modified. The *Drosophila* neuromuscular junction, unlike the static NMJs of mammals, is dynamic: as the larva develops, it sprouts new branches and boutons—varicosities at the sites of which the nerve synapses onto the muscle. During only four days of larval growth, the postsynaptic muscle surface area increases in size about a hundred fold and, to keep up with this expansion, there is about a ten-fold increase in the number of boutons. This process of synaptic growth is precisely regulated, resulting in the number and organization of boutons and branches that is consistent from animal to animal (Zito et.al., 1999). Therefore, defects in this stereotyped synaptic morphology can be easily detected and studied using all the incredible tools available at the NMJ.

Upon examination of the NMJs of hypomorphic *rab11* mutants, I found that they exhibited a strong synaptic overgrowth phenotype, with an increase

in the number and branching of boutons. Both aspects of this synaptic phenotype were significantly suppressed in *beach1*, *rab11* double mutants, demonstrating that *beach1* interacts with *rab11* during synaptic growth. This loss-of-function interaction between *beach1* and *rab11* at the synapse reveals a role for *beach1* in the regulation of nerve terminal morphology, as was suspected from its overexpression phenotype in the photoreceptor growth cones. Therefore, it is likely that overexpressed Beach1 protein exerts its effect on growth cone morphology by antagonizing Rab11 function.

Results

Rab11 was identified in a screen for modifiers of *beach1* overexpression

I was not able to detect a loss-of-function phenotype in *beach1* mutants; therefore, to help me gain insight into the function of this gene, I screened the *Drosophila* deficiency collection for modifiers of the *beach1* overexpression phenotype. To make the screen easier to carry out, I used the external phenotype of the reduced, glazed adult eye, which results from the overexpression of *beach1* in the photoreceptors. After screening through a collection of 200 deficiencies that covered upwards of 80% of the genome, I identified 13 deficiencies, which showed significant modification of the adult eye phenotype: 5 suppressors and 8 enhancers (Table 1). After screening through all the available reagents within each deficiency region, I was able to narrow the interaction down in the Deficiency 3340 interval to one gene--*rab11*. Decreased dosage of *rab11* strongly enhances the *beach1* eye overexpression phenotype (Figure 1A). Such enhancement was observed with every available *rab11* allele, suggesting that this interaction is specific to *rab11*. Furthermore, none of the *rab11* mutants had a rough eye independently of the *beach1* overexpression, demonstrating that the enhancement of the eye phenotype was not simply due to an additive effect.

Table 1: Deficiencies that Modify beach1 overexpression

Deficiency number (chromosome affected)	Cytologic interval	Eye modification
993 (X)	14C5-15B1	strong suppressor
970 (X)	17A1-18B1	strong suppressor
3133 (II)	22A-22E	suppressor
198 (II)	43F-44D	suppressor
3591 (II)	44F10-45E1	strong enhancer
442 (II)	49C1-50D1	strong enhancer
757 (II)	55E6-56C1	strong enhancer
3650 (III)	62F-63D	strong enhancer
3617 (III)	76B1-76D5	strong suppressor
4430 (III)	78C5-79A1	strong enhancer
3003 (III)	86E2-87C7	strong enhancer
3340 (III)	93B6-93D4	strong enhancer
2585 (III)	95A5-95D11	strong suppressor

rop is the only other gene found to modify the *beach1* gain-of-function phenotype

To try to gauge the specificity of the *beach1-rab11* interaction, I tested several other candidate genes for the ability to modify the *beach1* eye overexpression phenotype. Since *rab11* is a known regulator of membrane trafficking, I tested other genes that are involved in various aspects of vesicle trafficking, such as *shibire*, the *Drosophila* homolog of dynamin, which is required for vesicle fission and endocytosis, *rab5*—a GTPase that regulates trafficking to and from the early endosome, *hrs*—a late endosomal protein that plays a role in the degradation of the activated EGF receptor, and *syntaxin*, a target SNARE, which directly mediates vesicle fusion. I found that none of these genes modified the *beach1* eye phenotype. Because Beach1 protein is enriched at the synapse, I also tested *synaptotagmin*, a regulator of Calcium-dependent neurotransmitter release, but did not observe an interaction with *beach1*.

Since the cytoskeleton plays an important role in the regulation of growth cone morphology and function, I tested several known regulators of the actin cytoskeleton, such as *capping protein beta*, which caps actin filaments at their barbed ends, *chickadee*—the *Drosophila* homolog of profilin, which promotes actin polymerization, and alleles of the Rho family GTPases (Rho, Rac and Cdc42), the key regulators of actin cytoskeleton dynamics. In addition, I tested mutants in the *kinesin heavy chain*—a component of the microtubule-dependent motor kinesin, and found that neither the regulators of the actin nor the microtubule cytoskeleton caused modification of the *beach1* gain-of-function.

Finally, to assess the specificity of the *beach1* interaction with *rab11* among other rabs, I tested other available *rab* mutants, including *rab1, rab3, rab5, rab6, and rab14*. A reduction in the dosage of *rabs*, other than *rab11*, did not modify the *beach1* eye phenotype.

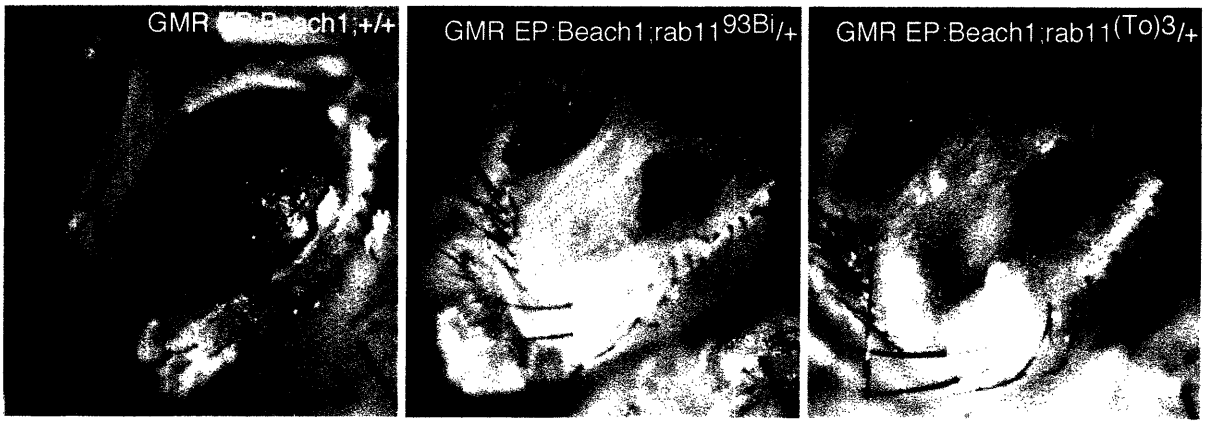
In fact, *rop*, the *Drosophila* homolog of yeast *sec1* and mammalian *munc18*, was the only other modifier of *beach1* overexpression that I was able to identify (Figure 1B). Rop is thought to play a role in vesicle docking during exocytosis by holding Syntaxin in a non-interacting state until it is appropriate to proceed with membrane fusion. However, there is still some controversy about its exact mechanism of function (Toonen and Verhage, 2003). *Rop* is not as strong an enhancer of *beach1* overexpression as *rab11*: only the null allele or a combination of two hypomorphic alleles is capable of modifying the eye (Figure 1B). Interestingly, *rop* mutants have a similar phenotype to *rab11* mutants in the localization of posterior determinants, such as oskar mRNA, to the posterior pole of the oocyte (Ruden et.al., 2000 and Jankovics et.al., 2001).

Figure 1. Reduced Dosage of *rab11* or *rop* Enhances the *beach1* Overexpression Phenotype

(A) Left panel: overexpression of *beach1* in the photoreceptors results in a reduced, glazed eye without distinct ommatidia (for comparison to wildtype see Chapter 2, Figure 2A). Reduction in *rab11* dosage, using hypomorphic alleles $rab11^{93Bi}$ and $rab11^{(To)3}$, enhances the *beach1* overexpression phenotype (middle and right panels, respectively). This enhancement is not due to an additive effect since $rab11^{93Bi}$/+ and $rab11^{(To)3}$/+ animals have a wildtype eye morphology (data not shown).

(B) Left panel: overexpression of *beach1* in the photoreceptors results in a reduced, glazed eye without distinct ommatidia (for comparison to wildtype see Chapter 2, Figure 2A). Reduction in *rop* dosage, using a null allele rop^{G27} or a combination of hypomorphic alleles rop^{A19} *and* rop^{G11}, enhances the *beach1* overexpression phenotype (middle and right panels, respectively). This enhancement is not due to an additive effect since rop^{G27}/+ and rop^{A19}/ rop^{G11} animals have a wildtype eye morphology (data not shown).

A.


GMR EP:Beach1;+/+
GMR EP:Beach1;rab11^{93Bi}/+
GMR EP:Beach1;rab11$^{(To)3}$/+

B.

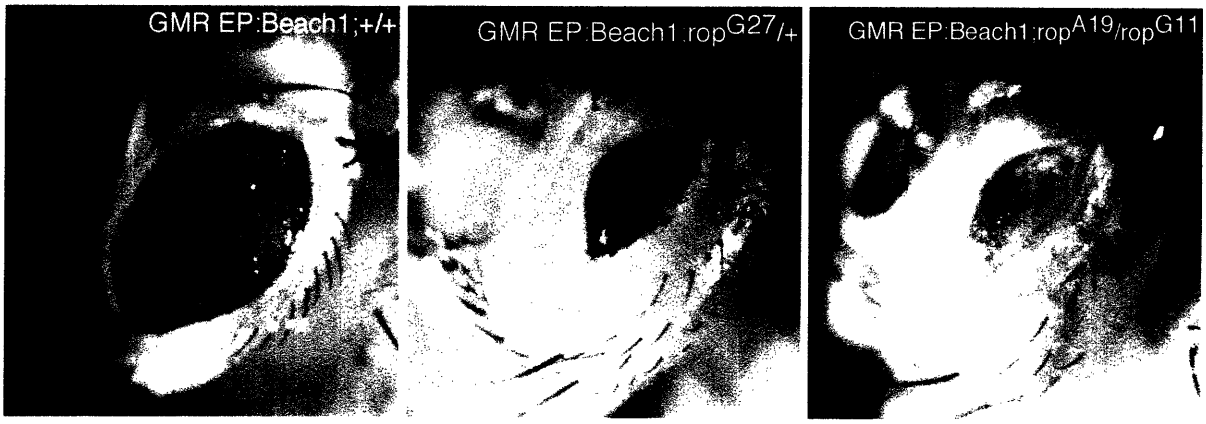

GMR EP:Beach1;+/+
GMR EP:Beach1;rop^{G27}/+
GMR EP:Beach1;rop^{A19}/rop^{G11}

Table 2: Candidate Genes Tested for Modification of *beach1* overexpression

Process	Gene	Modifies *beach1* overexpression?
vesicle trafficking, including Rabs	Syntaxin-1A	no
	Rop	yes
	Rab1	no
	Rab3	no
	Rab5	no
	Rab6	no
	Rab11	yes
	Rab14	no
cytoskeleton	capping protein	no
	profilin (chicadee)	no
	Rho	no
	Rac	no
	Cdc42	no
	kinesin heavy chain	no
synaptic vesicle exocysosis	Synaptotagmin	no

Table 3: Rab11 Alleles and Phenotypes

Rab11 Allele (lesion, if known) (paper reported)	93Bi (Jancovics et.al.)	E(T0)3 (Jancovics et.al.)	Df(3R)e-N19 (Jancovics et.al.)	ex1 (excision of 2nd exon including start codon) (Dollar et.al.)
Phenotype over $rab11^{93Bi}$	Viable, Moderate bristle defects, fertile	Viable, strong bristle defects, sterile	Lethal	Semi-lethal, strong bristle defects, sterile

Loss of *beach1* suppresses *rab11* lethality

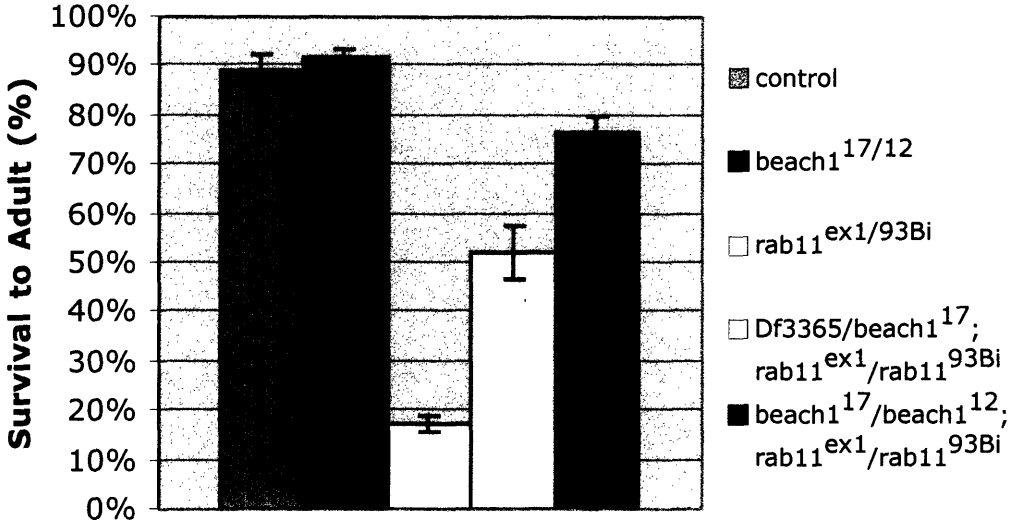
Decreased dosage of *rab11* enhances the *beach1* overexpression phenotype; therefore, I decided to test whether mutants in *beach1* would, in turn, modify *rab11* loss-of-function phenotypes. *Rab11* null mutants die in mid-embryogenesis; however, hypomorphic combinations have varying degrees of viability, sterility and bristle defects (Table 3). The combination of a hypomorphic allele $rab11^{93Bi}$ and a deficiency that completely removes the *rab11* locus was previously reported to be lethal (Jankovics et.al., 2001). However, when I tested an allelic combination of $rab11^{93Bi}$ over a putative null excision allele, $rab11^{ex1}$ (Dollar et.al., 2002), I found that, when raised separately from their heterozygous siblings, a small number of escapers survived to adulthood. Fewer $rab11^{93Bi}/rab11^{ex1}$ mutants were able to pupate compared to controls and, of those, most died as pharate adults. Upon my attempts to remove these pharate flies from the pupal cases, I found that they had soft, poorly formed cuticles, which were often adhered to the inside of the pupal case. Thus, one of the defects in the $rab11^{93Bi}/rab11^{ex1}$ mutants appears to be in the secretion of the cuticle. To test whether the loss of *beach1* function has an effect on the viability of *rab11* mutants, I introduced strong *beach1* alleles into the *rab11* mutant background. I used protein null alleles $beach1^{12}$, $beach1^{17}$ and an independent deficiency (Df3365) that, as I confirmed by PCR analysis (Chapter 2, Figure 7), removes the *beach1* locus. I found that the removal of one or both copies of *beach1* strongly suppresses *rab11* lethality (Figure 2A and B). For example, in one experiment, survival to adulthood was improved from 17+/-4% in $rab11^{93Bi}/rab11^{ex1}$ mutants to 77+/-3% in $beach1^{12}/beach1^{17}$; $rab11^{93Bi}/rab11^{ex1}$ double mutants (mean+/-SEM, n=3, $p<0.0005$). In a different experiment, survival was improved from 5+/-1% for $rab11^{93Bi}/rab11^{ex1}$ mutants to 48+/-6% for $beach1^{17}/+;rab11^{93Bi}/rab11^{ex1}$ animals (mean+/-SEM;n=8, $p<0.0005$).

Figure 2: Loss of Function in *beach1* Suppresses *rab11* Lethality

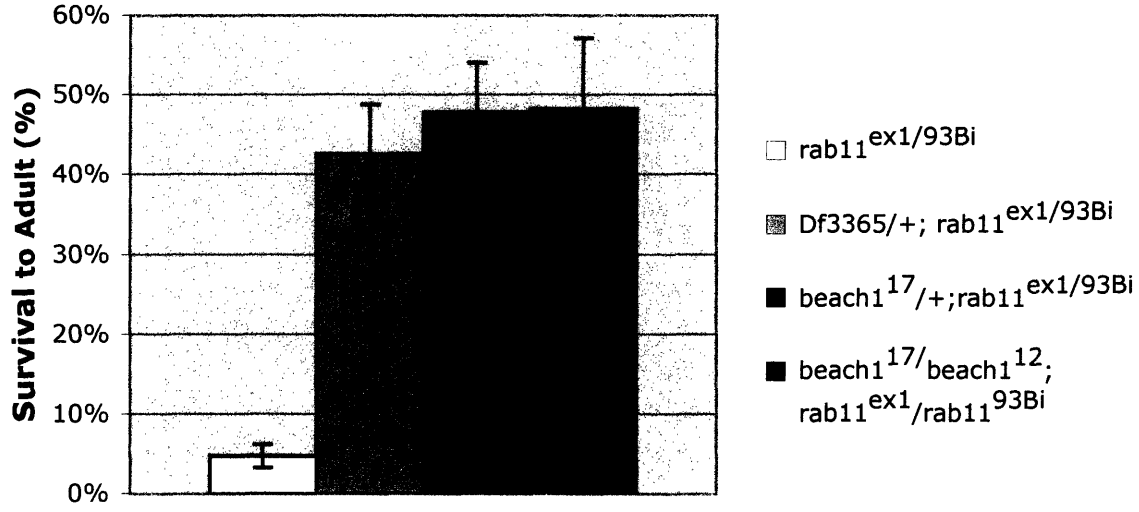
(A) Equal numbers (60) 2nd instar larvae of each indicated genotype were placed in identical vials and monitored for survival to adulthood. Fewer hypomorphic $rab11^{ex1}/rab11^{93Bi}$ mutant larvae made it to the pupal stage, and most of those died as pharate adults. Most double mutants for *beach1, rab11* were able to pupate, and the majority of those emerged as normal adults. Df3365 is a deficiency that removes the beach1 locus, alleles $beach1^{12}$ and $beach1^{17}$, are protein null alleles. Viability for control (wcs) was 89+/-3%, for $beach1^{17/12}$ 92+/-2%, p=0.5; for $rab11^{ex1/93Bi}$ 17+/-4, p<0.0001; for $Df3365/beach1^{17};rab11^{ex1}/rab11^{93Bi}$ 52+/-5%, p<0.01; for $beach1^{17}/beach1^{12};rab11^{ex1}/rab11^{93Bi}$ 77+/-3%; p<0.001; n=at least 3 for each genotype, mean+/-SEM is shown for each genotype).

(B) In an independent experiment, equal numbers (around 60) 2nd instar larvae of each indicated genotype were placed in identical vials and monitored for survival to adulthood. Fewer hypomorphic $rab11^{ex1}/rab11^{93Bi}$ mutant larvae made it to the pupal stage, and most of those died as pharate adults. More double mutants for *beach1, rab11* were able to pupate, and the majority of those emerged as normal adults. Df3365 is a deficiency that removes the *beach1* locus, alleles $beach1^{12}$ and $beach1^{17}$, are protein null alleles. Viability for $rab11^{ex1/93Bi}$ was 5+/-1%;; for $Df3365/+;rab11^{ex1}/rab11^{93Bi}$ 43+/-6%, p<0.005; for $beach1^{17}/+;rab11^{ex1}/rab11^{93Bi}$ 48+/-6%; p<0.005;for $beach1^{17}/beach1^{12};rab11^{ex1}/rab11^{93Bi}$ 48+/-9%, p<0.01; n=at least 5 for each genotype, mean+/-SEM is shown for each genotype).

A.


Survival to Adult (%)
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
control
beach1 17/12
rab11 ex1/93Bi
Df3365/beach1 17; rab11 ex1/rab11 93Bi
beach1 17/beach1 12; rab11 ex1/rab11 93Bi

B.


Survival to Adult (%)
60%
50%
40%
30%
20%
10%
0%
rab11 ex1/93Bi
Df3365/+; rab11 ex1/93Bi
beach1 17/+;rab11 ex1/93Bi
beach1 17/beach1 12; rab11 ex1/rab11 93Bi

Loss of *beach1* suppresses *rab11* bristle defects

Rab11 mutants have previously been reported to exhibit bristle shortening and loss phenotypes of varying severity (Jankovics et.al., 2001 and Table 3). In agreement with these data, I observed the loss of microchaetes, the small mechanosensory bristles on the fly's posterior abdomen, in all viable *rab11* allelic combinations (Figure 3A). I also observed the previously not described shortening of the posterior scutellar macrochaetes, the large mechanosensory bristles, in the semi-lethal $rab11^{93Bi}/rab11^{ex1}$ mutants (Figure 3B).

I also observed that the removal of *beach1* suppressed both the bristle shortening and the bristle loss defects of *rab11* mutants (Figure 3). To quantify this interaction, I calculated the fraction of empty sockets in the last row of abdominal tergites 2, 3 and 4 for two different allelic combinations of *rab11*—the viable $rab11^{93Bi}$ homozygote and the semi-lethal $rab11^{93Bi}/rab11^{ex1}$ heterozygote together with different allelic combinations of *beach1.* I found that the removal of *beach1* strongly suppresses bristle loss in both *rab11* allelic combinations in a dose-dependent manner (Figure 3C and D). Removal of one copy strongly suppresses the defect, while the removal of both copies of *beach1* returns the number of bristles to almost wildtype levels. For example, in $rab11^{93Bi}$ mutants, only 54+/-1% of sockets are filled with bristles, while the removal of one copy of *beach1* restores this number to 88+/-2% (n=13 and 12, respectively; mean+/-SEM; $p<0.0001$), and in the $beach1^{58}$, $rab11^{93Bi}$ double mutant it is restored it to 99% (n=13 and 10, respectively; mean+/-SEM;$p<0.0001$). Interestingly, $beach1^{8}$ allele, which encodes a missense mutation in the PH-BEACH interface is able to suppress bristle loss to the same extent as the protein null allele $beach1^{58}$ (Figure 3C, top panel). The semi-lethal *rab11* allelic combination has only 30+/-4% of bristle-filled sockets compared to wildtype, the removal of one copy of *beach1* via allele $beach1^{17}$ restores bristle number to 86+/-1%, $p<0.0001$, while the removal of

both copies of *beach1* restores the fraction of bristle-filled sockets to 99%; p<0.0001 (Figure 3C, bottom panel).

Allele $beach1^{17}$ suppresses bristle defects as completely as the deficiency that removes the entire *beach1* locus (Df3365), and the $beach1^{17}/beach1^{12}$ combination suppresses lethality and bristle defects at least as well at the Df3365/ $beach1^{17}$ combination; therefore, alleles $beach1^{17}$ and $beach1^{12}$ can be considered genetic nulls. This is in agreement with Western blot analysis, which shows that these mutants do not make any detectable Beach1 protein.

Figure 3. Loss of *beach1* Suppresses *rab11* Bristle Defects

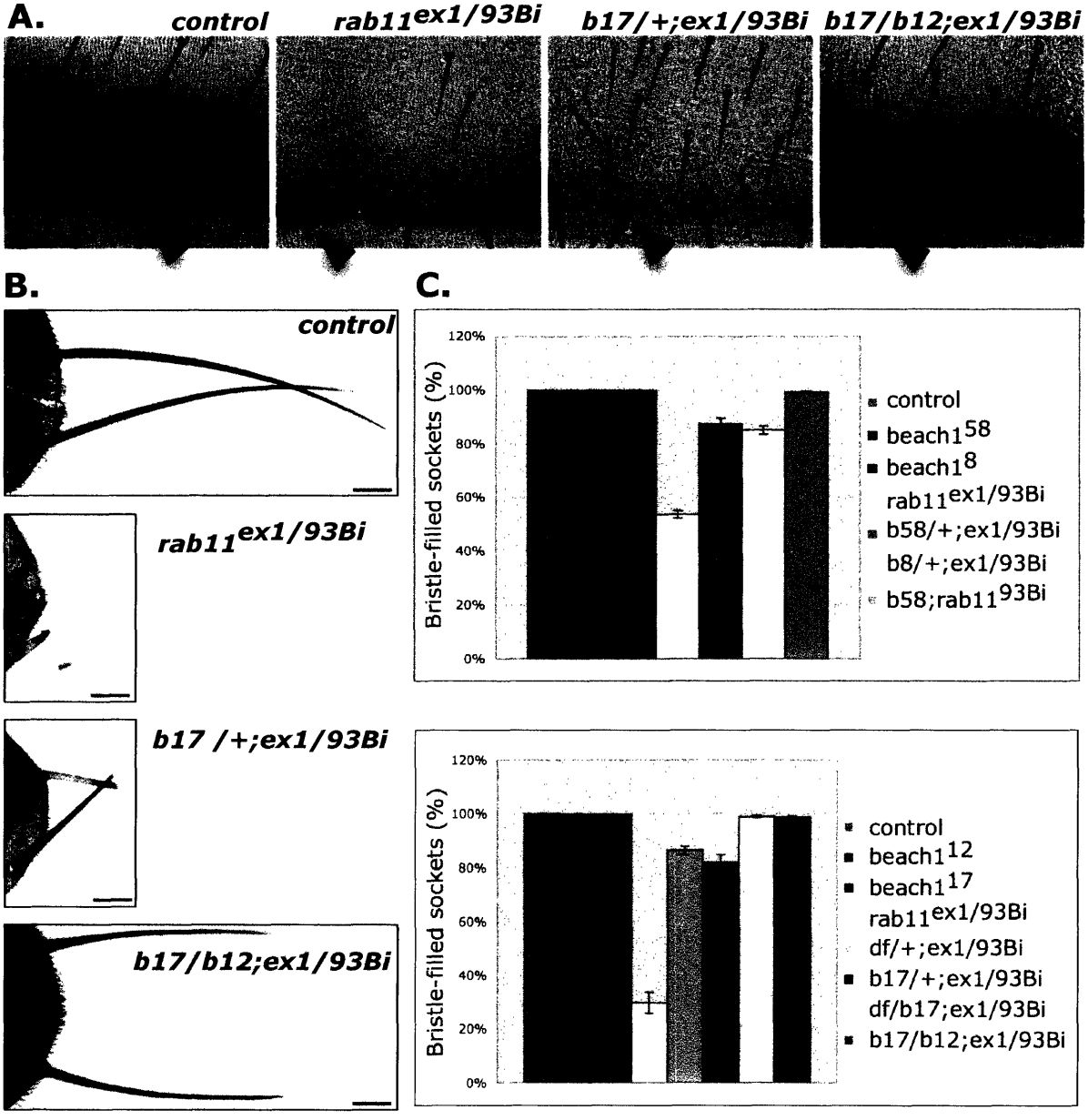
(A) Portions of posterior abdomens from animals of indicated genotypes are shown. In the control, every socket is filled with a bristle (a microchaete), while in the $rab11^{ex1}/rab11^{93Bi}$ mutant many empty sockets are seen. The removal of one copy of *beach1* in $beach1^{17}/+;rab11^{ex1}/rab11^{93Bi}$ restores most of the bristles, and the removal of both copies of the *beach1* gene in $beach1^{17}/beach1^{12};rab11^{ex1}/rab11^{93Bi}$ restores the bristles completely. Arrowheads indicate bristle-filled or empty sockets in the last row of the third abdominal tergite (segment). Scale bar is 30μm.

(B) Portions of the posterior scutellum (part of the thorax) for animals of the indicated genotype are shown. Posterior scutellar bristles in the $rab11^{ex1}/ rab11^{93Bi}$ mutant are severely shortened. The removal of one copy of *beach1* in $beach1^{17}/+; rab11^{ex1}/ rab11^{93Bi}$ partially restores the length, and the removal of both copies of the *beach1* gene in $beach1^{17}/beach1^{12}; rab11^{ex1}/rab11^{93Bi}$ restores the length of these bristles to about 3/4 of wildtype length. Scale bar is 50μm.

(C) Quantitation of the *rab11* abdominal bristle loss phenotype and its suppression by *beach1*. Bristle-filled and empty sockets were counted in the last row of abdominal tergites 2, 3 and 4 were counted and the fraction of bristle-filled sockets over the total number of sockets calculated. The mean with the error bars that represent SEM (Standard error of the mean) is shown.

Top panel: in $beach1^{58}$ and $beach1^{8}$ mutants, as in control, all the sockets are filled with bristles. In the hypomorphic *rab11* mutant $rab11^{93Bi}$ 54+/-1% of sockets are filled with bristles, In $beach1^{58}/+;rab11^{93Bi}/rab11^{93Bi}$ 88+/-2% of sockets are filled, in $beach1^{8}:rab11^{93Bi}/+:rab11^{93Bi}$ 85+/-2% of sockets are filled, in $beach1^{58};rab11^{93Bi}$ 99% of sockets are filled. (n=at least 10 for each genotype, mean+/-SEM, p<0.0001 for all genotypes).

Bottom panel: in $beach1^{12}$ and $beach1^{17}$ mutants, as in control, all the sockets are filled with bristles. In the hypomorphic *rab11* mutant $rab11^{ex1}/rab11^{93Bi}$ 30+/-4% of sockets are filled with bristles, in $Df3365/+;rab11^{ex1}/rab11^{93Bi}$ 86+/-1% of sockets are filled, in $beach1^{17}/+;rab11^{ex1}/rab11^{93Bi}$ 82+/-3% of sockets are filled, in both $Df3365/beach1^{17};rab11^{ex1}/rab11^{93Bi}$ and $beach1^{17}/beach1^{12};rab11^{ex1}/rab11^{93Bi}$ 99% of sockets are bristle filled. (n=at least 10 for each genotype, mean+/-SEM, p<0.0001 for all genotypes).


A.
control
rab11ex1/93Bi
b17/+;ex1/93Bi
b17/b12;ex1/93Bi
B.
control
rab11ex1/93Bi
b17 /+;ex1/93Bi
b17/b12;ex1/93Bi
C.
Bristle-filled sockets (%)
120%
100%
80%
60%
40%
20%
0%
control
beach158
beach18
rab11ex1/93Bi
b58/+;ex1/93Bi
b8/+;ex1/93Bi
b58;rab11 93Bi
Bristle-filled sockets (%)
120%
100%
80%
60%
40%
20%
0%
control
beach112
beach117
rab11ex1/93Bi
df/+;ex1/93Bi
b17/+;ex1/93Bi
df/b17;ex1/93Bi
b17/b12;ex1/93Bi

Beach1 and Rab11 partially co-migrate on a sucrose gradient

To try to learn more about the nature of the Beach1-Rab11 interaction, I examined the subcellular distribution of these proteins on a sucrose gradient, which was generously provided by Bill Adolfsen (Littleton lab, MIT) (Figure 4). Velocity gradient subcellular fractionation experiments were carried out by B. Adolfsen, who used 10–30% sucrose gradients to separate Canton-S head extracts. To determine the identities of fractions containing plasma membrane, synaptic vesicle, and cytosol compartments, known markers, such as Syntaxin 1A, n-Synaptobrevin, and ROP were used (Schulze et al., 1995; DiAntonio et al., 1993; Salzberg et al., 1993, respectively). Fractions positive for Beach1 were found to partially overlap with those positive for Rab11. However, Beach1 was not found in the Syntaxin 1A, Rop and Rab11-positive plasma membrane fraction, nor was it found in the Rop and Rab11-positive right-most fractions, which were collected last and most likely represent cytosolic proteins. Therefore, Beach1 and Rab11 localize to membrane fractions that partially co-migrate on a sucrose gradient, suggesting that they may localize to partially overlapping subcellular compartments. In addition, Beach1 does not co-migrate with Synaptotagmin1 and n-Synaptobrevin, which are synaptic vesicle proteins, suggesting that Beach1 is not enriched on synaptic vesicles.

Beach1 localizes to a distinct set of membranes at the synapse

To try to determine the identity of the Beach1-positive puncta at the neuromuscular junction, I co-labeled 3rd instar larvae with an antiserum against Beach1 and several markers for subcellular compartments that have been well characterized at the NMJ. To this end I drove the expression of early endosomal markers 2XFYVE-GFP and Rab5-GFP (Wucherpfennig et.al., 2003), dense-core vesicle marker Anf-GFP, and endocytic marker Clathrin-GFP in

neurons using a pan-neuronal driver Elav-Gal4 (Figure 5 A, B, C, and D, respectively). I found that Beach1 positive puncta do not show significant overlap with any of these markers (Figure 5). It is somewhat surprising that Beach1, which has a C-terminal FYVE domain, does not localize to the early endosomal compartment labeled with 2XFYVE-GFP and Rab5-GFP.

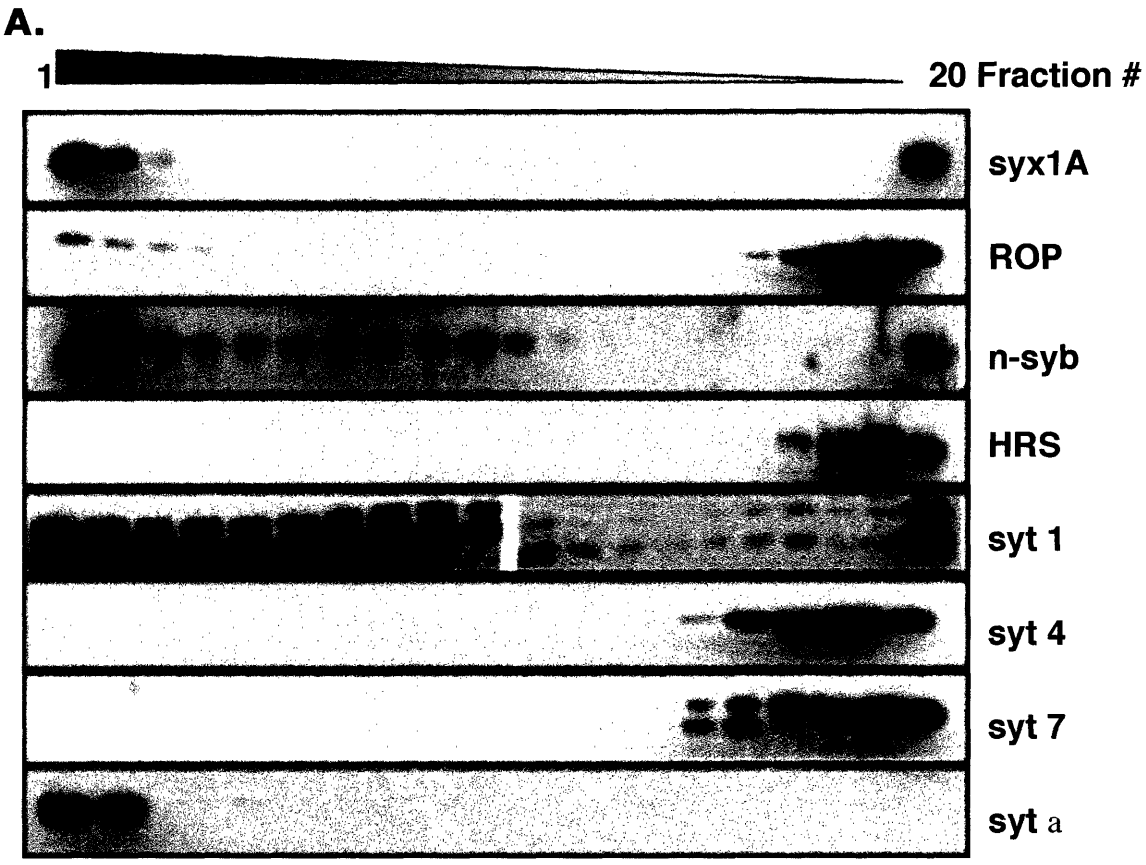
Rab11 and Beach1 compartments may partially overlap at the neuromuscular junction

Knowing that Beach1 is enriched at the synapse, I wanted to examine the localization of Rab11 at the 3rd instar larval neuromuscular junction. I found that Rab11 has a punctate staining pattern and localizes to the nerve, the synaptic boutons and the muscle (Figure 6A). The observed staining is specific to Rab11 since it is greatly reduced in *rab11*^{93Bi}/*rab11*^{ex1} mutants, which also show reduced protein levels compared to control by western blot analysis (Figure 6A and B). The morphology and size of Rab11 puncta are similar to those of Beach1 puncta; however, Beach1 seems to be more enriched in the boutons (Figure 6 in Chapter 2), while Rab11 is more abundant in the muscle.

Figure 4. Beach1 and Rab11 Partially Co-migrate on a Sucrose Gradient

(A) Modified from Adolfsen et.al., 2004. Post-nuclear fractions of Canton S head extracts were separated on 10–30% sucrose gradients. Isolated fractions were probed for subcellular markers by Western analysis, including antisera against Syx1A and ROP, which localize to the plasma membrane (left-most fractions). Synaptic vesicle fractions were identified using the Syt 1 and n-Synaptobrevin antibodies, cytosolic fractions were indicated by immunostaining for ROP, and endosomal fractions by staining for HRS (Lloyd et al., 2002). Syt 4 and Syt 7 were not detected in synaptic vesicle or plasma membrane fractions, but rather found near the top of the gradient (Adolfsen et.al., 2004).

(B) Fractions from the same sucrose gradient shown in (A) were re-probed for ROP, HRS, and syt1 to confirm that proteins had not been degraded over time. Fractions were also probed with antisera against Beach1 and Rab11. Beach1 and Rab11 localized to partially overlapping sets of fractions, which also partially overlap with those containing Rop and Hrs. However, Beach1 was not found on the Syntaxin 1A, ROP and Rab11-positive plasma membrane fraction 1. Notably, Beach1 does not co-migrate with Synaptotagmin1 and n-Synaptobrevin, which are synaptic vesicle proteins.


A.
1
20 Fraction #
syx1A
ROP
n-syb
HRS
syt 1
syt 4
syt 7
syt a
B.
1
20 Fraction #
Beach1
Rab11
ROP
HRS
syt 1

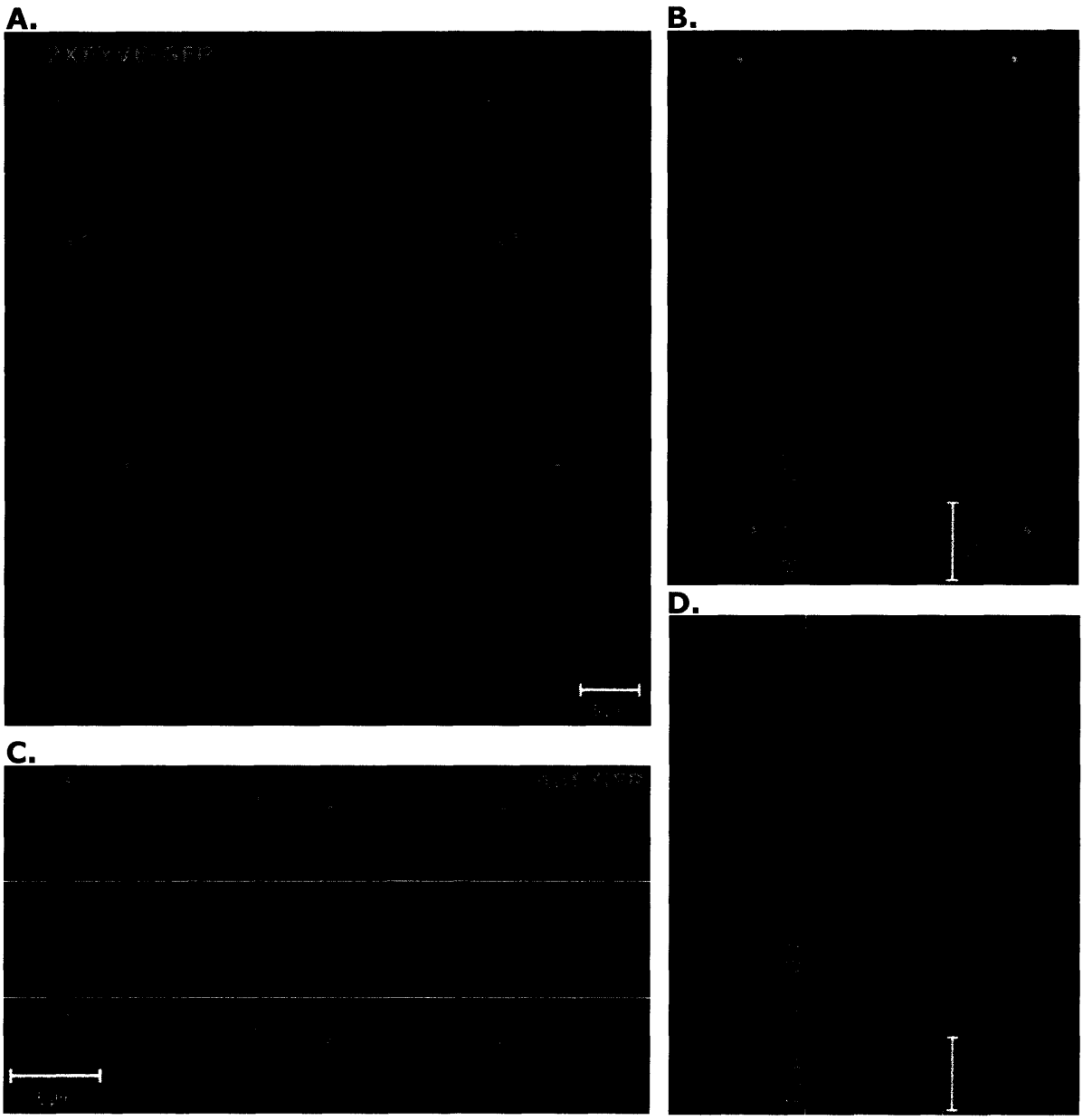


Figure 5. Beach1 Localizes to a Distinct Compartment at the NMJ
All GFP-tagged markers, shown in green, were expressed using a pan-neuronal Elav-Gal4 driver. Beach1 is shown in red, HRP, which labels neuronal membrane, is shown in blue.
A single confocal section at NMJ 6/7 is show in each panel. Scale bars a 5um.
Beach1 puncta do not show significant overlap with any of the following GFP markers:
(A) 2XFYVE-GFP and (B) Rab5-GFP mark the early endosomal compartment at the NMJ (Wucherpfennig et.al., 2003).
(C) Anf-GFP (a neuropeptide) is a dense-core vesicle marker.
(D) Clathrin-GFP (vesicle coat protein) marks areas actively undergoing endocytosis.

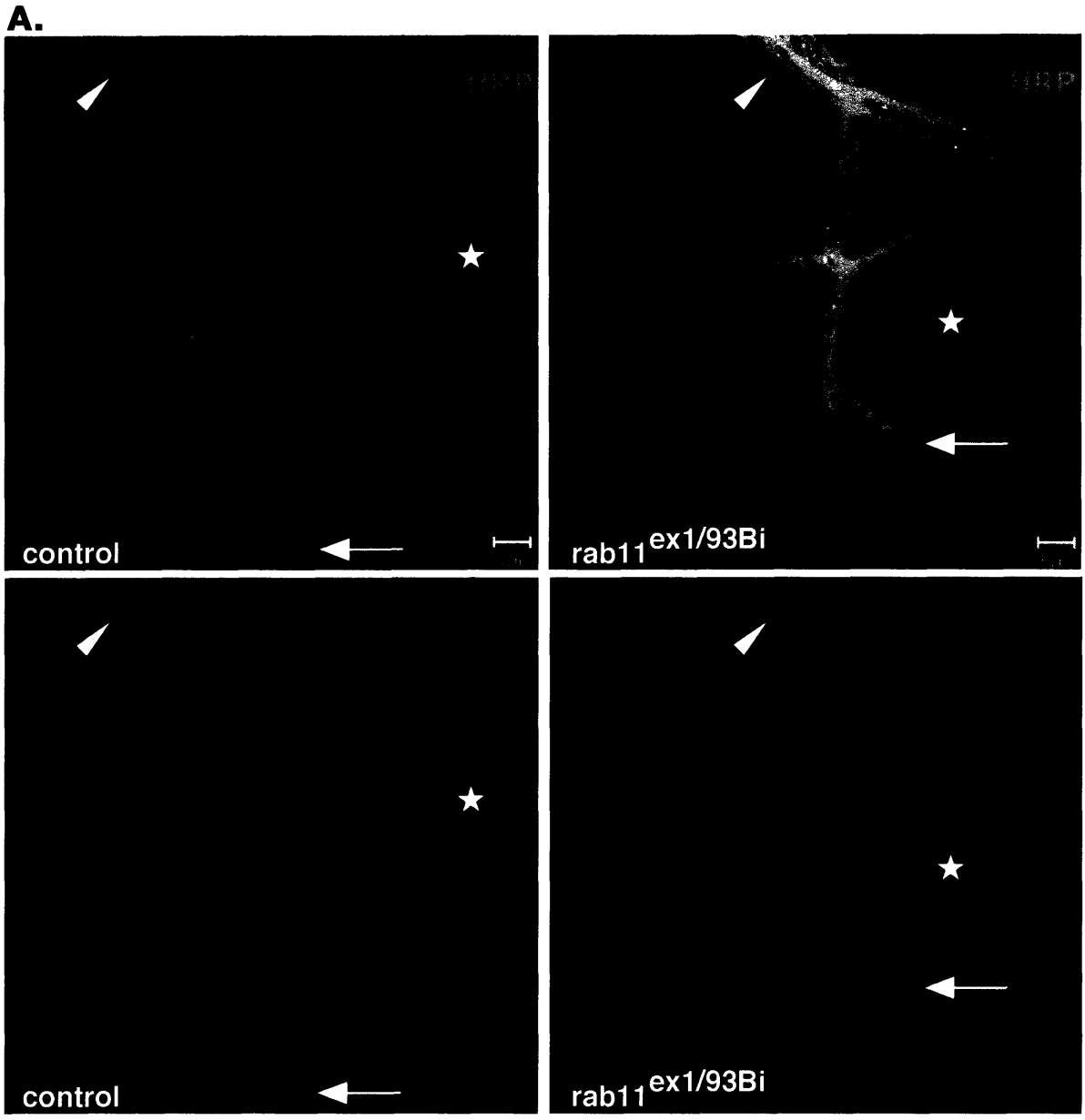
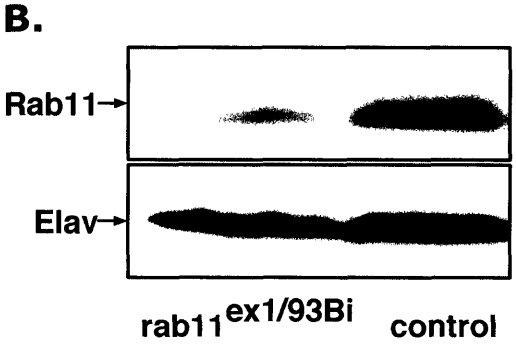


Figure 6. Rab11 has a Punctate Localization at the NMJ

(A) Rab11 positive puncta are present in the nerve (arrowhead), the boutons (arrow), and the muscle (star).
Both the number and the brightness of Rab11 puncta is reduced in *rab11*^{93Bi}/*rab11*^{ex1} mutants (right panels) compared to controls (left panels). Confocal stacks through NMJs at muscle 4 are shown. Scale bar is 5um.



(B) Western blot showing that rab11^{93Bi}/rab11^{ex1}mutants have reduced levels of Rab11 protein compared to controls. Antibody against a neuronal epitope Elav demonstrates approximately equal loading.

Figure 7. Beach1 and Rab11-positive Compartments Partially Overlap at the Neuromuscular Junction
A single confocal slice through muscle 4 NMJs of a 3rd instar larva, double labeled against Rab11 (green) and Beach1(red), reveals significant overlap between these compartments. However, interpretation of this result is made difficult by the potential cross-reactivity between the primary and secondary antibodies made in closely related species (rat anti-Beach1 and mouse anti-Rab11, and the respective secondary antibodies). Anti-HRP staining (blue) outlines neuronal membrane.

To examine the distribution of Rab11 with respect to Beach1, 3rd instar larvae were double-labeled with antibodies against these two proteins. A significant overlap was observed between the Rab11 and the Beach1-positive compartments at the NMJ (Figure 7). However, there is a caveat to this experiment: the only antibodies available against the *Drosophila* Rab11 and Beach1 are made in mouse and rat, respectively, which makes co-labeling technically challenging. With the reagents available to me, I was not able to completely rule out cross-reactivity; this precludes me from drawing definitive conclusions from these double-labeling experiments. Nevertheless, the similarities in morphology and size between Rab11 and Beach1 compartments, and the significant overlap between both the bright and the faint puncta seen in the double-labeling experiments, suggest that Beach1 and Rab11 partially co-localize. Experiments using an HA-tagged Beach1 construct are currently ongoing to circumvent cross-reactivity problems.

Loss of *beach1* suppresses the *rab11* overgrowth phenotype at the NMJ

Since both Beach1 and Rab11-positive puncta are present at the neuromuscular junction, I decided to examine synaptic morphology of the semi-lethal, $rab11^{93Bi}/rab11^{ex1}$ mutant. I found that all the synapses in 3rd instar larvae of this genotype are overgrown, with an increased number of closely clustered boutons. *Rab11* mutant NMJs have an appearance more akin to bunches of grapes than to the wildtype beads-on-a-string morphology (Figure 8A). To quantify this overgrowth phenotype, I counted the number of boutons at NMJ 6/7 of the 3rd abdominal segment. The nerve at the neuromuscular junction grows in proportion to muscle area; therefore, because *rab11* larvae are smaller than their developmentally matched controls, I normalized the bouton number to the estimated muscle size (Schuster et.al., 1996). I found that normalized bouton count in $rab11^{93Bi}/rab11^{ex1}$ mutants is increased to 225% of control ($3.32+/-0.22x10^{-3}$

versus 1.48+/-0.08x10^{-3} boutons/μm^2; mean+/-SEM; $p<5x10^{-7}$). I tested to see whether the loss of *beach1* modified this phenotype, and found that in *beach1*^{12}/*beach1*^{17}; *rab11*^{93Bi}/*rab11*^{ex1} double mutants the normalized bouton count is 169% of control (2.50+/-0.18 versus 3.32+/-0.22x10^{-3} boutons/μm^2; mean+/-SEM; $p<0.008$). This is a 44% suppression of the *rab11* overgrowth phenotype (Figure 8B). Upon careful examination of *rab11* mutants, I found that the abnormal bouton clustering was a consequence of an increase in the number of branching boutons—those connected to more than two neighboring boutons. Normally, only a small fraction of branching boutons is found at a given NMJ, but in *rab11* mutants this fraction is increased to 266% of control (22.2+/-0.8% versus 8.4+/-0.4% of total boutons; mean+/-SEM; $p<5x10^{-12}$ at NMJ 6/7). In *beach1*^{12}/*beach1*^{17}; *rab11*^{93Bi}/*rab11*^{ex1} double mutants the fraction of branching boutons is 187% of control (15.6+/-1.1% versus 22.2+/-0.8%; mean+/-SEM; $p<0.0001$), which is a 48% suppression of the *rab11* defect. Thus, the loss of *beach1* partially suppresses both the increase in bouton number and the increase in bouton branching exhibited by *rab11* mutants.

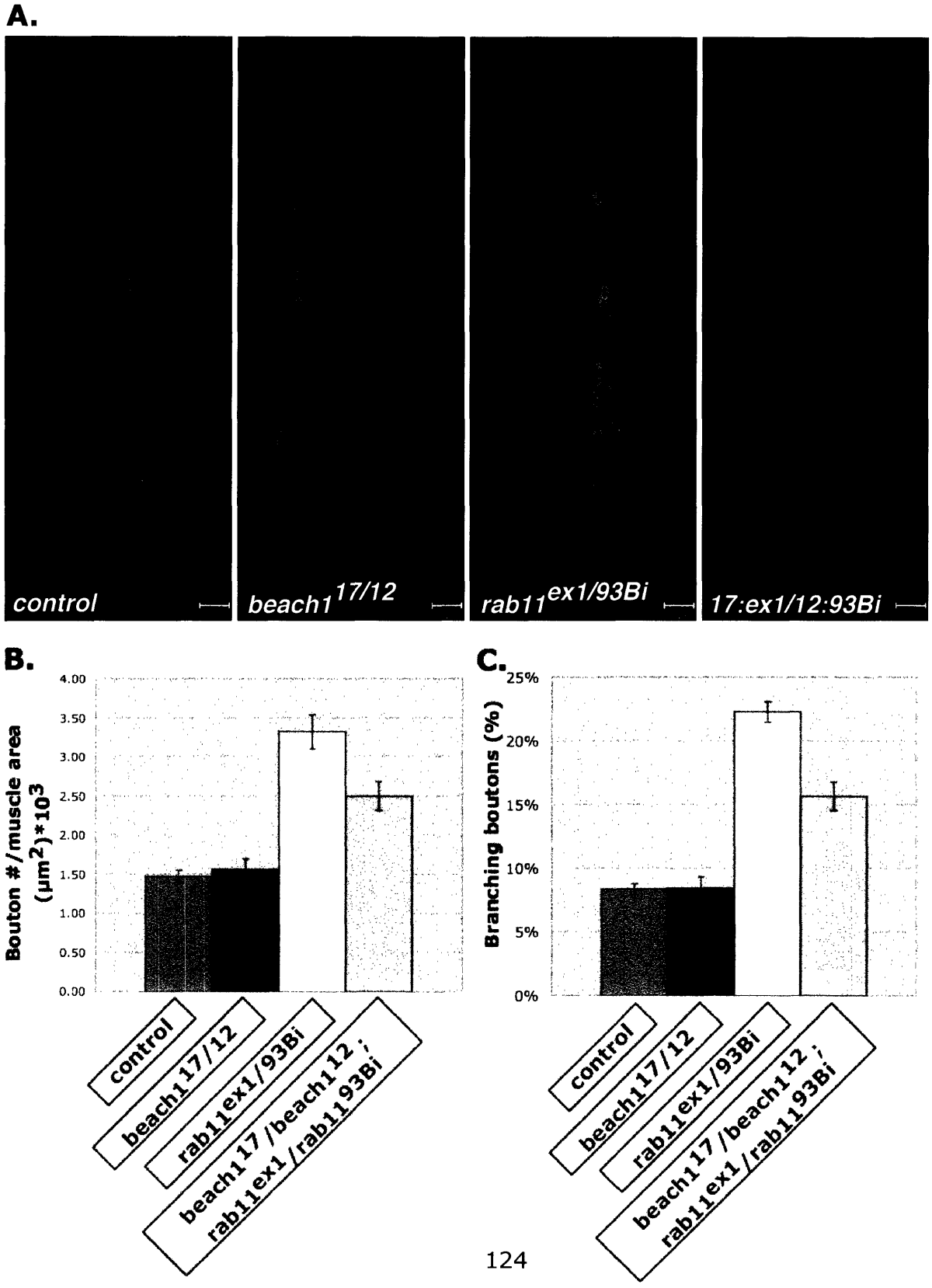
Figure 8. Loss of *beach1* Suppresses the *rab11* Overgrowth Phenotype at the Neuromuscular Junction

(A) Confocal images taken at NMJ 6/7 (abdominal segment 3) labeled with anti-HRP to outline neuronal membrane are shown. *rab11ex1/rab11*^{93Bi} mutants show an increase in the normalized bouton number per muscle area and in the number of branching boutons compared to controls. Both of these defects are suppressed in *beach117/beach112;rab11ex1/rab11*^{93Bi} double mutants. *beach117/beach112* null mutants do not have a defect in bouton number or branching. Scale bar is 10μm.

(B) Quantitation of *rab11* overgrowth defect and its suppresion by *beach1*. Normalized bouton number in *rab11* mutants is 225% of control (22.2+/-0.8% versus 8.4+/-0.4% of total boutons; mean+/-SEM; $p<5x10^{-12}$) and in *beach1, rab11* double mutants is 169% of control (2.50+/-0.18 versus 3.32+/-0.22x10^{-3} boutons/μm^2; mean+/-SEM; $p<0.008$). This is a 44% suppression of the *rab11* defect.

(C) Quantitation of the branching defect in *rab11* mutants and its suppression by *beach1*.

In *rab11* mutants the fraction of branching boutons is 266% of control (22.2+/-0.8% versus 8.4+/-0.4% of total boutons; mean+/-SEM; $p<5x10^{-12}$) and in *beach1, rab11* double mutants is 187% of control (15.6+/-1.1% versus 22.2+/-0.8%; mean+/-SEM; $p<0.0001$). This is a 48% suppression of the *rab11* branching defect.


A.
control
beach1 17/12
rab11 ex1/93Bi
17:ex1/12:93Bi
B.
Bouton #/muscle area (µm2)*103
4.00
3.50
3.00
2.50
2.00
1.50
1.00
0.50
0.00
control
beach1 17/12
rab11 ex1/93Bi
beach1 17/beach1 12 ; rab11 ex1/rab11 93Bi
C.
Branching boutons (%)
25%
20%
15%
10%
5%
0%
control
beach1 17/12
rab11 ex1/93Bi
beach1 17/beach1 12 ; rab11 ex1/rab11 93Bi

Discussion

Rab11 is a small GTPase that has been shown to regulate the delivery of membrane and cargo to the plasma membrane via both the recycling and the biosynthetic pathways (Ren et.al., 1998; Pelissier, et.al., 2003; Satoh et.al., 2005). I have demonstrated that loss-of-function mutations in *beach1* strongly suppress bristle defects, synaptic overgrowth, and lethality of *rab11* mutants. Since Beach1 antagonizes Rab11 in multiple processes and cell types in *Drosophila*, it may be involved in every *rab11*-requiring process. Because the interaction is strong, it is tempting to suggest that Beach1 and Rab11 interact directly with each other. However, since I have not demonstrated direct binding, and there are some caveats to the co-localization experiments, other explanations for their interaction must also be considered.

Possible models for the Beach1-Rab11 interaction

There are three possible mechanisms by which Beach1 might antagonize Rab11: first, by functioning in a parallel pathway to the *rab11* pathway, second, by functioning in the same pathway with *rab11*, or, third, by interacting with Rab11 either directly or as part of same complex.

Parallel pathways

If *beach1* and *rab11* contributed to the same process, such as the addition of membrane to the cell surface, but functioned in parallel pathways, null mutations in *beach1* could, theoretically, bypass the requirement for *rab11*. This could be the case if both pathways had similar capacities to contribute to the process, and if the alternative to *rab11* pathway was under strong inhibitory control by *beach1*. In my observations, loss-of-function in *beach1* suppresses multiple phenotypes of hypomorphic *rab11* mutants, but *beach1, rab11* null double mutants do not survive. Therefore, the fact that

beach1 can suppress *rab11* defects, but cannot bypass the requirement for *rab11*, suggests that *beach1* and *rab11* are likely to function in the same pathway. Of course, one cannot rule out the possibility that they function in parallel pathways, one of them (the *rab11*-requiring pathway) being much more essential for the process of interest than the *beach1* pathway. Then, even the null mutations in *beach1* would not eliminate the requirement for *rab11*.

Same pathway

Another possibility is that *beach1* and *rab11* antagonize one another by functioning at different points in the same pathway. For example, while *rab11* is responsible for membrane addition by exocytosis, *beach1* could control membrane internalization at the same site by endocytosis. Then, in double mutants, the rates of both of these processes would be reduced, resulting in the suppression of phenotypes that require a net overall increase in membrane addition. However, such a model cannot explain how *beach1* suppresses bristle extension and cuticle deposition defects of *rab11* mutants, because neither process has a significant component of endocytosis. Bristle extension occurs during a small window in time, between 32 and 48 hours of pupal development, and requires significant membrane addition at the tip of the growing bristle. Once extension is completed, a cuticle is secreted to support the bristle cell (Tilney et.al., 1996). Both the formation of the cuticle around the bristle, and the deposition of cuticle that covers the entire fly, require high amounts of secretion of cuticle proteins (Moussian et.al., 2005). However, it is not thought that, during these periods, significant endocytosis is simultaneously taking place at the sites of membrane and cuticle addition. Thus, it would be difficult to explain the strong suppression of *rab11* defects in these processes by suggesting a role for *beach1* in endocytosis.

Another line of evidence against the primary role for *beach1* in endocytosis is the fact that the *beach1* gain-of-function is not enhanced by

mutations in genes required for endocytosis, such as *shibire*, a small GTPase, which is required for vesicle fission during endocytosis. Finally, double-labeling experiments show that Beach1 does not colocalize with Clathrin-GFP—a marker for zones of endocytosis, at the neuromuscular junction. Taken together, these lines of evidence point against a role for *beach1* in endocytosis downstream of *rab11*-mediated membrane addition.

The recycling pathway

Since Rab11 has an established role in endocytic recycling (Dollar et.al., 2001; Riggs, et.al., 2003), another explanation for the *rab11-beach1* interaction could be that *beach1* functions upstream of *rab11* in the endocytic recycling pathway. However, the inability of a decreased dosage of *rab5*, which regulates vesicle traffic to and from the early endosome, to modify the *beach1* gain-of-function phenotype, points against this model. Moreover, the Beach1-positive compartment at the NMJ does not overlap with the early endosomal compartment labeled with 2XFYVE-GFP and Rab5-GFP. Therefore, it is unlikely that Beach1 antagonizes Rab11 by functioning in the early endosomal compartment upstream of the rab11 endosomes.

Rop is an enhancer of *beach1* overexpression

Rop, a *Drosophila* homolog of the yeast Sec1p and the mammalian Munc-18 (SM) genes, is the only other gene found to modify the *beach1* overexpression phenotype. SM genes are thought to confer specificity to SNARE-mediated vesicle fusion via their interaction with a target-SNARE Syntaxin; however, there is no consensus on their exact mechanism of function (Toonen and Verhage, 2003; Toonen et.al., 2005). SM proteins have been shown to genetically and physically interact with Rabs and their effectors; furthermore, it has been suggested that Rabs might modulate the interaction between SM proteins and Syntaxin (Misura et.al., 2000). Rabs might signal the arrival of a vesicle to its target membrane via SM proteins,

which, in turn, set in motion the steps that lead to membrane fusion, such as changing the conformation of Syntaxin to an active form (Misura et.al., 2000).

In *Drosophila*, *rop* is required for both neurotransmission and secretion. Moreover, it has a similar phenotype to *rab11* mutants in the organization and maintenance of the posterior membrane compartment of the oocyte (Ruden et.al., 2000). Rop and Rab11 co-migrate on a sucrose gradient, suggesting that they localize to the same subcellular compartment or compartments. It is therefore possible that the ability of *rop* to enhance *beach1* overexpression is due to an interaction between Rop and Rab11.

It is also interesting that, while *rop* modifies the *beach1* gain-of-function, *syntaxin* does not. It is possible that Beach1 interacts with Rop in a Syntaxin-independent process, since SM proteins are thought to have syntaxin-independent roles in vesicle trafficking (Toonen and Verhage, 2003). Alternatively, Beach1, Rop, and Rab11 might function in a pre-fusion step in vesicle trafficking, such as the tethering of a vesicle to a membrane, rather than the actual fusion step, which is executed by Syntaxin and other SNAREs. A role in such pre-fusion step would be consistent with the fact that Rab11 has been shown to physically interact with members of the exocyst complex--a tethering complex that targets vesicles to sites of membrane addition (Zhang et.al., 2004; Beronja et.al., 2005).

Direct interaction

Finally, strong genetic interactions between *beach1* and *rab11* could be an indication that these proteins interact directly, as part of the same complex, or as part of the same domain on a vesicle or an organelle. This model is supported by their partial co-migration on a sucrose gradient and the overlap between the Beach1 and the Rab11-positive compartments at the neuromuscular junction. Still, further experiments are needed to confirm this hypothesis. First, an unambiguous co-localization must be established using reagents that are not prone to cross-reactivity: such experiments are currently

underway. Second, co-immunoprecipitation experiments will be needed to show that these proteins can form a complex. I have attempted to co-immunoprecipitate Beach1 and Rab11 from *Drosophila* S2 cells, in which both these proteins were overexpressed. Unfortunately, the results of these experiments were ambiguous, since I could not establish a clean negative control. Similar experiments will need to be repeated, preferably from endogenously expressed proteins, to show co-immunoprecipitation.

The demonstration of a direct binding between Beach1 and Rab11 will be difficult due to the large size of Beach1. Information about the domains in Beach1 that are required for binding, which could be gained from co-immunoprecipitation experiments with various deletion and truncation constructs, will be needed prior to the direct binding assays.

Some highly-speculative models

There are many models one can come up with to explain how Beach1 and Rab11 interact; however, it is difficult to give one of them more weight than the other without knowing more about the molecular function of Beach1. One can imagine that Beach1 is a Rab11GAP—a GTPase activating protein, or that it is a scaffolding protein that recruits a GAP to the Rab11 compartment (Figure 9). A GAP regulates the activity of a GTPase by putting it in a GDP-bound or inactive state. Thus, overexpression of *beach1* would lead to a reduction in *rab11* function, while a loss of Beach1 would increase Rab11 activity. Thus, a reduction in or a loss of *beach1* function could suppress *rab11* phenotypes by permitting the remaining Rab11 molecules to stay in an activated state. According to this model, a loss of *beach1* function would not be able to bypass a requirement for *rab11*, which is consistent with my results.

In an alternative model, Beach1 could act as a negative regulator of the interaction between Rab11 and Rop. This may, in turn, negatively regulate the fusion between Rab11-positive vesicles and the plasma membrane. A loss of

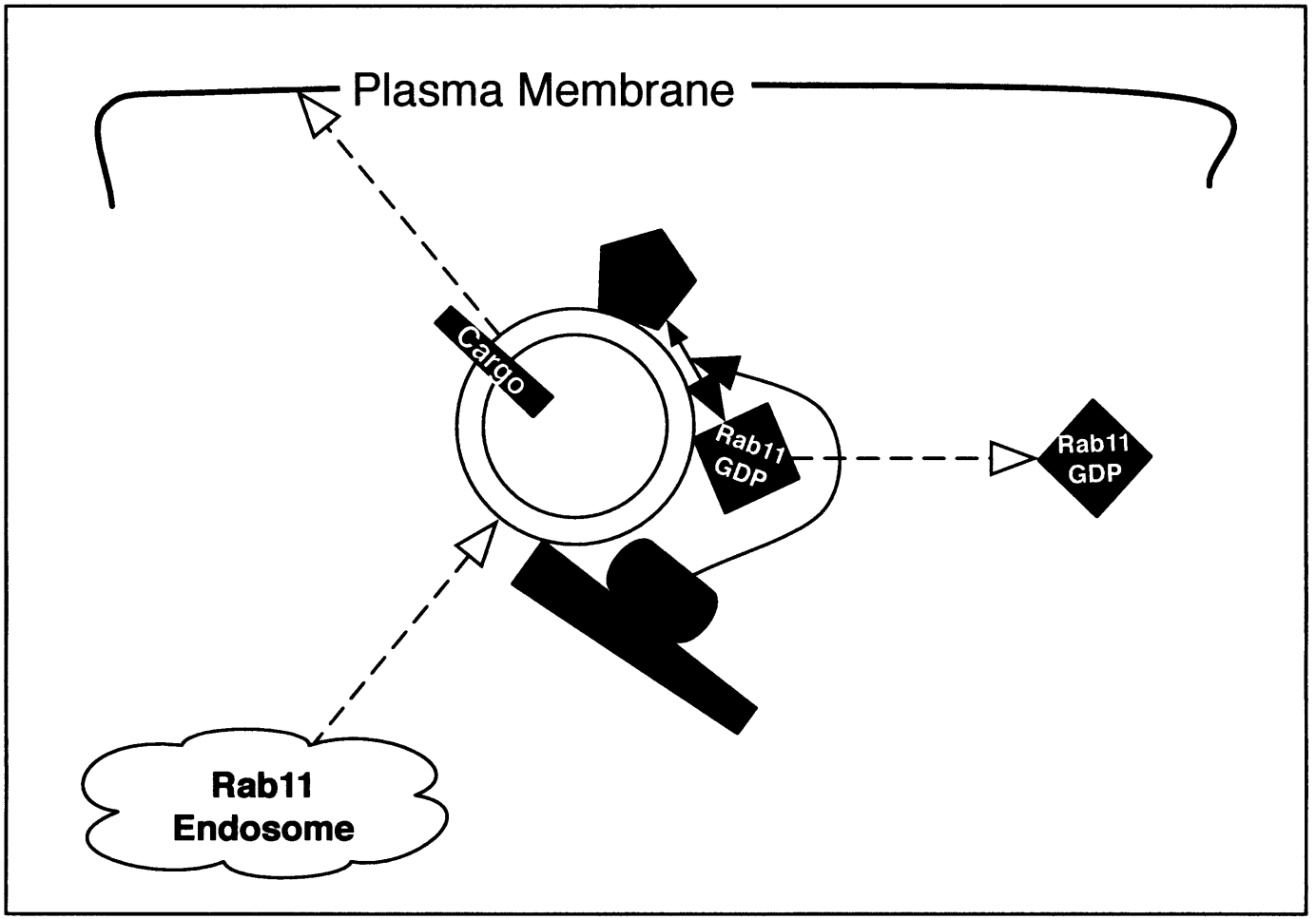
beach1 would relieve such an inhibition and suppress phenotypes caused by a decrease in the level of Rab11.

There are many other possible explanations for how Beach1 and Rab11 might interact. Beach1 could regulate various steps in the Rab11 cycle, such as its localization to the correct membrane, its activation, or the recruitment of its effectors. Interestingly, I made an observation that *beach1* mutants have more *rab11* puncta at the NMJ than controls, while there was no observable increase in the total level of Rab11 protein. If this result is confirmed, it will be interesting to look into the cause behind this observation.

Beach1 is enriched in the presynaptic terminal, which is equivalent to the apical membrane domain, and Rab11 is known to deliver newly synthesized or recycled cargo to such sites. Therefore, it appears that Beach1 is likely to function in one of the plasma membrane-proximal steps of Rab11-dependent trafficking, rather then the steps of vesicle formation from the *trans*-Golgi or the perinuclear recycling endosome. However, it is difficult to know precisely where Beach1 fits in without a better understanding of both its molecular mechanism of function and the mechanisms behind all the steps in the Rab11-dependent trafficking pathways.

Figure 9. A Potential Model for the Mechanism of Interaction between Beach1 and Rab11

Beach1 could recruit a Rab11 GAP--GTPase activating protein to a Rab11-positive compartment, leading to an increase in the rate of conversion from an active form, Rab11 GTP, to an inactive form, Rab11GDP. GDP-bound Rab11 would then be extracted from the membrane by a GDI--GDP dissociation inhibitor, and recycled.


Plasma Membrane
Cargo
Rab11
GDP
Rab11
GDP
Rab11
Endosome

What does the NMJ phenotype tell us

The viability and the bristle phenotypes of *rab11* mutants served well to establish an interaction between *rab11* and *beach1*; however it would have been difficult to further analyze the mechanism behind their interaction in these systems. The neuromuscular junction, another place where these proteins interact, is a much better place for follow-up analysis, because it combines all the advantages of *Drosophila* genetics with a well-described morphology and anatomical, as well electrophysiological, accessibility.

Rab11 mutants exhibit an overgrowth defect at the NMJ. Specifically, they show a significant increase in the number and branching of synaptic boutons. Branch formation is a part of normal synaptic growth: branches form from pre-existing boutons in a process that superficially resembles budding in yeast (Zito et.al., 1999). Synaptic growth is a highly regulated process and, normally, most boutons at a given NMJ, excluding the terminal boutons, connect to only two neighbors. A small fraction of boutons (fewer than 10%), termed branching boutons, have three or more connections. *Rab11* mutants exhibit synaptic overgrowth and an increase in bouton branching, suggesting that they have misregulated synaptic growth.

Mechanisms of synaptic growth

Several mechanisms have been implicated in the regulation of synaptic growth and morphology at the *Drosophila* NMJ, such as activity, protein turnover, the cytoskeleton, signaling cascades, and endocytosis (Jin, 2002 and Koh et.al, 2000). However, many of the previously described mutants that affect these processes have phenotypes that do not resemble those seen in *rab11* mutants. For example, increased electrical activity that results from mutations in Shaker—a type I_A Potassium channel, causes an increase in bouton number. However, this phenotype is unlike that seen in *rab11* mutants; in *Shaker* mutants the synapse expands over a broader muscle area, but bouton and branch morphology remains relatively normal (Jarecki and

Keshishian, 1993 and Mosca et.al., 2005). Similarly, mutations in *highwire*, a ubiquitin ligase, as well as overexpression of *fat facets*, a deubiquitinating protease, lead to an expansion of the synapse over a larger than normal muscle area (Wan et.al., 2000 and DiAntonio, 2001). Mutants in the BMP receptor *wishful thinking*, which is required presynaptically, and the gene encoding its ligand *glass bottom boat*, which is the retrograde signal coming from the muscle, have a slightly reduced number of boutons (McCabe et.al. 2003, Aberle et.al., 2002, Marques et.al., 2002). Similarly, in the animals mutant for *wingless*, a *Drosophila* Wnt, synaptic boutons are poorly defined and reduced in number (Packard et.al., 2002). Thus, none of these mutants cause a defect in synaptic morphology similar to that observed in *rab11* mutants.

Mutants with defects in endocytosis have phenotypes similar to *rab11* mutants

Interestingly, phenotypes observed in mutants that perturb endocytosis resemble overgrowth defects seen in animals with reduced *rab11* function. Loss-of-function mutants in the *dynamin-associated protein 160kD* (*dap160*) and other endocytic proteins result in an increased number of satellite boutons--small boutons, which are clustered around the larger boutons (Koh et.al., 2004, Marie et.al., 2004, and Dickman and Schwarz, unpublished data). Although it is not completely understood how a defect in endocytosis leads to this overgrowth phenotype, it is possible that a signal transducton pathway, which requires endocytosis, is involved. This is not an unlikely hypothesis, since it is becoming increasingly clear that endocytosis is not just a mechanism for the downregulation of signals, but is also important for the temporal and special control of signal transduction (Miaczynska and Zerial, 2004).

Why would a mutant in *rab11*, which has been implicated in the delivery of membrane and proteins to the plasma membrane, have a phenotype

reminiscent of endocytosis mutants? Perhaps, the defect in *rab11* mutants lies in the failure to deliver essential components of the endocytic machinery to the synaptic terminals, thus having a secondary effect on endocytosis. Satoh et.al. gave an analogous explanation for the paucity of multivesicular bodies (MVBs) in the *rab11* mutant photoreceptors, a phenotype usually attributed to a defect in endocytosis. They demonstrated that in *rab11* mutants the labyrinthine channels in garland cells, which are the sites of very active endocytosis, are absent, a phenotype opposite to the elongation of these channels observed in endocytic mutants, such as *shibire*. This *rab11* phenotype is consistent with a decrease in the delivery of membrane and components essential for sustaining high rates of endocytosis, which is caused by diminished apical delivery (Satoh et.al., 2005). Thus, although the primary defects in *rab11* and endocytic mutants, are different, the downstream manifestations of these defects, such as the absence of MVBs in the photoreceptors, can be the same.

Alternatively, *rab11* could be required in the muscle for the secretion of a signal that regulates presynaptic growth. However, such a model would make it difficult to explain how Beach1, which is enriched presynaptically, suppresses the *rab11* overgrowth defect.

Synaptic morphology and the cytoskeleton

Signaling cascades that transduce extracellular cues through transmembrane receptors to the regulators of the cytoskeleton are known to control neuronal migration and growth cone morphology in both mammals and insects (Dent and Gertler, 2003; Korey and Van Vactor, 2000). The actin cytoskeleton has also long been suspected in the regulation of synaptic growth and morphology; however, until recently, this has not been demonstrated. Recently, a mutant in *nervous wreck* (*nwk*) was shown to have an aberrant synaptic morphology, with an increase in bouton number and bouton branching Of all the known mutants that affect synaptic morphology, the *nwk*

phenotype is most similar to the defects caused by *rab11*; however, the overgrowth in *rab11* mutants is much more dramatic. Nwk is an SH3 adaptor protein that shows genetic interactions with the *Drosophila* ortholog of Wasp (Wsp) and biochemically interacts with Wasp via its SH3 domain (Coyle et.al., 2004). Wasp and its homologs regulate actin assembly via the ARP2/3 complex--a central regulator of actin cytoskeletal dynamics (Dent and Gertler, 2003). Thus, Nwk could regulate synaptic growth and branching as part of a signaling cascade that begins with an extracellular cue and culminates in the reorganization of the actin cytoskeleton by Wasp (Collins and DiAntonio, 2004).

Since the NMJ phenotype in *rab11* mutants most resembles that of *nwk*, perhaps the best explanation for the *rab11* synaptic overgrowth is a misregulation of the actin cytoskeleton. Rab11 has been previously implicated in processes where both membrane addition and cytoskeletal rearrangements are involved, such as cytokinesis in *C.elegans* and mammalian cells and cellularization of the *Drosophila* embryo (Skop et.al., 2001; Wilson et.al., 2005; Riggs et.al., 2003; Pelisser et.al., 2003). Moreover, bristle extension in *Drosophila*, which is aberrant in *rab11* mutants, is another instance in which both of these processes are required. Bristle growth is driven by the assembly of short modules of actin filaments into long actin bundles; however, significant membrane addition is also required to support this rapid growth (Tilney et.al., 1996). The mechanism by which Rab11 might simultaneously coordinate both of these processes is not understood. One possibility is that it is simply required for the delivery of cytoskeletal regulators and components of the signaling pathway that lie upstream (Riggs et.al., 2003). It has even been suggested that Rab11 vesicles might bring membrane and actin as a unit to sites where both are needed (Rothwell et.al., 1999). Furthermore, Rab11 might interact with cytoskeletal regulators, such as Arf GTPases, via dual Rab-Arf effectors (Turner and Brown, 2001; Prekeris, 2003).

Beach1 suppresses *rab11* defects in processes where both membrane and actin dynamics are at play, such as bristle extension and synaptic growth. Furthermore, *beach1* overexpression causes a defect in growth cone morphology, another place where these processes might meet. Thus, it is likely that Beach1, together with Rab11, is somehow involved in the coordinate regulation of membrane traffic and the cytoskeleton. However, none of the regulators of the actin cytoskeleton that were tested in the modifier screen, such as the Rho family of GTPases, the Capping Protein, and the *Drosophila* homolog of Profilin, had an effect on the *beach1* overexpression phenotype. There are many reasons why this could have been the case. For instance, *beach1* and *rab11* could lie too far upstream of these regulators to show a genetic interaction, or they could affect the cytoskeleton via a different set of regulators. Another possibility is that the *beach1* overexpression phenotype is too strong to be modified by these genes. Finally, it is possible that the actin cytoskeleton does not have a role in the growth cone phenotype. It will be interesting to see whether the *rab11* phenotype at the NMJ can be modified by genes involved in the regulation of the actin cytoskeleton, such as *wasp*. The NMJ might be a good system to gain a better understanding of how the *beach1-rab11* pathway may tie in with the regulation of the cytoskeleton.

Materials and Methods

Fly stocks

The following stocks were obtained from the Bloomington stock center: the *Drosophila* deficiency kit and all the smaller deficiencies and reagents in the intervals of interest, $rab11^{P2148}$, $rab11^{93Bi}$, rop^{G27} Df1128 (BL1128-Df(2L)GpdhA/CyO breakpoints 25D7-E1;26A8-9 and Df3365 (BL3365-Df(2L)cl7, pr[1] cn[1]/CyO, breakpoints 25E1-2;26A7), Rab1 dominant negative, P-element insertion in Rab14-12457, a deficiency uncovering Rab3, a Rab5 mutant (k08232), a Rab6 mutant (stock 5821-d23dmutant).

yw; *hrsl(2)*^{Ad028} (Lloyd et.al., 2002), bw; st *Rop*^{A19}/TM6B, and bw; st *Rop*^{G11}/TM6B (Wu et.al., 1998) were a gift from the Bellen lab (Baylor College of Medicine).

rab11^{ex1}/TM3, Sb and *rab11*^{ex2}/TM3, Sb (Dollar et.al., 2002) were a gift from the Cohen Lab (University of Kansas).

The *beach1, rab11* double mutants were made by first making stocks that have an allele of *beach1* and an allele of *rab11* over a fused second-third chromosome balancer, S:T, which is marked with Cy and Tb-. These were then crossed to one another to create the desired double mutant combinations.

Screen for modifiers of *beach1* overexpression

The Deficiency Kit contained 195 lines with 75-80% genome coverage. Each line from the Deficiency collection was crossed to flies carrying a recombinant GMR Gal4, EP:Beach1 chromosome. The resulting progeny, which carried one copy of the GMR:Beach1 chromosome and were heterozygous for the deficiency, were examined for modification of the adult eye phenotype. To rule out enhancement due to an additive effect, each deficiency was also examined for the ability to cause a rough eye in the absence of *beach1* overexpression. To identify the modifying lesion smaller deficiencies, which covered the original deficiency region, were obtained from the Bloomington stock center and screened. Once interaction was narrowed down to the smallest available deficiency, all reagents encompassed by that deficiency, including alleles of known genes, lethals, and p-element insertions, were screened. Individual candidate genes were screened in a similar manner.

Quantitation of the *beach1* suppression of *rab11* lethatity and bristles defects

In the viability experiment, equal numbers (60) 2nd instar larvae of each genotype were placed in identical vials at 25^{o}C and monitored every day for survival to adulthood.

To quantify the suppression of bristle loss, the number of bristle-filled and the number of empty sockets was counted in the last row of abdominal tergites (segments) 2, 3, and 4 in newly-eclosed animals. At least 10 animals of each genotype were counted. All the counting was done under the dissecting microscope. P-value was determined using a two-tailed unpaired Student t-test.

Subcellular fractionation and Western blotting

Sucrose gradient was prepared by Bill Adolfsen (Littleton Lab, MIT) according to the protocol described in Adolfsen et.al., 2004. For western blot analysis, each lane of a 7, 10, or 12% SDS-polyacrylamide gel was loaded with a 15μl of each gradient fraction. The gel was run, then transferred to Hybond-P membrane (Amersham Pharmacia). Membranes were blocked in 5% nonfat milk and probed with antibodies at following dilutions: Beach1 antisera (3775) 1:1,000 (generated as described in Chapter2), mouse Rab11 (BD Biosciences, Parmingen) 1:1000, Rop (4F8) mouse monoclonal (Harrison, et.al., 1994) 1:1000 (gift from the Bellen Lab), HRS#GP30 guinea pig policlonal (Lloyd et.al., 2002) (a gift from the Bellen Lab) 1:20,000 and Synaptotagmin (rabbit) 1:500 (gift from Littleton lab), anti-Elav (Hybridoma bank) 1:1,000. All HRP-conjugated goat anti-rat, mouse, rabbit, guinea pig secondary antibodies (Jackson Laboratories) were diluted at 1:5,000.

Immunohistochemistry

3rd instar body wall dissections were done in PBS and fixed in 4% PFA in PBS. Rat anti-Beach1 antiserum (3775) was preabsorbed against *beach1* null animals and used at 1:500. Goat anti-rat Cy3 secondary (Jackson Laboratories) was used at 1:500. Subtracted goat anti-rat Cy3 (Jackson Laboratories) was used at 1:100. Mouse anti-Rab11 antibody (BD Biosciences) was used at 1:200, goat anti-mouse Alexa-488 was used at 1:100. Cy5- and FITC-conjugated anti-HRP were used at 1:100. Confocal data

was acquired as single images or image stacks of multi-tracked, separate channels with a Zeiss LSM 510 microscope.

Quantitation of the overgrowth and bouton branching phenotypes

Confocal stacks through 3rd instar NMJ 6/7 were taken and flattened into projections using the LSM 510 software. Each bouton was marked on the image by a colored dot using Adobe Photoshop and the resulting dots were counted using ImageJ software. The number of branching boutons was counted in the same manner. Muscle surface area was estimated at described in Schuster et.al., 1996. P-value was determined using a two-tailed unpaired Student t-test.

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Chapter 4: Discussion

Beach1 and Rab11 interact

In my graduate work, I identified Beach1, a member of the BEACH family of proteins with putative roles in membrane trafficking, in an overexpression screen for regulators of axon guidance and growth cone morphology. Although *beach1* overexpression causes a strong alteration in growth cone shape, loss of *beach1* function has no obvious phenotype. However, a screen for modifiers of *beach1* overexpression uncovered *rab11*, a member of a family of small GTPases, which are known regulators of vesicle trafficking. Examination of *beach1*, *rab11* double mutants revealed a requirement for *beach1* during development and in bristle extension. Finally, examination of synaptic morphology revealed a requirement for *rab11* and *beach1* in the regulation of synaptic growth. Beach1 functionally antagonizes Rab11 in all of the processes I examined; furthermore, fractionation and double-labeling experiments suggest that these proteins may function in the same subcellular compartment. However, further experiments are needed to determine whether Beach1 and Rab11 interact directly, function in the same protein complex, or closely cooperate in the same molecular pathway.

Why am I not surprised?

Evidence presented in this thesis is the first demonstration of an interaction between Rab11 and Beach1; however, a functional link between these two proteins should not come as a complete surprise. Rab11 and the only well-characterized homolog of Beach1, the *Dictyostelium* LvsA, have been previously shown to function in some of the same processes. Both LvsA and Rab11 are required for cytokinesis—a highly conserved process of cell division following mitosis (Kwak et.al., 1999; Skop et.al., 2001; Riggs et.al., 2003; Pelisser et.al., 2003; Wilson et.al., 2005). Furthermore, both Rab11 and LvsA are involved in the regulation of the contractile vacuole (CV) in *Dictyostelium* (Harris et.al., 2001; Gerald et.al., 2002). Notably, it has been suggest that the CV compartment in amoebae might be an evolutionary precursor of the

recycling endosome (Harris et.al., 2001). Indeed, some internalized proteins in *Dictyostelium* are recycled back to the plasma membrane via the contractile vacuole. This is an interesting parallel, since Rab11 is known to regulate membrane trafficking through the recycling endosome to the plasma membrane in mammalian cells and *Drosophila* (Ren et.al., 1998; Riggs et.al., 2003). One possibility is that the contractile vacuole has evolved into the recycling endosomal compartment. Alternatively, after the CV became obsolete, Rab11 and, likely, Beach1, could have retained their functions in directing membrane proteins from the recycling compartment back to the plasma membrane.

From growth cone to synapse

In my research I seem to have come full circle from the growth cone phenotype caused by *beach1* overexpression to the role of *beach1* in the regulation of synaptic bouton morphology. Both processes affect terminal regions of neurons, and both are thought to involve membrane and cytoskeletal reorganization. Finally, it is likely that, as in the regulation of synaptic growth, a *rab11*-dependent pathway is at the root of the *beach1* overexpression phenotype.

My results place Beach1 in a pathway with Rab11 and provide strong evidence that these two proteins interact very closely, perhaps even directly. However, I was unable to determine the mechanism by which Beach1 antagonizes Rab11, or to establish exactly at which point during membrane trafficking these two proteins interact. Before this is possible, we will need to fill in the gaps in our understanding of the *rab11*-regulated membrane traffic and to gain some knowledge about the molecular function of Beach1 and other BEACH proteins.

Help is on the way from the NMJ

I found that *beach1* and *rab11* are involved in the regulation of synaptic morphology at the neuromuscular junction (NMJ). The *Drosophila* NMJ, which is amenable to genetic, anatomical, and electrophysiological analysis, is a useful system for delving into the mechanisms behind *rab11* phenotypes, and for investigating how *beach1* may be involved. For example, experiments to test for genetic interactions between *rab11* and *nervous wreck* (*nwk*) or *wasp (wsp)* may help to determine how closely the *rab11* phenotype is linked to the actin cytoskeleton. Similar studies were done at the NMJ to demonstrate that *nwk* and *wsp* function in the same pathway. Double mutants of *nwk*, *wsp* have a much higher incidence and complexity of hyperbranched boutons, those generating over three branches, than the *nwk* single mutants (Coyle et.al., 2004). It will be interesting to see if similar interactions are observed for *rab11* and *wasp*, or *rab11* and *nwk*.

The *rab11* NMJ overgrowth phenotype, which is suppressed by *beach1*, also resembles those of endocytic mutants, such as *dap160* (Marie et.al., 2004; Koh et.al., 2004). One possibility is that in *rab11* mutants the delivery of components of the endocytic machinery to the plasma membrane is compromised, resulting in a secondary defect in endocytosis. This hypothesis can be easily tested by immunohistochemistry experiments at the NMJ, using antibodies against various endocytic proteins. Another possibility is that *rab11* is required for the secretion of a postsynaptic signal that subsequently requires endocytosis at the presynaptic terminal. The question of whether *rab11* is required on the side of the muscle or the neuron can be answered with rescue constructs driven in either the muscle or the nerve.

Some regulators of vesicle trafficking, such as the SNARE proteins, are involved in both membrane addition and neurotransmission in neurons (Sudhof, 2000). Others, like the exocyst complex, are required for membrane addition in the growing neurite, but not for neurotransmitter release (Murthy et.al., 2003). Since Rab11 has been shown to physically interact with certain

members of the exocyst complex (Zhang et.al., 2005; Beronja et.al., 2005), it will be interesting to learn whether *beach1* and *rab11*, like the exocyst, are also selectively involved in membrane addition. This can be accomplished by examining the electrophysiology of *rab11* mutants and of *beach1, rab11* double mutants.

Synaptic ultrastructure of the neuromuscular junction has been extensively examined both in wildtype animals and in numerous mutants. Therefore, defects in various aspects of this ultrastructure, such as the number and morphology of synaptic vesicles, the shape of boutons, and the number and distribution of active zones, can provide interesting insight into the mechanisms behind synaptic phenotypes. Thus, EM analysis of *rab11* mutants and *beach1, rab11* double mutants could yield further information about the subcellular basis of the *rab11* defect and its modification by *beach1*.

Getting a clue to BEACH protein function

BEACH proteins have long been suspected of being involved in membrane trafficking; however, a mechanism for their involvement had previously not been described. Therefore, my discovery of the interaction between Beach1 and Rab11 may provide the first explanation of how Beach1 and other BEACH proteins fit in. It is possible that all BEACH proteins regulate membrane trafficking via interactions with Rab GTPases. It has already been suggested that LvsB, a homolog of the Chediak-Higashi protein, might regulate lysosomal dynamics by interacting with a lysosomal Rab (Harris et.al., 2002). Moreover, since membrane trafficking, especially endocytosis, plays an important role in signal transduction (Miakzynska and Zerial, 2004), it would not be a stretch to reason that BEACH proteins that regulate signaling, such as the mammalian FAN and LRBA, and the *Drosophila* AKAP550, do so by regulating vesicle trafficking events in conjunction with Rabs. Of course, much work remains to be done to determine whether and how other BEACH proteins and Rab GTPases interact.

I hope that my discovery of a link between Beach1 and Rab11 will lead to further investigation into the interactions between these two families of proteins. Such research is likely to provide insight not only into the mechanism of function for BEACH proteins, but for Rab GTPases as well.

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