The HOPS complex and Vps33 in *Dictyostelium discoideum*

Nicolas Caffarelli

The University of Texas at Austin

2009

_____ _____

Dr. Arturo De Lozanne — Date
Supervising Professor

Table of Contents

Abstract

In order for cells to eat, they must detect food at their cell surface and pull it in to form a vesicle known as an endosome. This endosome then becomes acidified to become a lysosome and eventually becomes neutral again, as a postlysosome, so that waste can be expelled from the cell. Collectively, this is referred to as the endocytic pathway. Several proteins are involved in this process – Rabs are known to mediate specificity of fusing vesicles, and SNARES catalyze the actual vesicle fusion. In this study we look at the protein Vps33, a subunit of the HOPS complex. The HOPS complex is known to interact with Rabs and SNARES, and we are interested as to where it acts within the cell. By tagging Vps33 with Green Fluorescent Protein (GFP), we can visualize its localization under the microscope. We observe here that Vps33 localizes primarily to the cytoplasm, with sparse localization to intracellular vesicles.

Introduction

All organisms require an energy source to survive, and in consumers such as animals and fungi this energy must come in the form of food. Thus, these organisms require a method for ingesting and processing macromolecules, and Eukaryotes have developed intracellular pathways for this purpose.

Upon making contact with potential food, the cell's plasma membrane invaginates and forms an endosome. Within a minute, ATPase proton pumps are shuttled to the endosome and acidify it such that it becomes a lysosome. The lysosome contains hydrolytic enzymes activated at low pH, and will undergo several fission and fusion events. Eventually the lysosome is de-acidified and becomes a neutral post-lysosome, which can then exocytose remaining waste at the plasma membrane. (Maniak 2003)

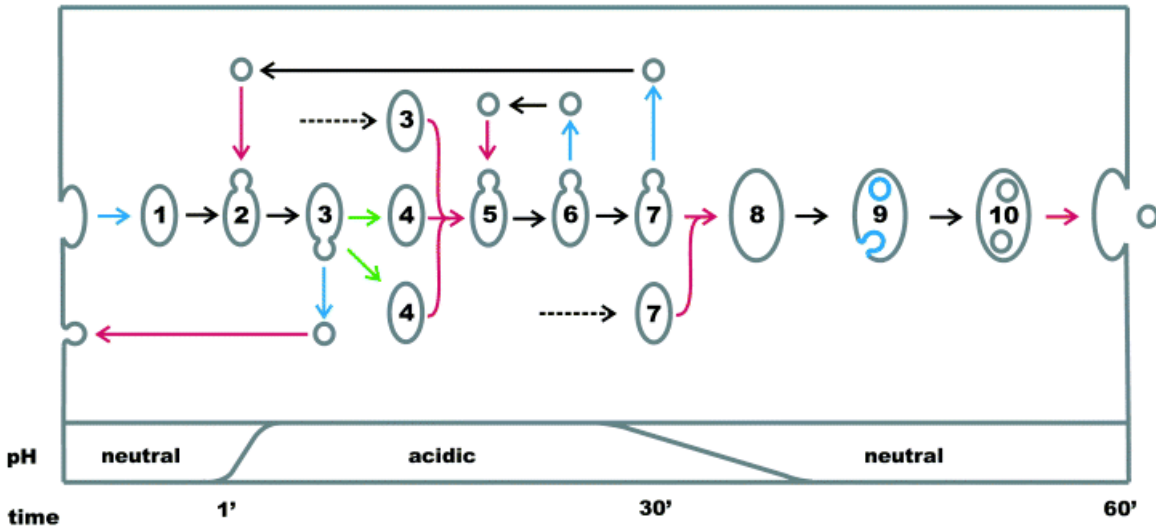


Figure 1 (adapted from Maniak 2003) The *Dictyostelium* endocytic pathway **1.** A new endosome is formed within the cell and **2.** becomes a lysosome through acidification. **3.** Endocytosed plasma membrane proteins are sent back through small vesicles and several **4.** fission and **5.** fusion events are undertaken. **6.** Hydrolytic enzymes are recycled to earlier lysosomes followed by **7.** de-acidification and recycling of proton pumps. **8.** The resulting postlysosome will often become a **9.** multivesicular body that is eventually **10.** exocytosed.

Many proteins are involved in this pathway, including the well-characterized Rabs and SNARES. Rabs are GTPases, meaning that they are capable of binding GTP and hydrolyzing it to GDP. GTP-Rabs are bound to an organelle membrane through a

prosthetic lipid group, and are capable of executing several functions. Subsequent hydrolysis to GDP leads to sequestration of the prosthetic group, possibly by another protein, such that the Rab becomes soluble in the cytoplasm. The GDP can then be exchanged for new GTP in order to re-initiate the cycle. While the classical role of Rabs is defining membrane specificity during vesicle fusion, they are also thought to be involved in vesicle cargo sorting, vesicle transport, and tethering. (Grosshans et al. 2006)

SNAREs (soluble N-ethylmaleimide sensitive factor attachment protein receptors) are integral membrane proteins that mediate membrane fusion through the formation of coiled coils, in which four unique snares will form a complex to induce fusion. While the coiled-coil region generally contains hydrophobic residues, it will also contain either an arginine or glutamine residue, such that SNAREs are classified as either R or Q, respectively. This residue is found at a point within the coiled coil termed the '0' layer, and generally tetrameric SNARE complexes will contain 3 Q-SNAREs and 1 R-SNARE. Like Rabs, SNAREs have been demonstrated to play an important role in vesicle fusion specificity. Often, they contain a region that binds to SM (Sec1/Munc18) proteins, which are necessary for vesicle fusion. SM proteins are thought be important for SNARE assembly and/or SNARE proofreading. (Dacks et al. 2009)

Recently studies have shown that vesicle fusion and specificity is also regulated by the HOPS complex. This complex was first characterized by Seals et al. (2000) and was shown by immunoprecipitation to be composed of six proteins: Vps39, Vps41, Vps11, Vps16, Vps18, and Vps33. The latter 4 proteins are all classified as class C Vps proteins, such that their absence results in loss of vacuoles, whereas Vps39 and Vps41 are class B Vps proteins that display many small vacuoles when absent (Banta et al., 1988).

Price et al. (2000) found that the HOPS complex is normally bound to SNAREs such as Vam3p, Vti1p, and Ykt6p, but is then released in the presence of NSF/Sec18p and ATP. This dissociation is followed by binding to the Rab Ypt7p in its GTP-bound state (Price at al., 2000). A later study by Stroupe et al. (2006) further revealed the HOPS complex's ability to bind phosphoinositides and the SNARE vam7p. Lastly, Peplowska et al. (2007) found that Vps41 and Vps39 can dissociate from the HOPS complex, while Vps8 and Vps3 will associate with remaining C-class protein core to form a CORVET complex. They also found that the CORVET complex tends to localize mostly on newly-formed endosomes whereas the HOPS complex is primarily found on the yeast vacuole, an organelle analogous to the vertebrate lysosome but with additional osmotic function.

Of all the proteins within the HOPS and CORVET complexes, perhaps the best characterized is vps33. Mutations in vps33 have been shown to cause ARC syndrome in humans (Gissen et al., 2004, 2006), resulting in muscle degradation, kidney failure, and infant mortality. Vps33 is capable of binding and hydrolyzing ATP and will localize to punctuate structures during ATP depletion (Gerhardt et al., 1998). It also binds SNAREs and shares homology with the SM proteins. Lastly, Vps33-null yeast have been shown to missort and secrete hydrolytic enzymes normally destined for the lysosomal vacuole (Banta et al., 1990).

While the majority of studies on the HOPS complex have been performed in yeast, there is little known on its function in more complex systems such as vertebrates. Here we use the social amoeba, *Dictyostelium discoideum*, as a model organism for studying the HOPS complex. Whereas yeast contain a common osmotic vacuole/lysosome as the final endpoint of endocytosed material (Baba et al., 1997), the

Dictyostelium endocytic pathway is disjunct from the contractile vacuole (Maniak 2003) and thus is similar to the vertebrate system. In this study, we tag the HOPS complex subunit vps33 with Green Fluorescent Protein (GFP) to observe its localization within *Dictyostelium*. We find that vps33 localizes primarily to the cytoplasm, with some localization to vesicles.

Methods

Cloning

The *Dictyostelium* vps33 gene was cloned from two segments using a unique BglII restriction site within the gene. The N-terminal segment was amplified from the cDNA clone dda19p04.3, generously provided by Dr. Hideko Urushihara (Dictyostelium cDNA project, University of Tsukuba, Japan). The upper primer (AO-731), designed with an additional 5' XhoI restriction site and to be in frame with GFP, was ACTCGAGAGATGTTTAAAAAACCAGCATCAC AAGC. The lower primer (AO-732) was AAGATCTTTACGAATTGATGGGAATGTAGG. The C-terminal segment was amplified from genomic DNA using upper primer (AO-730) TTGAAACCTACATTCCCATCAATT and lower primer (AO-733) GTCTAGACTGATGAAAAGGTACCACCATTATCTCC, which introduces a 5' XbaI restriction site. Each PCR-amplified segment was incubated 15 min with Taq polymerase to add A-overhangs and cloned into the Invitrogen TA vector pCR 2.1 and proper orientation was confirmed by restriction analysis. The C-terminal segment was then cloned into the N-terminal segment vector using BglII, such that the entire gene, without introns, was contained in the pCR 2.1 vector. The Vps33 gene was then extracted using XhoI and XbaI and ligated into the pTX-GFP vector, containing Green Fluorescent Protein to be tagged at the N-terminus of the insert. The integrity and veracity of the GFP-vps33 construct was confirmed by sequencing. All constructs were electroporated into AX2 *Dictyostelium* cells to be grown in HL-5 media with the selective marker G-418. GFP was detected by a western blot using anti-GFP primary antibody and HRP secondary.

Fixing

All cells were incubated 1 hr in Lo-flo media prior to fixing. 1-step methanol fix was performed by incubating cells in anhydrous MeOH with 1% formaldehyde at -20° C for 5 min. 1-step formaldehyde fix consisted of 15 min RT incubation in 1X PDF, 1mM $CaCl_2$, 2.5 mM $MgSO_4$, and 2% HCHO. 2-step fixing was performed by 15 min RT incubation in formaldehyde fixative followed by 5 min -20° C in methanol fixative. After fixing, all cells were rinsed in 1X PDF, washed with water, and mounted onto slides with MOWIOL + DABCO. For flattening, agarose squares were placed on cells previous to fixing and liquid was drained by wicking until cells appeared flat.

Microscopy

All images were taken on a Nikon Eclipse TE200 microscope with a Photometrics Quantix camera. Unless otherwise noted exposure time for all images was 200ms. Grayscale was aligned between control and experimental groups such that brightness is representative of signal strength.

Results

Bioinformatics of the *Dictyostelium* HOPS Complex and Vps33

The full genome of *Dictyostelium* has been sequenced and can be found online at Dictybase.org. Dictybase also contains the aligned sequences of cDNA clones, representing endogenous mRNA strands that were reverse transcribed and sequenced ("*Dictyostelium* cDNA project in Japan", http://dictycdb.biol.tsukuba.ac.jp/). The alignments were performed by H. Zhang (2003). Using these alignments as indicators of gene transcription sites, all 6 members of the HOPS complex have been identified, through homology with other organisms, and labeled within the Dictybase genome.

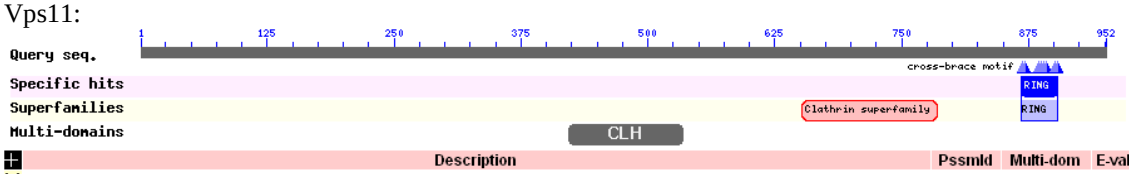
A Dictybase search for "HOPS" reveals the six proteins identified by Seals et al., (2000). Each of the DNA coding sequences for these proteins was submitted to NCBI nucleotide blast (http://blast.ncbi.nlm.nih.gov/Blast.cgi) for search within the Nucleotide collection database using discontiguous megablast. For each vps protein, this process yielded several appropriately labeled homologs (data not shown), suggesting that the Dictybase labels were accurate. Table 1 displays characteristics for the *Dictyostelium* genes encoding HOPS complex proteins.

Gene	Dictybase Gene ID	Genomic Length (bp)	Number of Introns	Protein length (residues)	Molecular Weight (kDa)
Vps11	DDB_G0278141	2945	1	952	108.28
Vps16	DDB_G0270754	2595	1	832	94.87
Vps18	DDB_G0269924	3836	3	1077	124.27
Vps33	DDB_G0291097	2055	1	644	72.60
Vps39	DDB_G0279169	2730	2	851	97.62
Vps41	DDB_G0286803	3362	1	1,087	124.67

Table 1 The six members of the HOPS complex in *Dictyostelium.*

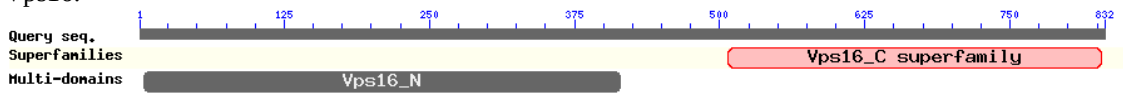
Each protein was then submitted into NCBI "Conserved Domains" tool (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) to search for potential characterized regions and domains, such that the following results were obtained:

Vps11:



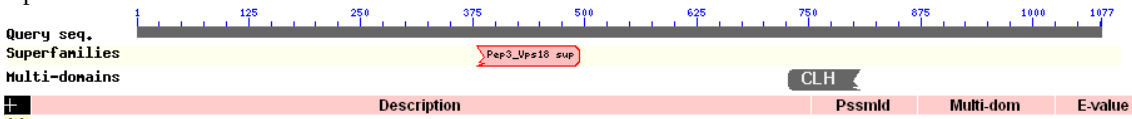
Description	Pssmid	Multi-dom	E-value
[+] pfam00637, Clathrin, Region in Clathrin and VPS	109685	N/A	5e-06
[+] cd00162, RING, RING-finger (Really Interesting New Gene) domain, a specialized type of Zn-finger of 40...	29102	no	0.003
[+] smart00299, CLH, Clathrin heavy chain repeat homology;	47627	yes	0.003

Vps16:



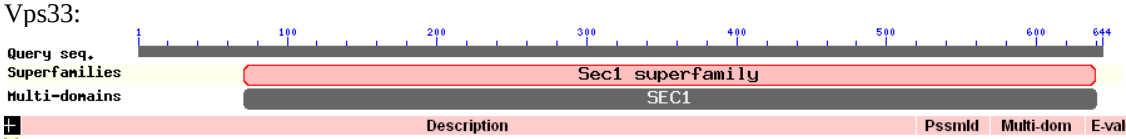
Description	Pssmid	Multi-dom	E-value
[+] pfam04840, Vps16_C, Vps16, C-terminal region	113606	N/A	2e-67
[+] pfam04841, Vps16_N, Vps16, N-terminal region	113607	yes	5e-60

Vps18:



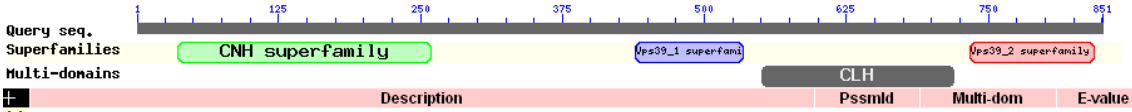
Description	Pssmid	Multi-dom	E-value
[+] pfam05131, Pep3_Vps18, Pep3/Vps18/deep orange family	113886	N/A	3e-22
[+] smart00299, CLH, Clathrin heavy chain repeat homology;	47627	yes	0.005

Vps33:



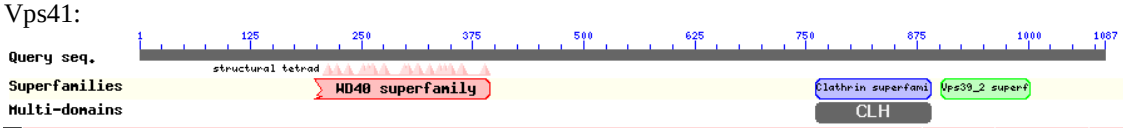
Description	Pssmid	Multi-dom	E-value
[+] pfam00995, Sec1, Sec1 family	110026	N/A	2e-63
[+] COG5158, SEC1, Proteins involved in synaptic transmission and general secretion, Sec1 family [...	34759	yes	2e-37

Vps39:



Description	Pssmid	Multi-dom	E-value
[+] pfam10367, Vps39_2, Vacuolar sorting protein 39 domain 2	118888	N/A	3e-35
[+] pfam10366, Vps39_1, Vacuolar sorting protein 39 domain 1	118887	N/A	1e-21
[+] cl02434, CNH, CNH domain	121308	N/A	7e-07
[+] smart00299, CLH, Clathrin heavy chain repeat homology;	47627	yes	7e-04

Vps41:

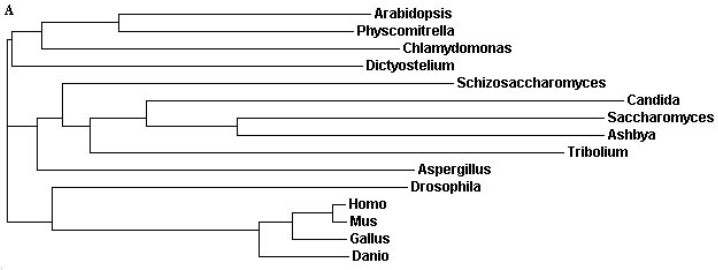


Description	Pssmid	Multi-dom	E-value
[+] cl02567, WD40, WD40 domain, found in a number of eukaryotic proteins that cover a wide variety of...	121405	N/A	2e-07
[+] pfam00637, Clathrin, Region in Clathrin and VPS	109685	N/A	1e-06
[+] pfam10367, Vps39_2, Vacuolar sorting protein 39 domain 2	118888	N/A	6e-04
[+] smart00299, CLH, Clathrin heavy chain repeat homology;	47627	yes	4e-17

The E-value, an indicator of confidence, is highly variable among putative domains – a smaller e-value indicates a higher degree of confidence. We see that some of the proteins contain self-identifying domains, such as Vps16 and to a lesser extent Vps18 and Vps39. Several proteins seem to contain a clathrin heavy chain homology domain. Appropriately, Vps33 displays a strong homology with Sec1, a well-characterized SM protein.

In order to observe the evolutionary history of Vps33, protein homologs from different species were obtained from NCBI and a phylogenetic tree was constructed using EBI clustalW alignment software (http://www.ebi.ac.uk/Tools/clustalw2/) with default settings. The resulting phylogram is displayed in Fig2A, with NCBI and Dictybase accession numbers in Fig2B. Vps33 is found only in Eukaryotes, although it covers a large spectrum with multiple phyla. Dictyostelium Vps33 shows an early and progressed divergence with closest relation to plants (Arabidopsis, Physcomitrella) and algae (Chlamydomonas).

A



B

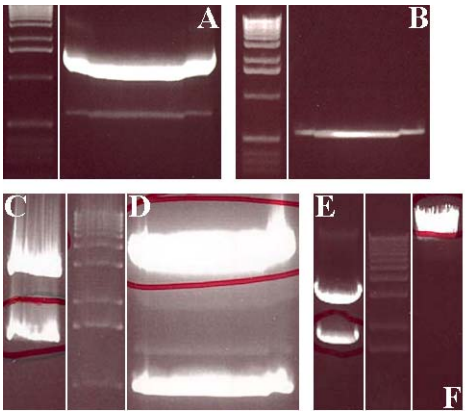
Organism	Accesion Number
Arabidopsis thaliana	AAK48903.1
Physcomitrella patens	EDQ77981.1
Chlamydomonas reinhardtii	XP_001703227.1
Dictyostelium discoideum	DDB_G0291097
Schizosaccharomyces pombe	CAB66459.1
Candida albicans	XP_710907.1
Saccharomyces cerevisiae	AAB82354.1
Ashbya gossypii	AAS52748.1
Tribolium castaneum	XP_971387.1
Aspergillus niger	XP_001399674.1
Drosophila melanogaster	NP_523410.1
Homo sapiens	NP_075067.2
Mus musculus	NP_084205.2
Gallus gallus	XP_415153.1
Danio rerio	NP_001093443.1

Figure 2 **A**. Phylogram for Vps33 proteins across different taxa. Branch length is representative of divergence. **B**. Accession numbers for protein sequences used in constructing the phylogram. All proteins are from NCBI except for *Dictyostelium*, which is from Dictybase.

Cloning and Expression of *Dictyostelium* Vps33

N-terminal and C-terminal segments (Fig3A and B, respectively) of Vps33 were amplified through PCR, gel extracted, and cloned into pCR 2.1. Dividing the gene into

two allowed us to use an intron-free yet incomplete cDNA clone for the N-terminus while supplementing the rest of the gene with genomic DNA. The C-terminal segment insert (Fig3C) was cloned into the N-terminal segment vector (Fig3D), and the entire intron-free VPS-33 gene (Fig3E) was then ligated into the pTX-GFP vector (Fig3F).



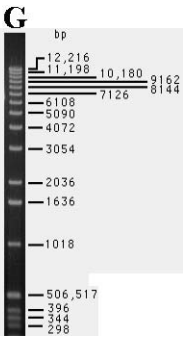


Figure 3 **A.** N-terminal segment amplified from clone dda19p04.3 (top band) **B.** C-terminal segment amplified from *Dictyostelium* genomic DNA **C.** C-terminal segment plus a section of pCR 2.1 vector as an insert (bottom band) **D.** N-terminal segment with remaining pCR 2.1 (top band) **E.** Vps33 gene without introns, constructed by ligating C and N-terminal segments (bottom band) **F.** pTX-GFP vector **G.** Invitrogen 1kb ladder used to determine molecular weight of DNA fragments.

GFP-Vps33 was transformed into *Dictyostelium*, and the pTX-GFP vector producing soluble GFP was transformed as a control. A western blot was performed to assess protein levels, and the GFP-Vps33 protein had an apparent molecular weight slightly greater than the predicted weight of 102 kDa (Fig4).

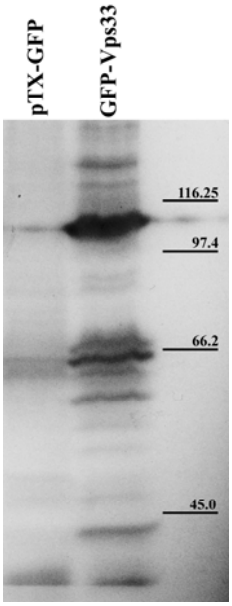


Figure 4 Western blot. Molecular weights (kDa) determined by BioRad Broad Range standard marker

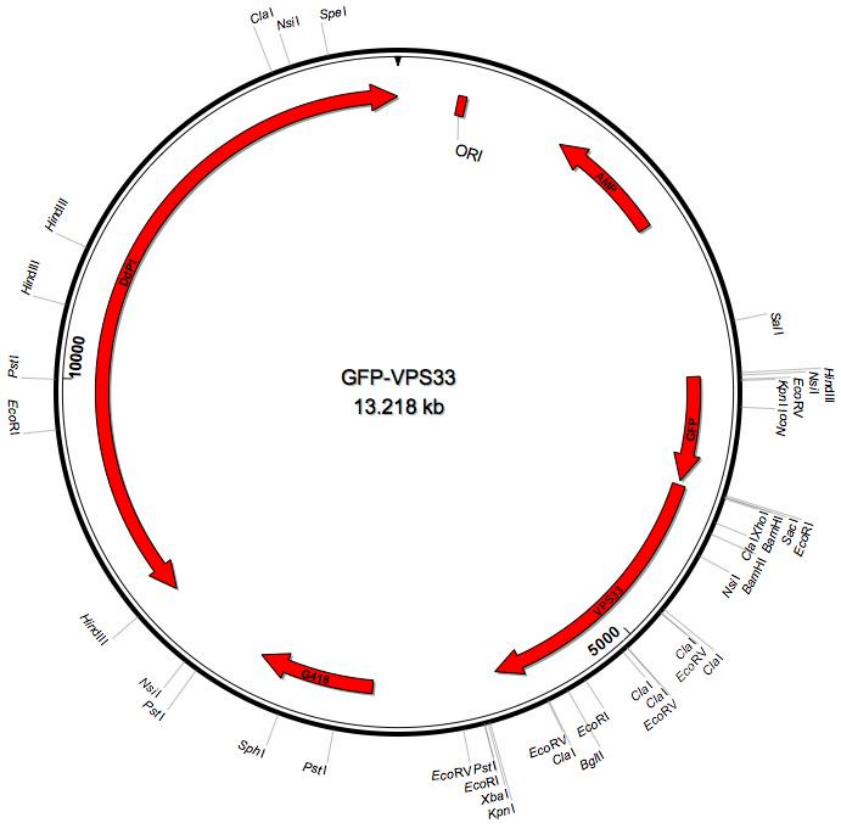


Figure 5 The GFP-Vps33 vector containing G418 and Ampicillin (AMP) resistance as well as a bacterial origins of replication site (ORI) and part of an endogenous *Dictyostelium* plasmid (DdP1), permitting replication within *Dictyostelium*.

Localization of Vps33

Live cells were incubated 1 hour in Lo-Flo media and observed for fluorescence (Fig6). In live cells Vps33 localizes to the cytoplasm, with occasional light vesicular staining. Control cells on average had slightly stronger expression, although both groups expressed strongly.

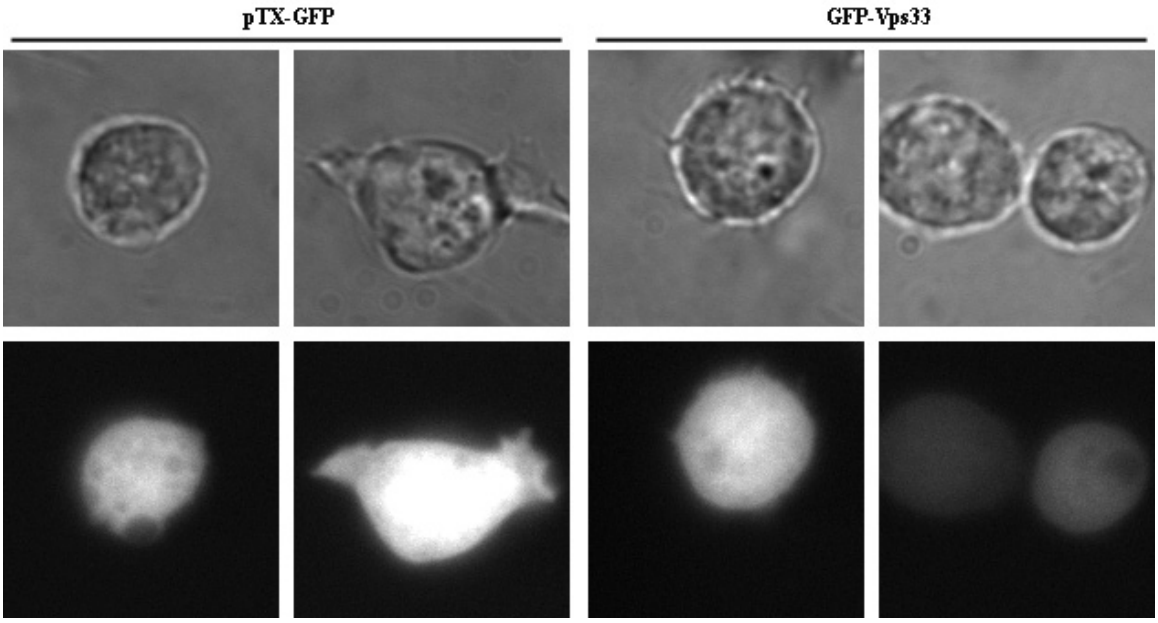


Figure 6 Live AX2 *Dictyostelium* cells transformed with either pTX-GFP control (left) or GFP-Vps33 (right). Images taken with 100ms exposure.

In order to reduce the cytoplasmic concentration and increase the relative signal of non-soluble Vps33, cells were fixed through various methods. Cells fixed in formaldehyde continued to show cytoplasmic localization, and sparse vesicle localization (Fig7). They also displayed a stronger signal relative to the control. In methanol-fixed cells (Fig8), the signal was vastly stronger than the control, and vps33 continued to localize to the cytoplasm. Methanol-fixed cells also showed vps33 localization to some vesicles as well as the plasma membrane. Cells that were sequentially fixed in formaldehyde and methanol continued to show cytoplasmic and vesicle localization (Fig9).

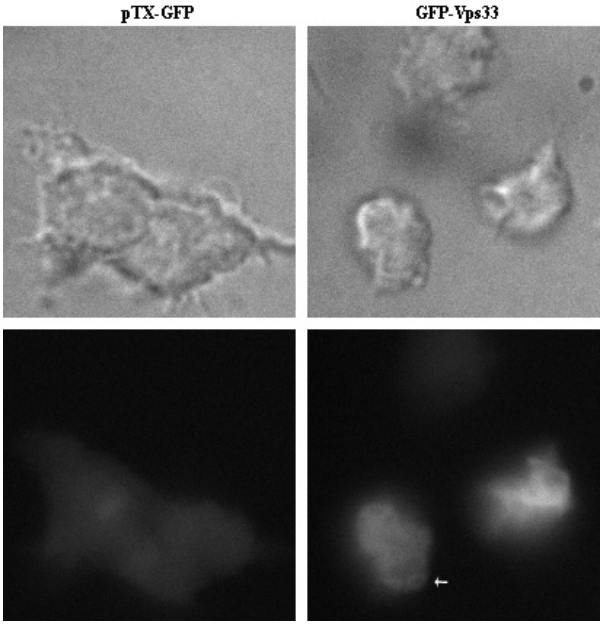


Figure 7 1-step formaldehyde fix. Vps33 shows a relatively stronger signal than soluble GFP and some localization to vesicles (arrow).

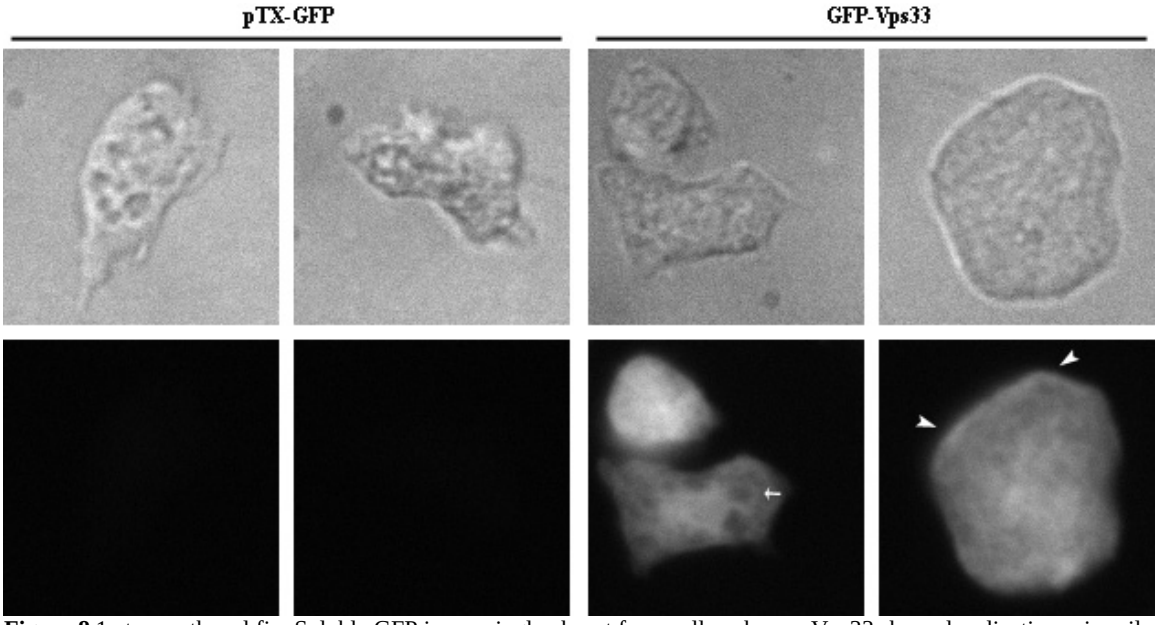


Figure 8 1-step methanol fix. Soluble GFP is seemingly absent from cells, whereas Vps33 shows localization primarily to the cytoplasm but also to vesicles (arrow) and the plasma membrane (arrowheads).

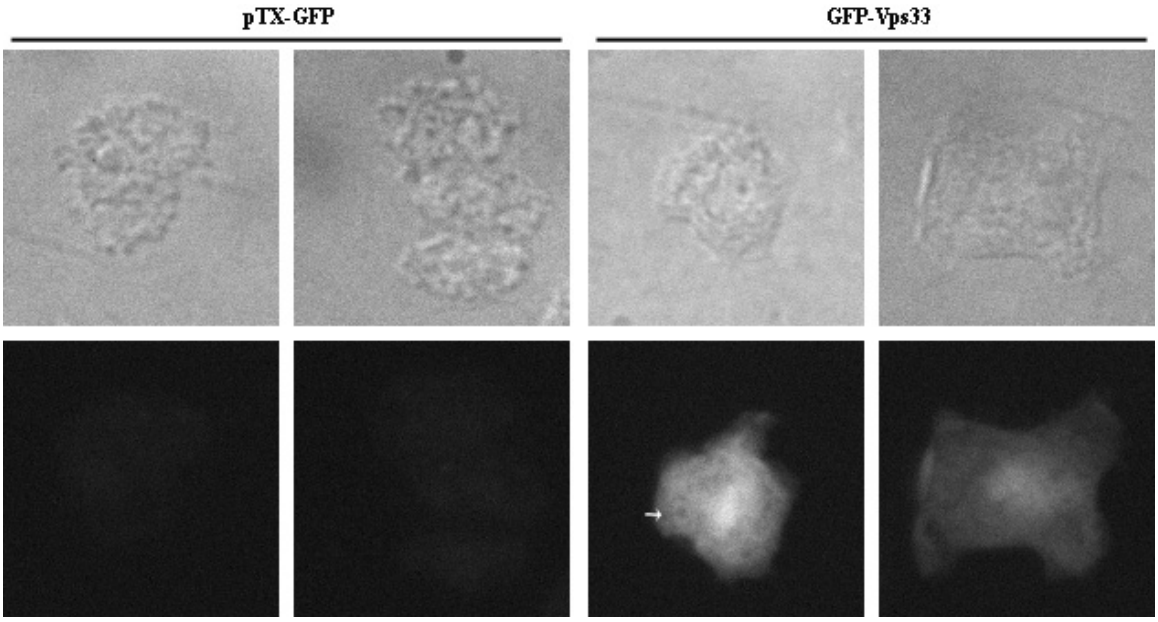


Figure 9 2-step formaldehyde and methanol fix. Vps33 localizes to the cytoplasm and some vesicles (arrow).

In order to constrict the focal range of cells and to possibly elucidate otherwise unseen structures, cells were flattened with agarose (Yumura et al., 1984) prior to fixing with methanol. While control cells do not show fluorescence, flattened GFP-Vps33 cells show cytoplasmic localization and occasional vesicle localization (Fig10). All cells observed also showed a single punctum adjacent to the nucleus. In order to verify the effectiveness of fixation, immunostaining for p80 and vacuolin, markers of the lysosome and postlysosome (respectively) was performed (data not shown). While these stains were effective in marking their appropriate vesicles, there was no overlap with GFP-staining, suggesting that Vps33 does not localize to these vesicles. Lastly, live cells were exposed to sodium azide in order to determine the effect of ATP depletion. A previous experiment by Gerhardt et al. (1998) described punctuate localization of Vps33 in yeast under ATP depletion but our results were inconclusive due to variable outcomes.

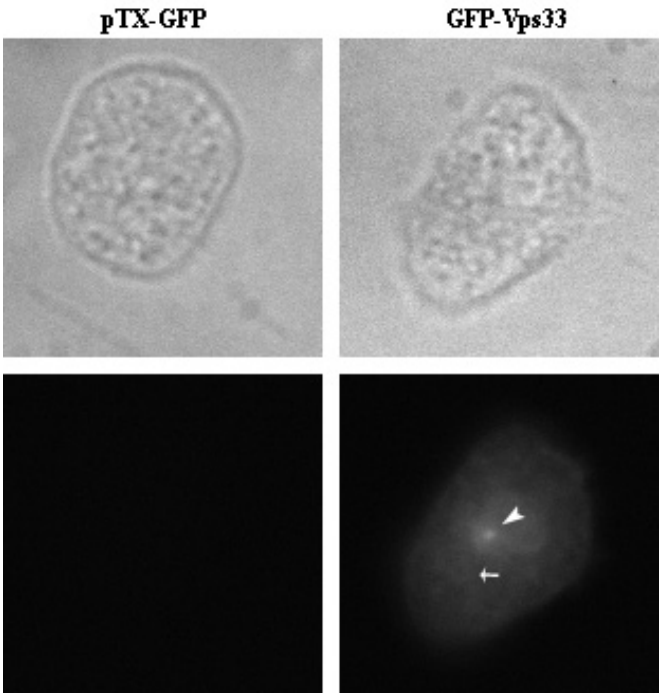


Figure 10 Cells flattened and fixed in methanol. Vps33 showed localization to the cytoplasm and some vesicles (arrow). All cells also displayed a single punctuate perinuclear stain (arrowhead).

Discussion

In this study, we looked at features of the HOPS complex in *Dictyostelium discoideum* and tagged one of its subunits, Vps33, with Green Fluorescent Protein. We found that all 6 subunits of the HOPS complex were represented for, and that several contained characterized domains in the NCBI database. A phylogeny of Vps33 protein revealed a near ancestral form within *Dictyostelium*, with some relation to plants and algae. Our GFP-Vps33 construct showed localization primarily to the cytoplasm and some vesicles in fixed cells, along with localization to the plasma membrane and perinuclear puncta under certain conditions.

The putative *Dictyostelium* HOPS proteins were first characterized through a large-scale cDNA sequencing project, in which all mRNAs from cell lysates are reverse transcribed, sequenced, aligned to genomic DNA, and identified through homology with other organisms. The presence and location of introns is inferred from consistent gaps within the mRNA sequence, along with characterized splice sites. While sequence homology is generally a robust indicator of identity, sequences can diverge greatly while retaining protein functionality, such that misidentifications are possible. Thus, while the putative proteins presented within Dictybase are the best candidates for the HOPS complex subunits, there may be other players involved. Mammals, for example, are known to have two versions of Vps33 (Gissen et al., 2005) such that either or both could be interacting with the complex. Differences in phenotype between mammalian Vps33a and Vps33b mutants further suggest nonredundancy, such that the significance of homology is again not without exceptions.

Protein domain searches within NCBI pull up several results, including some self-identifying domains that serve to bolster confidence for proper identification. Interestingly, 4 of the proteins (Vps11, 18, 39 and 41) contain one or more types of clathrin homology. While the E-values are relatively large for some of these, its broad presence suggests significance. Clathrin is a coat protein involved shown to play an important role in the formation of vesicle, and interacts with several proteins including cargo-bound adaptor proteins (Takei and Haucke, 2001). The presence of these clathrin homologies in the HOPS Vps proteins may indicate shared or similar binding partners, and merits further investigation. While Vps33 does not display a clathrin homology, it contains Sec1 homology with a very high degree of confidence, which is consistent with studies finding that it binds SNAREs and has ATPase activity. However Dictybase does identify a separate Sec1 protein coded from another region – thus Sec1 and Vps33 may play separate roles and display different specificity despite a high degree of similarity and likely common ancestry.

In order to asses the relation of *Dictyostelium* Vps33 to its xenospecific homologs, a phylogram was constructed using other characterized Vps33 proteins. The resulting branching pattern is reflective of evolutionary relationships, with animals, protists, and plants for the most part clustering together. Although the *Dictyostelium* branch length is fairly long indicating a high degree of divergence, it is clustered with the plants and mosses, contrary to evidence suggesting ancestry shared with animals and fungi. Thus *Dictyostelium* may retain a more ancestral form of the Vps33 protein, or may cluster with plants due to convergence, perhaps through functional constrains or simple coincidence. Likewise, the red flour beetle *Tribolium castaneum* seems to contain a

Vps33 protein more similar to that of fungi than animals – again highlighting questions of functionality versus sequence homology.

A GFP-Vps33 construct was designed to observe localization, by fusing together N and C terminal-segments of the gene. All bands appeared at their appropriate molecular weight, and the integrity of the construct was verified by sequencing. The N-terminal segment PCR reaction did have a dim additional band, likely a result of low efficiency binding of one of the primers to an internal site. Expression of the construct was verified by western blot, with the protein expressing strongly at an apparent molecular weight slightly above the predicted. This may be due to a slight negative charge, as inferred by amino acid content (not shown) as well as one or more protein modifications, such as phosphorylation or acetylation. The presence of other bands within the sample is likely due to degradation products during lysis as well as non-specific antibody binding.

Live and fixed cells showed largely cytoplasmic localization of GFP-Vps33. While live control cells seemed to show slightly stronger expression, formaldehyde fixation resulted in dampening of soluble GFP, while methanol fixation altogether removed the signal. Although formaldehyde is capable of slightly permeabilizing cells, it functions primarily by crosslinking intracellular molecules. Methanol, however, is a strong permeabilizing agent that permits for smaller molecules to be washed out. Thus, the relatively small (25 kDA) GFP can be washed out far more easily in methanol fixative than the larger GFP-Vps33 (102 kDa) or the fully-assembled GFP-tagged HOPS complex (650 kDa), accounting for the disparity of signal strength between the control and experimental groups. While the cytoplasmic localization was mostly homogeneous throughout the cell, some cases did display variable signal strength. This could

potentially be indicative of localization to the endoplasmic reticulum, but is more likely due to crowding and an unequal distribution.

Fixed GFP-Vps33 cells also display some localization to vesicles, suggesting there is a slight decrease in cytoplasmic concentration, perhaps through washing. This is expected of Vps33 and the HOPS complex, which are known to play an important role in vesicle fusion and interact with fusion proteins. Fixed cells were immunostained for p80 and vacuolin, endocytic vesicle markers, but colocalization was not observed. In some cases the cytoplasmic GFP signal was strong enough that presence or absence of vesicular GFP could not be determined conclusively, thus opening the possibility for unobservable localization. However, most cases displayed clear absence of colocalization, suggesting that Vps33 is not found on lysosomes or postlysosomes.

Interestingly, methanol fixation showed possible plasma membrane localization, perhaps as a result of exocytosis or impending endocytosis. Also, flattening of cells followed by fixation resulted in a constant single punctum appearing near the nucleus. This may be staining the centrosome, which is typically of smaller size and found by the nucleus. However, due to the specificity of conditions leading to these results, the localization may be merely an artifact of the procedure and not representative of localization within a live, healthy cell.

In summary, our studies showed that Vps33 localizes primarily to the cytoplasm and some vesicles in *Dictyotelium discoideum*. Because of its many interactions, these observations could be of Vps33 by itself or as part of the larger HOPS complex, or in some cases even the CORVET complex. In any study involving GFP tagging, one must also be aware of the possibility of affecting the native protein conformation, and thus its

functionality and localization. Rescue of a Vps33 knockout strain with GFP-Vps33 could rule out this possibility, and would be an ideal avenue for future studies, along with further vesicular staining in an attempt to identify Vps33-marked vesicles.

Acknowledgements

I would like to thank Dr. Hideko Urushihara for his generous provision of the cDNA clone dda19p04.3, as well as my labmates Rebecca Brady, Hilary Graham, Kevin Bersuker, Yujia Wen, Thomas Sosa, Kristin Benson, and Ying Cheng for their help and guidance. I would also like to thank Dr. Terri O'Halloran for her input and most of all Dr. Arturo De Lozanne for his invaluable mentorship. This research was funded in part by the University of Texas Undergraduate Research Fellowship, for which I am also thankful.

References

Baba et al. 1997 “Two Distinct Pathways for Targeting Proteins from the Cytoplasm to the Vacuole/Lysosome”. The Journal of Cell Biology.139:7, 1687-1695.

Banta et al. 1988. “Organelle assembly in yeast: characterization of yeast mutants defective in vacuolar biogenesis and protein sorting”. The Journal of Cell Biology. 107:4, 1369-1383.

Banta et al. 1990. “Characterization of yeast vps33p, a protein required for vacuolar protein sorting and vacuole biogenisis”. Molecular and Cellular Biology. 10:9, 4638-4649.

Dacks at al. 2009. “Evolution of specificity in the eukaryotic endomembrane system”. Int J Biochemical Cell Biol. 41:2, 330-340.

Gerhardt et al. 1998. “The vesicle transport protein vps33p is an ATP-binding protein that localizes to the cytosol in an energy-dependent manner”. The Journal of Biological Chemistry. 273:25, 15818-15829.

Gissen et al. 2004. “Mutations in VPS33B, encoding a regulator of SNARE-dependent membrane fusion, cause arthrogryposis-renal dysfunction-cholestasis (ARC) syndrome”. Nature Genetics. 36:4, 400-404.

Gissen et al. 2005. “Comparative evolutionary analysis of VPS33 homologues: genetic and functional insights” Human Molecular Genetics. 14:10, 1261-1270.

Gissen et al. 2006. “Clinincal and molecular genetic features of ARC syndrome”. Human Genetics. 120, 396-409

Grosshans et al. 2006. “Rabs and their effectors: Achieving specificity in membrane traffic”. PNAS 103:32, 11821-11827.

Maniak. 2003. “Fusion and Fission Events in the Endocytic Pathway of *Dictyostelium*”. Traffic. 4:1-5

Peplowska et al. 2007. “The CORVET tethering complex interacts with the yeast Rab5 homolog vps21 and is involved in endo-lysosomal biogenesis”. Developmental Cell. 12, 739-750.

Price et al. 2000. “The Docking Stage of Yeast Vacuole Fusion Requires the Transfer of Proteins from a cis-SNARE Complex to a Rab/Ypt Protein” The Journal of Cell Biology. 148:6, 1231-1238.

Seals et al. 2000. “A Ypt/Rab effector complex containing the Sec1 homolog Vps33p is required for homotypic vacuole fusion”. PNAS. 97:17, 9402-9407.

Stroupe C. et al. 2006. “Purification of Active HOPS complex reveals its affinities for phosphoinositides and the SNARE Vam7p” The EMBO journal. 25:8, 1579-1589.

Takei and Haucke. 2001. “Clathrin-mediated endocytosis: membrane factors pull the trigger”. Trends in Cell Biology. 11:9, 385-391.

Wurmser et al. 2000. “New Component of the Vacuolar Class C-VPS Complex Couples Nucleotide Exchange on the Ypt7 GTPase to SNARE-dependent Docking and Fusion”. J. Cell Biol. 151:3, 551-562.

Yumura s. et al. 1984. “Localization of actin and myosin for the study of ameboid movement in Dictyostelium using improved immunofluorescence”. J Cell Biol. 99:3,894-899.

Zhang H. 2003. “Alignment of BLAST High-scoring Segment Pairs Based on the Longest Increasing Subsequence Algorithm”. Bioinformatics. 19:1391-1396.