Characterizing Adaptor Protein Complex 3 and Its Interactions with Cellular Proteins

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Abstract

Adaptor Protein Complex 3 (AP-3) is a heterotetrameric protein complex involved in the transport of proteins from the Golgi to the lysosome. AP-3 is known to interact with other cellular proteins during its activity, and other interactors are anticipated. This study investigates additional candidate AP-3 interactors in the budding yeast *Saccharomyces cerevisiae* that were identified by three screening methods: mass spectrometry of proteins in yeast cell extracts that copurify with recombinant AP-3 proteins, genetic screening for mutations in yeast that missort an AP-3 reporter cargo protein, and in-vivo proximity-labeling of yeast cellular proteins by AP-3 subunits fused to biotin ligase. The candidate AP-3 interactors are evaluated using bimolecular fluorescence complementation (BiFC), which revealed 17 positive AP-3 interactors out of 102 potential candidates. One of these interactors is Vma10, a subunit of the vacuolar ATPase (V-ATPase). The interaction between AP-3 and the V-ATPase is investigated through location-specific V-ATPase subunits Vph1 and Stv1. BiFC revealed that Vph1 is a strong AP-3 interactor, whereas Stv1 is not. Using confocal microscopy, we show that Vph1 is transported to the vacuole (the yeast lysosome) via the VPS Pathway, which is an alternative transport pathway that parallels the route of delivery via the AP-3 pathway. Additionally, this study shows that expression of the Apl5 subunit of AP-3 in vivo requires expression of each of the other 3 AP-3 subunits, suggesting that Apl5 might be the last subunit to coassemble during AP-3 complex formation.

Introduction

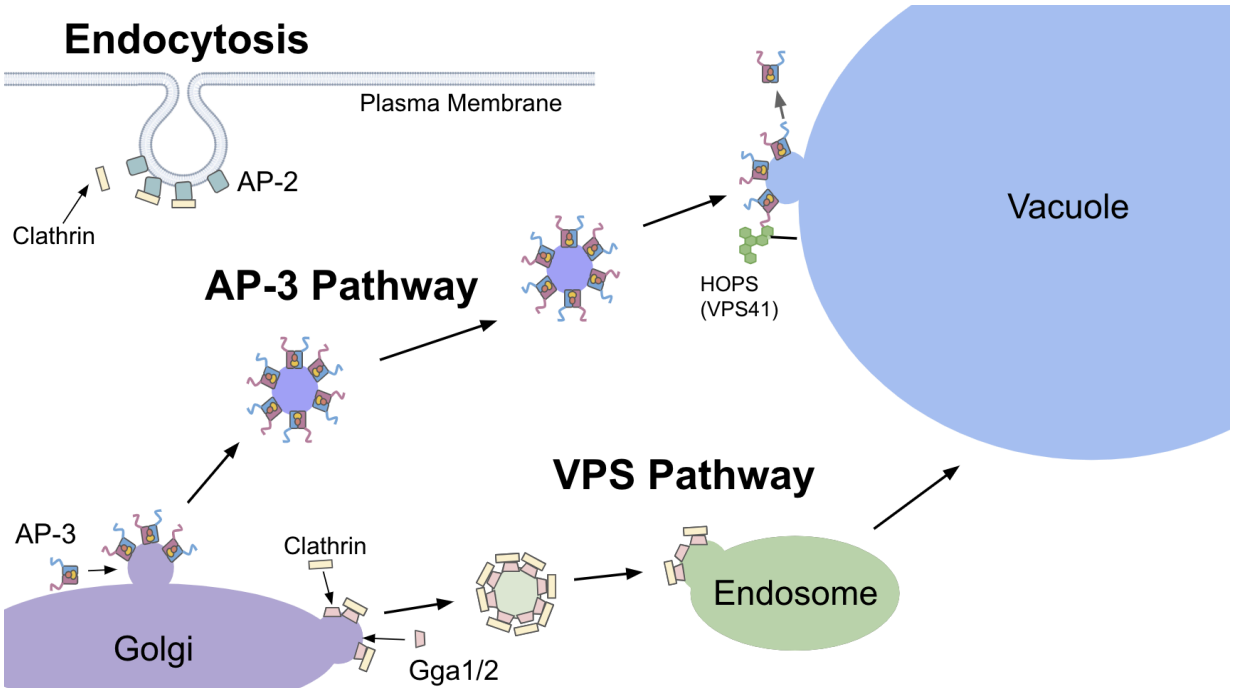
Adaptor Protein Complexes

Membrane trafficking is a cellular mechanism that utilizes vesicle transport to bring proteins to their target destination (Rothman and Lenard, 1984). Adaptor protein (AP) complexes are critical for eukaryotic cell survival and are involved in various cellular processes including molecule and protein transport, signal transduction, and more (Reviewed in: Park and Xiaoli, 2014). APs facilitate intracellular membrane trafficking, select transmembrane protein cargoes, and curve membranes to facilitate budding and vesicle formation (Ohno et al., 1995; Reviewed in: Park and Xiaoli; Angers and Merz, 2009). APs specifically select cargo by recognizing amino acid sequences in the cargo protein cytosolic domain (Ohno et al., 1995). There are five AP complexes; AP-1, AP-2, and AP-3 are conserved from yeast to humans, whereas AP-4 and AP-5 are in higher eukaryotes (Schoppe et al., 2021). AP-1 and AP-2 bind clathrin coat proteins to facilitate the budding process (Reviewed in: Kirchhausen et al., 2014). However, in *Saccharomyces cerevisiae,* the model organism in my research, the interaction between AP-3 and clathrin is not characterized (Dell'Angelica et al., 1998).

The vacuole is a lysosome-like organelle in yeast that is responsible for various cellular processes, including macromolecule degradation, which requires proteins such as lipases, proteases, and nucleases (Broach et al., 1991; Li and Kane, 2009). Vacuolar proteins can reach their destination from the Golgi to the vacuole through the VPS Pathway, facilitated by the GGA adaptors (Reviewed in: Eising et al., 2022, Nagano et al., 2023). The vesicles from the Golgi fuse with an endosome before they arrive at the vacuole by endosome-vacuole fusion. The AP-3 Pathway bypasses endosomes and is another pathway for vacuolar proteins to reach the vacuole (Eising et al., 2022). The AP-3 pathway is a simplified trafficking pathway whereby AP-3 coated

vesicles bud from the Golgi, traffic through the cytoplasm, and fuse directly to the vacuole (Figure 1). AP-2 is involved in the endocytosis of membrane proteins from the plasma membrane (Reviewed in: Park and Xiaoli, 2014). This process is essential for bringing proteins into the cell and for membrane turnover (Reviewed in: Goode et al., 2015).

Figure 1. Adaptor Protein Pathways



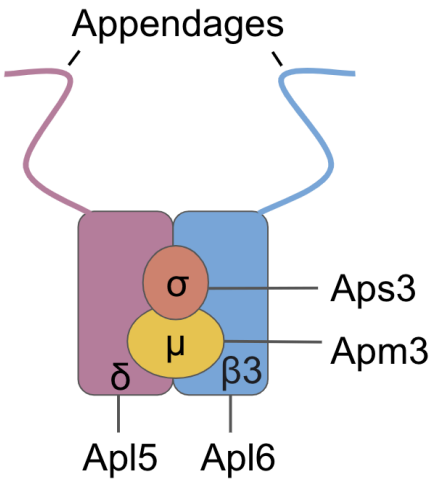
Note. Endocytosis is the process of proteins being brought into the cell from the extracellular space. In endocytosis, the Adaptor Protein 2 (AP-2), together with clathrin, will create a vesicle from the plasma membrane to bring proteins into the cell. The AP-3 Pathway uses Adaptor Protein 3 (AP-3) to bring vacuolar proteins from the Golgi to the vacuole. AP-3 facilitates budding from the Golgi, traffics the AP-3-coated vesicle through the cytoplasm, and fuses the vesicle with the vacuole through its interaction with the HOPS complex subunit Vps41. Similar to the AP-3 complex, Gga will facilitate vesicle budding from the Golgi with clathrin. Gga and clathrin-coated vesicles will fuse with the endosome and reach the vacuole by endosome-vacuole fusion.

Adaptor Protein 3 Subunits and Functions

Adaptor protein complex 3 (AP-3) is a heterotetrameric protein complex that consists of two large subunits (δ, the Apl5 protein in yeast, and β3, Apl6) a medium subunit (μ, Apm3), and a small subunit (σ, Aps3) (Simpson et al., 1997) (Fig. 2). The medium subunit is responsible for

cargo recognition, and the small subunit is proposed to be responsible for the stability of the complex (Collins et al., 2002; Ohno et al., 1995). The large subunits contain three domains; the N-terminal domain, which interacts with the medium and small subunits, and the C-terminal domain, containing the appendages (Reviewed in: Ohno, 2006). The large subunit appendages are thought to interact with proteins that facilitate vesicle budding and fusion (Angers and Merz, 2009).

Figure 2. Adaptor Protein Complex 3



Note. The Adaptor Protein 3 complex is a heterotetrameric protein complex. It consists of two large subunits, δ (Apl5) and β3 (Apl6), a medium subunit, μ (Apm3), and a small subunit, σ (Aps3).

Golgi Dynamics

The Golgi apparatus is a membrane-bound organelle responsible for modifying, and packaging proteins for transport in eukaryotic cells (Dunphy and Rothman, 1985). The Golgi consists of disk-shaped membranous compartments called Golgi cisternae (Farquhar and Palade, 1981). In *Saccharomyces cerevisiae*, Golgi cisternae are dispersed throughout the cell and go through a maturation program whereby the protein and lipid constituents of individual cisternae change over time - from early/cis to late/trans (Reviewed in: Suda and Nakano, 2012). Cargo proteins synthesized in the endoplasmic reticulum are transported to the cis Golgi (Reviewed in:

Papanikou and Glick, 2009; Hecht et al., 2014). Enzymes in the Golgi modify these cargo proteins, and once processing is complete, the cargoes are packaged into vesicles for transport (Reviewed in: Suda and Nakano, 2012).

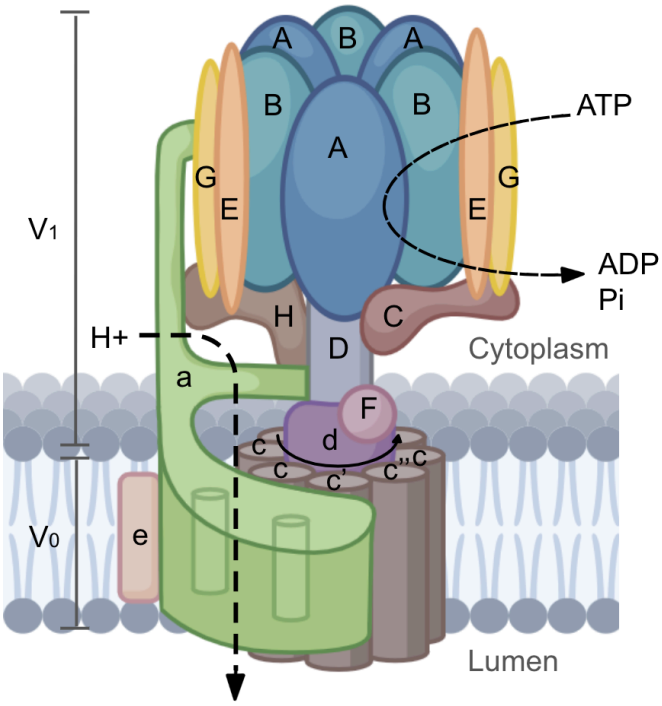
Vacuole Dynamics

The yeast vacuole is functionally similar to the mammalian lysosome, but can take up over 20% of yeast cell volume (Li and Kane, 2009). The vacuole is a membrane-bound organelle responsible for pH, protein, and ion homeostasis, ion and nutrient storage, degradation of macromolecules, and cellular detoxification (Reviwed in: Kane, 2006; Hecht et al., 2014). This organelle contains various proteins and enzymes that assist in vacuolar functions. The vacuolar proteins are cargoes transported from the Golgi to the vacuole via the endosome-dependent VPS pathway or via the endosome-independent AP-3 pathway (Cowles et al., 1997). The AP-3 pathway is responsible for transporting vacuole membrane protein (Ihrke et al., 2004).

Vacuolar-ATPase and Function

The vacuolar proton-translocating ATPase (V-ATPase) is responsible for acidifying the vacuole, which is important for vacuolar functions, such as protein degradation (Stevens and Forgac, 1997). The V-ATPase is a multi-protein complex that is divided into two domains (Fig. 3). The peripheral V1 domain, facing the cytoplasm, is responsible for ATP hydrolysis, which powers conformational change in the integral membrane V0 domain to enable the transport of protons (H+) from the cytosol to the lumen of the vacuole, resulting in vacuole acidification (Reviewed in: Pamarthy et al., 2018; Collins and Forgac, 2020). The V-ATPase, like many transmembrane proteins, reaches the vacuole by the VPS Pathway (Hirst et al., 2000). In yeast, subunit “a” has two location-specific isoforms (Manolson et al., 1992; Manolson et al.,1994). The V-ATPase subunit Stv1 localizes at the Golgi/endosome and Vph1 localizes at the vacuole.

Figure 3. Vacuolar ATPase Subunits and Function



Subunit	Gene	Function
A	*VMA1*	ATP hydrolysis
B	*VMA2*	Regulatory, ATP binding?
C	*VMA5*	Stator
D	*VMA8*	Rotor
E	*VMA4*	Stator
F	*VMA7*	Rotor
G	*VMA10*	Stator
H	*VMA13*	Stator
a	*VPH1/STV1*	Proton pore, stator, sorting
c	*VMA3*	Proton pore, rotor
c'	*VMA11*	Proton pore, rotor
c"	*VMA16*	Proton pore, rotor
d	*VMA6*	Rotor
e	*VMA9*	?

Note. The vacuolar ATPase consists of a peripheral V1 domain, facing the cytoplasm, and an integral membrane V0 domain, embedded in the membrane. The V1 domain consists of proteins (Vma1, Vma2, Vma5, Vma8, Vma4, Vma7, Vma10, and Vma13) that are involved in the hydrolysis of ATP to ADP and inorganic phosphate, which powers rotation of the complex. The integral membrane V0 domain consists of proteins (Vph1/Stv1, Vma3, Vma11, Vma16, Vma6, and Vma9) that respond to complex rotation by transporting proton (H+) from the cytoplasm to the lumen of the vacuole (diagram adapted from: Kane, 2006; Chen et al. 2022).

AP-3 Candidate Interactors

The Apl5 large subunit of AP-3 binds to the Vps41 subunit on the HOPS complex on the vacuole to facilitate vesicle fusion (Angers and Merz, 2009). This discovery makes it apparent that the large subunit appendages, specifically Apl5, interact with proteins. An unpublished mass spectrometry experiment was performed by the lab of our collaborator, Alex Merz, which used mass spectrometry to identify proteins in yeast cell extracts that bind recombinant fusion proteins containing the appendage of either Apl5 or the other AP-3 large subunit, Apl6. Mass spectrometry is a qualitative and quantitative analysis used to identify and quantify a substance

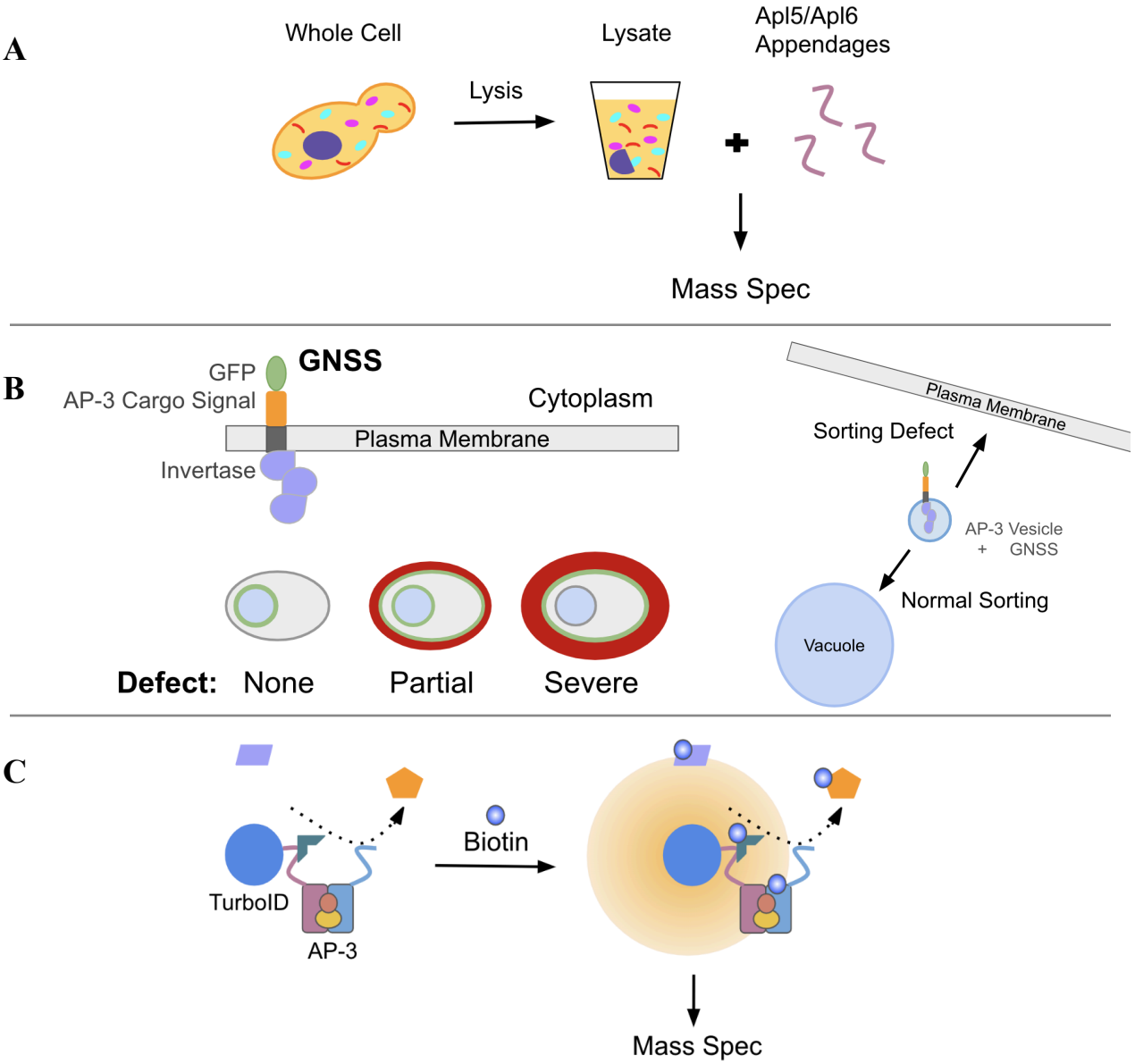
with unknown chemical components (Garg and Zubair, 2023). In order to identify protein candidates that could bind with AP-3, purified Apl5/Apl6 appendages were used to isolate these proteins from a yeast whole cell lysate (Fig. 4A). In the screening, 447 proteins were identified.

In parallel, an unpublished genetic screen in yeast was performed by the lab of our collaborator, Elizabeth Conibear, based on the sorting of GNSS, an cargo protein normally transported by AP-3 to the vacuole (Plemel et, al., 2020). A defective AP-3 Pathway results in GNSS cargo mislocalization to the plasma membrane, where it can easily be detected by a visible color change derived from invertase activity fused to the GNSS exoplasmic domain (Fig. 4B). The green fluorescence protein (GFP) fused to the cytosolic domain of GNSS provides a localization analysis through fluorescence microscopy. In a whole-genome knockout screen, the Conibear lab identified 1539 genetic mutations that caused a GNSS mislocalization phenotype.

A published proximity-based biotinylation study was performed by the lab of Christian Ungermann to identify potential AP-3 interactors (Schoppe et al., 2020). This approach used TurboID, a biotin ligase, fused to the Apl5 appendage domain. Biotin ligase adds the small molecule, biotin, to proteins that are in close enough proximity to interact with AP-3 (Fig. 4C). Additionally, proteins that transiently interact with the TurboID-tagged protein will also be biotinylated (Cheerathodi and Meckes, 2023). Proteins biotinylated by Apl5-TurboID were identified by mass spectrometry, resulting in 516 candidate AP-3 interactors.

A cross-reference of these three screening methods together with BiFC analysis I performed in the Odorizzi lab converged on the V-ATPase as an interactor of AP-3. In this study, I am investigating the V-ATPase and trafficking.

Figure 4. AP-3 Potential Interactors Screening



Note. The figures depict the three screening methods used to identify AP-3 interactor candidates. A: Whole-cell yeast is turned into lysate, which was mixed with purified recombinant Apl5 or Apl6 appendages; proteins in the lysate that bound Apl5/Apl6 were identified by mass spectrometry. B: GNSS is an AP-3 cargo that contains the invertase enzyme to identify proteins that cause an AP-3 sorting defect. AP-3 missorting causes the reporter to go to the plasma membrane, whereas normal sorting via the AP-3 pathway results in GNSS delivery to the vacuole. GNSS mislocalization to the plasma membrane is detected using a substrate that changes color in response to invertase activity. C: A Turbo-ID (Biotin Ligase) is attached to the Apl5 subunit of the AP-3 Complex. Proteins that interact with AP-3, including transient interacting proteins, and are within close proximity (~10 nm) are biotinylated (Kim et al., 2014). Biotinylated proteins are purified, then identified by mass spectrometry.

Project Justification

Saccharomyces cerevisiae is an excellent model for studying human disease because human and yeast cells have many conserved pathways in common (Gastelum et al., 2023). Mutations in the AP-3 complex in humans cause Hermansky-Pudlak syndrome (Eising et al., 2022). Hermansky-Pudlak syndrome is characterized by oculocutaneous albinism, reduced pigment in hair, skin, and eyes, bleeding tendency, and ceroid, lipid accumulation in the cell. This research will help us understand the AP-3 complex and its interactors, which have the potential to help people with this syndrome.

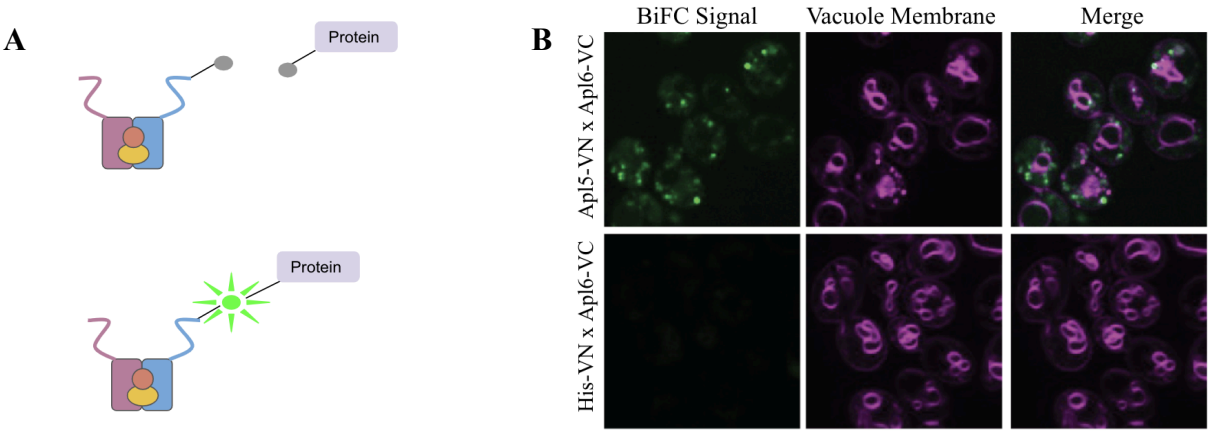
Additionally, the human V-ATPase is involved in various normal and pathophysiological processes and directly influences human health (Eaton et al., 2021). Some of these influences include cancer, neurodegenerative diseases, and diabetes. Expanding our knowledge about both of these proteins and their interactions could provide valuable information on these diseases and their effect on humans and human diseases.

Results

Bimolecular Fluorescence Complementation (BiFC) analysis of candidate AP-3 interactions

I used bimolecular fluorescence complementation (BiFC) to view interactions between candidate proteins with AP-3 in living yeast cells. The N- versus C-terminal fragments of the Venus variant of green fluorescent protein (VN and VC) are not fluorescent unless they coassemble with one another to form the full Venus protein (Reviewed in: Kerppola, 2008). Two proteins attached to either VN or VC will allow Venus assembly if the proteins of interest are in close proximity (7 nm), which results in Venus fluorescence emission under observation by microscopy (Fan et al., 2008) (Fig. 5). When combining data sets from the Merz, Conibear, and Ungermann studies described above, I identified 24 proteins as strong candidates for AP-3 interactions. Our lab was given access to a genome-wide BiFC library, where these 24 candidates were acquired alongside 78 candidates identified with high confidence by BioID (Schoppe, 2020) (Table 1).

Figure 5. Positive and negative controls of BiFC experiment



Note. A: Cartoon representation of two VC and VN fragments of the BiFC, and fluorescence with interaction. B: Positive control Apl6-VC and Apl5-VN, the large subunits of the AP-3 Complex, show fluorescence and are expected to be in close enough proximity to interact. Negative control Apl6-VN and His2-VN show no green fluorescence and are not known to interact. Imaged by Mitchell Leih, a graduate student in the Odorizzi lab.

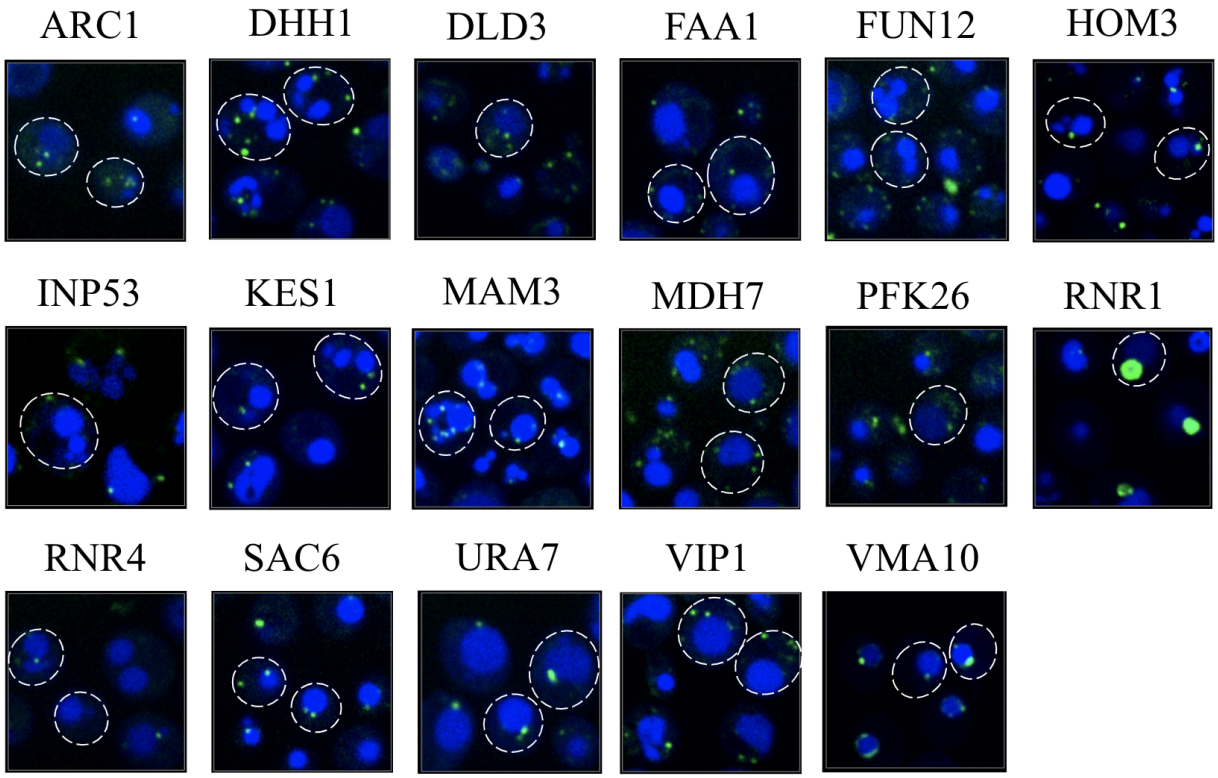
Table 1. Proteins Identified in Cross-reference Screening with GNSS, BioID, and Mass Spectrometry

Triple Screening	ARC1	FAA1	IMH1	PSP2	SPE3	VMA13
	APL5	FUN12	INP53	RNR1	TMA7	VPS35
	APL6	GIR2	KES1	RNR4	URA7	VPS74
	DLD3	HOM3	PBP1	SAC6	VIP1	YPR1
BioID Screening	ACK1	CPA1	ENT5	NAM7	RME1	VMA10
	AGE2	CRZ1	GLO3	NAP1	SCH9	VPS1
	AIM21	CSI1	IWR1	NET1	SCY1	VPS33
	AIM3	CWC15	LSB1	NEW1	SEC18	VPS41
	APM3	DBF2	LSG1	NMT1	SER2	VTC2
	ATG20	DEF1	MAM3	NVJ1	SKY1	WHI4
	ATG9	DHH1	MDG1	PBS2	SST2	YCK3
	BNI4	DRE2	MDH3	PFK26	STB3	YHR097C
	BNI5	EAP1	MEH1	PIB2	SUI1	YKL023W
	BRO1	EIS1	MLF3	PRE9	TOD6	YKR023W
	BSP1	ENT1	MSN4	RET3	TRX1	YKT6
	CAF20	ENT2	MTC1	REX2	TRX2	YLR257W
	CEX1	ENT3	MXR1	RIO2	VAC7	YPL225W

Note. Cross-referencing GNSS, BioID, and mass spectrometry revealed 24 proteins. Two proteins highlighted in yellow above, Apl5 and Apl6, were known to interact because they are AP-3 subunits. Of the 24 proteins, 12 novel proteins were identified to interact with Apl6-VC, highlighted in green. An additional 78 proteins from BioID screening, with high confidence, were added. There were 5 positive interactors identified. Of the total 102 proteins screened, 17 proteins were positive.

For the BiFC experiments, the VC fragment was attached to the AP-3 large subunit in haploid MAT alpha yeast cells, Apl6, and the VN fragment was attached to the different candidate AP-3 interactors in haploid MAT a cells. I mated haploids to create diploids coexpressing Apl6-VC together with one of the candidate-VN fusions. I then sporulated diploids to isolate haploid strains expressing both BiFC (VC and VN) fusion proteins. Yeast strains that had a positive BiFC signal contained green fluorescence from the interaction between the fragments (Fig. 6).

Figure 6. Positive BiFC Interaction with Apl6-VC



Note. BiFC images of positive interactors from the 102 candidates. There are 17 positive protein interactors with Apl6-VC. Each of the proteins (Arc1, Dhh1, Dld3, Faa1, Fun12, Hom3, Inp53, Kes1, Mam3, Mdh7, Pfk26, Rnr1, Rnr4, Sac6, Ura7, Vip1, Vma10) contained the VN fragment. The images show GFP puncta (green), CMAC-stained fluorescent dye vacuoles (blue), and cell borders (white dotted lines). The images were normalized to a known BiFC interaction by ImageJ to confirm the validity of the signal. Imaged by Mitchell Leih.

In cells that exhibit BiFC fluorescence, most of the Venus (GFP) puncta are small and localized adjacent to the vacuole, though there were exceptions. In the Apl6-VC + Rnr1-VN strain, a large accumulation of GFP was observed adjacent to the vacuole. Coexpression of Apl6-VC with VN fused to Mdh7, Pfk26, Dhh1, and Fun12, resulted in punta that also appear to be dispersed in the cytoplasm, or potentially on an unmarked organelle like the Golgi or nucleus. The BiFC experiment revealed that 17 out of the 102 candidate interactors had GFP puncta, including the two large subunits of AP-3, further validating the experimental approach. This is an

indicator that the proteins were in close enough proximity to interact with one another and should further be pursued as potential AP-3 interactors.

Table 2. Positive BiFC Apl6-VC Interactors and Their Function

Protein	Key Terms	Function
Arc1	Protein Synthesis	Methionyl- and glutamyl-tRNA synthetases; binds tRNA involved in tRNA delivery, catalysis, and localization
Dhh1	Translation	ATP-dependent RNA helicase; mRNA decaping, unwinding RNA, mRNA processing, translation regulation
Dld3	Metabolite Regulation	D-Lactate Dehydrogenase; converts D-2-hydroxyglutarate to alpha-ketoglutarate, overall lactate biosynthesis
Faa1	Lipid Synthesis	Long-chain fatty acid-CoA ligase; involved in long-chain fatty acid metabolism and import
Fun12	Translation	Translation initiation factor; GTPase promotes iMet-tRNA binding to ribosomes, regulates translational initiation
Hom3	Amino Acid Synthesis	Aspartate kinase; involved in the biosynthesis of methionine, threonine, and homoserine
Inp53	Phosphoinosidite Regulation	Phosphatidylinositol 4,5-bisphosphate 5-phosphatase; dephosphorylates phosphatidylinositol
Kes1	Golgi	Golgi protein; involved in exocytosis, endocytosis, transport of sterols, and maintenance of cell polarity
Mam3	Mitochondria	Mitochondrion organization and cellular homeostasis of magnesium and manganese
Mdh3	Metabolite Regulation	Peroxiosome L-malate dehydrogenase; binds mRNA, involved in fatty acid beta-oxidation and NADH regneration
Pfk26	Kinase	6-phosphofructo-2-kinase; involved in fructose 2,6-bisphosphate metabolism
Rnr1	Nucleotide Synthesis	Ribonucleotide-diphosphate reductase subunit; involved in deoxyribonucleotide biosynthesis
Rnr4	Nucleotide Synthesis	Ribonucleotide-diphosphate reductase small subunit; involved in deoxyribonucleotide biosynthesis
Sac6	Cytoskeleton	Actin binding protein; involved in actin filament organization and actin cable assembly
Ura7	Metabolite Regulation	CTP synthase; involved in phospholipid and pyrimidine biosynthesis
Vip1	Kinase	Inositol polyphosphate kinase; involved in inositol phosphate biosynthesis
Vma10	Vacuole	Subunit of V1 peripheral membrane domain of V-ATPase; involved in vacuolar acidification by H+ transport

Note. Positive BiFC interactors with AP-3, key terms, and their function (*Saccharomyces Genome Database).*

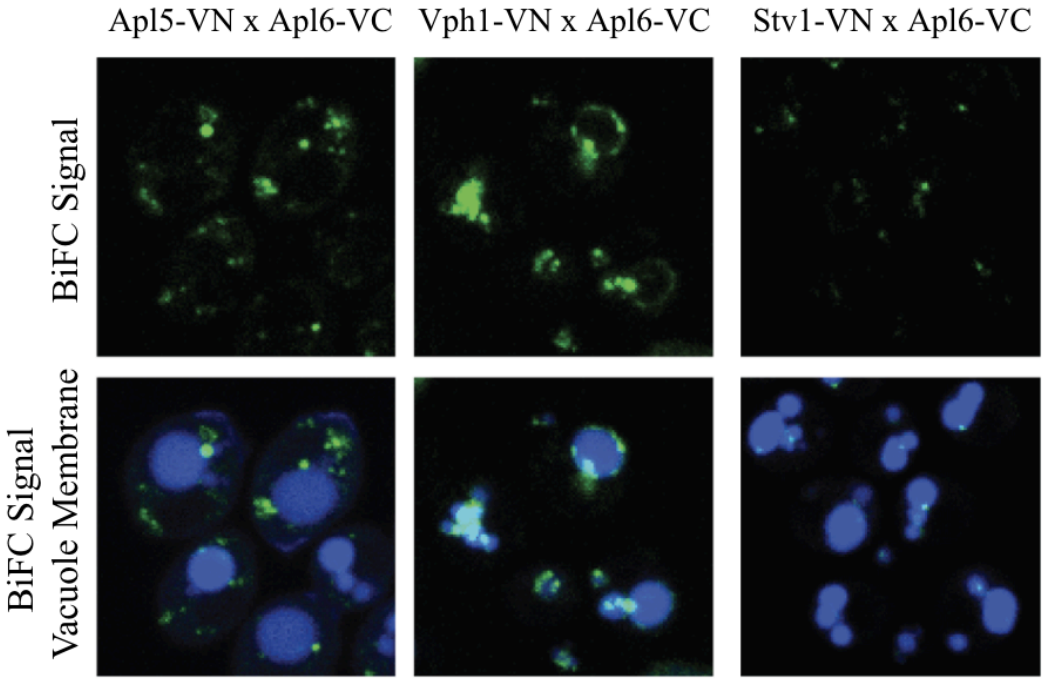
The proteins identified as Apl6-VC interactors using BiFC screening have various functions in the cell (Table 2). Although there is some overlap between functions, the majority of the proteins identified in the screening do not appear to have an overall similar location or mechanism of action. The ribonucleotide-diphosphate reductase protein complex had two subunits, Rnr1 and Rnr4, both of which were positive interactors with Apl6-VC. Vma13 and Vma10 are subunits of the vacuolar ATPase. Interestingly, Vma13 was found in the triple screening but did not interact in the BiFC experiment, whereas Vma10 was found in the BioID screening and had a positive interaction.

Vacuolar ATPase Subunits Interact with AP-3

My BiFC screening yielded 17 novel proteins with close enough proximity to AP-3 in vivo to allow their interaction. One of the positive interactors identified in the screening was Vma10, the "G" subunit in the peripheral V1 domain (cytoplasm facing) of the Vacuolar ATPase (V-ATPase)(Fig. 3). To investigate if other subunits of the V-ATPase interact with the AP-3 Complex, our lab obtained Vph1-VN and Stv1-VN from the genome-wide BiFC library. These homologous proteins are isoforms of subunit "a" and are location-specific: Stv1-containing ATPases localize at the Golgi/endosome, whereas and Vph1-containing V-ATPases localize at the vacuole (Manolson et al., 1992; Manolson et al., 1994) (Fig. 3). I mated these strains with Apl6-VC, sporulated, and identified haploids positive for both fragments to identify where the AP-3 complex could interact with the V-ATPase.

The BiFC experiment revealed that both Vph1 and Stv1 interacted with Apl6 with varying intensity (Fig. 7). This indicates that at least 3 V-ATPase subunits, Vma10, Vph1, and Stv1, are close enough to interact with AP-3, although Vma13 was negative and in the triple screening. To further investigate this interaction I wanted to know if defects in the VPS pathway or AP-3 pathway affect the localization of the V-ATPase subunits.

Figure 7. V-ATPase Subunit Vph1 interacts with AP-3 in BiFC



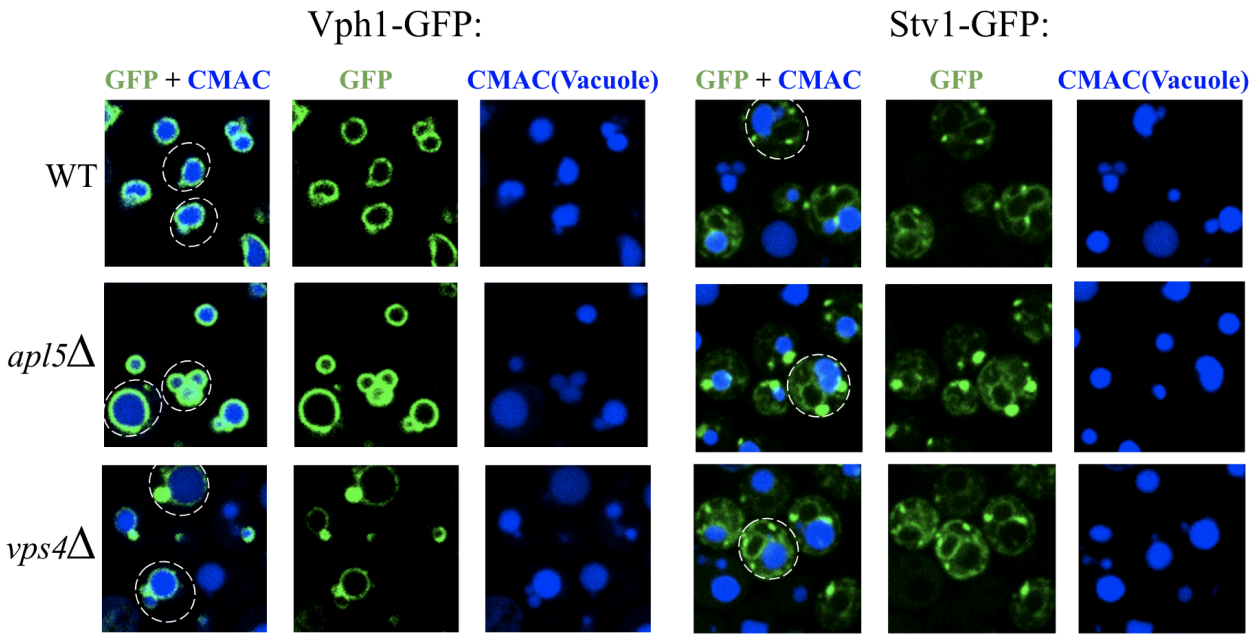
Note. The BiFC interaction between Apl6-VC and the V-ATPase subunits Vph1 and Stv1 showed fluorescence. Apl5-VN was used as a control for the experiment to normalize the interactions. Vph1-VN appears to have a stronger BiFC signal than Stv1-VN. GFP, positive BiFC is in green, and the vacuole labeled with CMAC fluorescent dye is in blue. Imaged by Mitchell Leih

Plasmids were used to express proteins of interest fused to a GFP fluorophore for visualization by fluorescent microscopy. I transformed Vph1-GFP and Stv1-GFP plasmids into wild-type, and knockout (Δ) strains of Apl5 and Vps4. A knockout of Apl5, an AP-3 subunit, disrupts the AP-3 pathway. A knockout of Vps4 disrupts the VPS pathway. Disruption of these pathways is informative because differing results from wild-type can provide useful information about which pathway the protein goes through.

Vph1-GFP consistently localizes to the vacuole in wild-type and in *apl5Δ* cells (Fig. 8). In *vps4Δ* cells, a large fluorescent punctum near the vacuole is apparent, which is consistent with a characteristic called a class E compartment (Russell et al., 2012). The proteins that go through the VPS pathway, when it is disrupted, will get trapped in the endosome/class E compartment

due to a change in its morphology. The endosome/class E compartment ends up next to the vacuole instead of fusing with the vacuole. The Vph1-GFP signal on the class E compartment suggests that Vph1 travels through the VPS pathway to reach the vacuole. Stv1 is an alternate "a" subunit that functions at Golgi and endosomes before being exchanged for Vph1 at the vacuole (Manolson et al., 1994). To visualize Stv1, we used an overexpression plasmid because our attempts using a more low-copy plasmid revealed no signal. The Stv1-GFP in the WT background showed a wide range of signal in puncta, which we assume are Golgi or endosomes and amore distributed fluorescence that looks like the endoplasmic reticulum (ER). In the, apl5Δ, and vps4Δ strains, we saw similar localization. This localization differs from previous studies of Stv1 and might stem from overexpression of plasmid-borne Stv1-GFP; because of this aberrant localization pattern,the Stv1-GFP result cannot be interpreted (Kawasaki-Nishi et al., 2001).

Figure 8. Vph1-GFP and Stv1-GFP with AP-3 Pathway and VPS Pathway Disruption

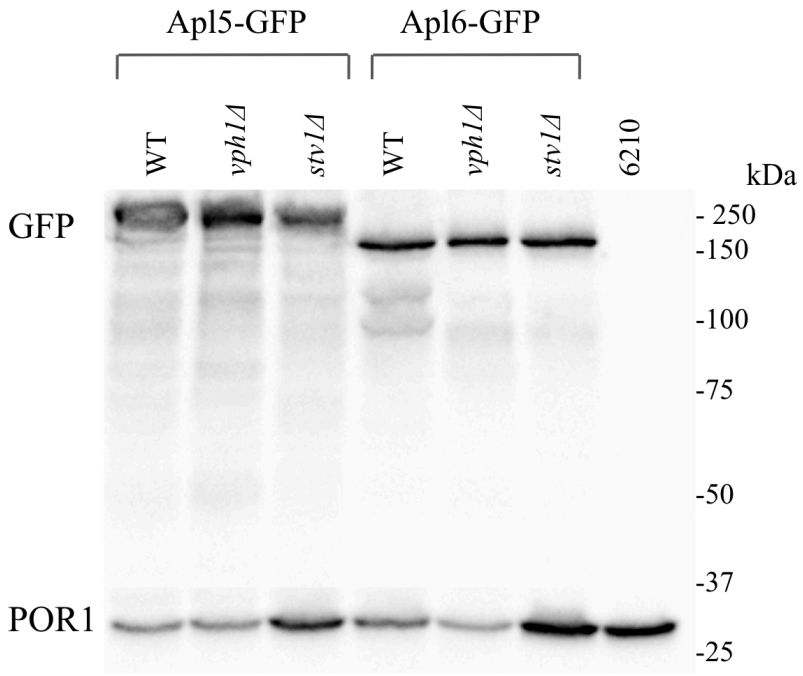


Note. The two plasmids containing Vph1-GFP and Stv1-GFP in wild-type (WT), *apl5Δ*, and *vps4Δ* strain. In WT and *apl5Δ*, the Vph1-GFP, in green, was consistently on the vacuole, in blue. The Vph1-GFP plasmid with a *vps4Δ* had a ring around the vacuole and a collection of GFP. The Stv1-GFP mislocalized and accumulated on the ER in WT, *apl5Δ*, and *vps4Δ* strain. Imaged by Mitchell Leih.

The effects of AP-3 on the V-ATPase, or the effect of the V-ATPase on AP-3, have not been explored. One possibility is that V-ATPase subunits affect AP-3 assembly. To investigate the effects of the V-ATPase on AP-3, I examined the abundance of the large AP-3 subunits by western blot of Vph1 and Stv1 knockout strains. To visualize the protein abundance in the sample, I transformed a GFP-tagged Apl5 and Apl6 into *vph1Δ* and *stv1Δ* strains and used Roche anti-GFP antibodies (1:3000) to blot against the GFP-fused large subunits.

The western blot revealed minor changes in Apl5-GFP expression (Fig. 9). The wild-type band appeared to have the most abundant Apl5, indicated by the white blot which is consistent with overexposure. The Vph1 knockout showed less protein abundance than the wild-type, however, it appeared to have more expression than the Stv1 knockout. The loading control, Por1, bands were somewhat inconsistent. It does not appear that the V-ATPase has significant impacts on AP-3 expression, however, this western blot should be repeated without overexposure and with consistent loading controls. Additionally, I want to validate these results by fluorescence microscopy.

Figure 9. Minimal Changes in AP-3 Large Subunit Abundance with *vph1Δ* and *stv1Δ*



Note. Western blot of AP-3 large subunits, Apl5 and Apl6, in wild-type and in V-ATPase subunit Vph1 and Stv1 knockouts. The Apl5 subunit in wild-type (Apl5-GFP) is the most abundant because the white spot indicates over-blotting, followed by *vph1Δ*, then *stv1Δ* as the least abundant. The abundance Apl6 in WT, *vphΔ*, and *stvΔ* appears to be consistent. The loading control, Por1, is inconsistent with the 6210 control sample. I blotted for Apl5-GFP (269kDa), Apl6-GFP (171kDa), and Por1 (30 kDa).

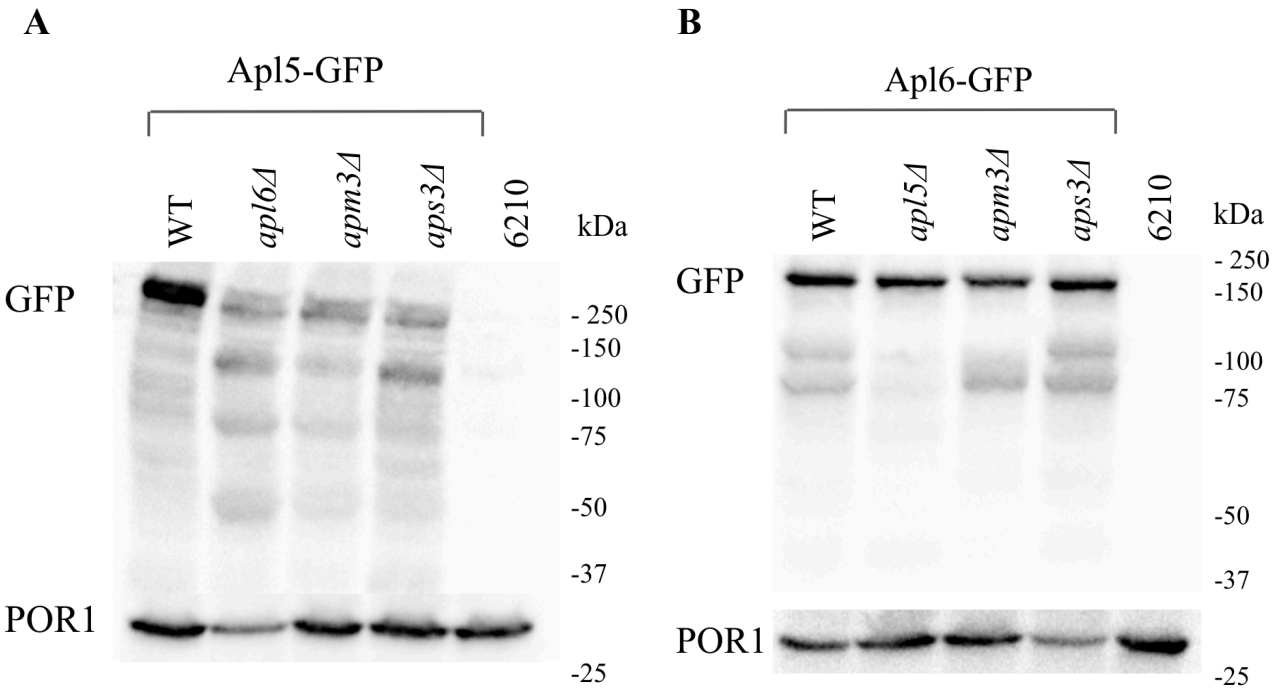
AP-3 Large Subunit Expression Dependency on the AP-3 Complex

In AP-1 and AP-2, homologs to AP-3, we see specific subunit assembly. These heterotetrameric protein complexes assemble in two steps (Wan et al., 2021). First, the γ/α large subunit and σ small are formed, followed by the β large subunit and μ medium subunit. Homology suggests that the AP-3 complex could also have a similar characteristic.

To test AP-3 subunit dependance, I performed a western blot of the AP-3 large subunits and examined the protein abundance in other AP-3 subunit knockouts. We see that the Apl5-GFP wild-type contains abundant protein, but this abundance is not present in the AP-3 subunit knockout strains (Fig. 10A). Additionally, there are non-specific cleaved bands below the

expected size of the Apl5-GFP. This could indicate that Apl5 depends on the other subunits' presence or it will be degraded. Apl6-GFP has consistent bands in the knockout strains and in the wild-type. This could indicate that Apl6 does not have subunit dependence and is unaffected by the presence of the other subunits. The Por1 has consistent abundance, however, Apl5-GFP *apl6Δ* and the Apl6-GFP *aps3Δ* do not contain as much protein as the 6210 control. This western blot should be repeated to confirm the results and improve the loading control, Por1.

Figure 10. Apl5-GFP Appears to Depend on the AP-3 Subunits and Apl6 Does Not.



Note. Western blot of the AP-3 large subunits, Apl5 and Apl6, in wild-type and 3 AP-3 subunit knockouts (*apm3Δ*, *aps3Δ*, and large subunits *apl5Δ/apl6Δ*). Apl5 subunit (Apl5-GFP) in wild-type is the most abundant compared to the other subunit knockouts. There is some cleavage of the protein shown, and is more prominent in the knockout strains. Apl6 subunit (Apl6-GFP) appear to be consistent in wild-type and the subunit knockout strains. The loading control, Por1, is consistent in the majority of the blot, but is weak in the Apl5-GFP *apl6Δ* and the Apl6-GFP *aps3Δ* compared to the 6210 control. I blotted for Apl5-GFP (269kDa), Apl6-GFP (171kDa), and Por1 (30 kDa).

Discussion

This study aimed to identify adaptor protein complex 3 (AP-3) interactors by exploring AP-3's interactions with the V-ATPase, which have not been characterized. Additionally, this study identified the subunit assembly dependence of AP-3's large subunits. It is important to better understand and characterize AP-3 and its interactors because we could help those suffering from adaptor protein pathway-deficient diseases, like Hermansky-Pudlak disease.

Screening methods of AP-3 candidate interactors done by the Merz, Conibear, and Ungermann Labs enabled our lab to narrow down the potential AP-3 interactors from ~6200 yeast proteins to 102. Mass spectrometry identified protein binding affinity to Apl5/Apl6 appendages, which were known to interact with the HOPS complex subunit Vps41 (Angers and Merz, 2009). GNSS identified functional defects in the AP-3 pathway by analysis of a synthetic cargo missorting to the plasma membrane (Plemel et, al., 2020). BioID identified proteins that interact or are in close enough proximity with AP-3 (Schoppe et al., 2020). Cross-referencing these three screenings provided us with 24 strong candidates for AP-3 interaction. The remaining 78 proteins were chosen from the BioID screening based on confidence ordering done by the Ungermann lab. I performed a BiFC analysis of these 102 proteins, obtained from a genome-wide BiFC library.

There was not a consistent pattern of location or mechanism of action for the 17 proteins that were identified in the BiFC screening (Table 2). The ribonucleotide-diphosphate reductase protein complex has two subunits, Rnr1 and Rnr4, both of which were positive interactors with Apl6-VC. Although these two proteins are on the same complex, they have significantly different BiFC results. Rnr1 has a very large accumulation of GFP located at the vacuole, whereas Rnr4 has a very small GFP puncta located between two vacuoles within one cell. Rnr1 and Rnr4 came

out in the triple screening that revealed 24 candidates. Vma13 and Vma10 are subunits of the vacuolar ATPase (V-ATPase) and are candidate AP-3 interactors analyzed with BiFC. Vma13 was found in the triple screening but did not interact in the BiFC experiment, whereas Vma10 was found in the BioID screening and had interacted with Apl6-VC. This was an unexpected result because Vma13 was more likely to interact with AP-3 than Vma10, based on the screenings, and they are members of the same multi-protein complex, like Rnr1 and Rnr4. The individual interactions with AP-3 and the proteins of interest should be further investigated. Experiments with BiFC and all of the V-ATPase subunits should be done to determine if there is a commonality between certain subunits that do not. It could further reveal the nature of AP-3 and V-ATPase interaction. Additionally, a western blot of AP-3 large subunit abundance with positive BiFC interactor knockouts should be investigated to further understand the interactions and if they affect AP-3 function.

I identified that V-ATPase subunit Vma13, “H” in the V1 peripheral cytoplasmic-facing domain, one of the 24 candidates, did not interact with AP-3 by BiFC (Fig. 3). However, V-ATPase subunit Vma10, “G” in the V1 peripheral cytoplasmic-facing domain, in BioID high confidence, did interact with AP-3 by BiFC. This result led me to investigate the interactions between AP-3 and other V-ATPase subunits. In this study, I examined the location-specific homologous protein isoforms of subunit “a” Vph1 and Stv1.

Our lab obtained Vph1 and Stv1 strains containing VN fragments from a genome-wide BiFC library. Vph1-VN and Stv1-VN had a confirmed BiFC interaction with Apl6-VC (Fig. 7). Vph1 had a strong interaction, and it was similar to the control Apl5-VN and Apl6-VC, whereas Stv1 had a weaker interaction. Stv1 is associated with the V-ATPase at the Golgi/endosome, and Vph1 is associated with the V-ATPase as the vacuole (Manolson et al., 1992; Manolson et al.,

1994). This could indicate that Vph1-VN could go through the AP-3 pathway, because of its persistent interaction with the AP-3 complex. Another possibility is that Vph1 interacts with the AP-3 complex and its interaction could be involved in V-ATPase or AP-3 function.

To further investigate this interaction and the pathway of Vph1 and Stv1, I transformed plasmids containing a GFP fluorophore fused to Vph1 and Stv1 into pathway-deficient strains to visualize the expression in the cells. Plasmids are small, circular fragments of DNA and are a useful genetic tool, ideal for carrying a new gene into a host cell (NCRBA, 1984). The strains containing plasmids are easily selected for because of a selection marker within the plasmid. Strains are transformed with a plasmid and spread on a selective plate, without the plasmid the strains will not survive.

I transformed Vph1-GFP and Stv1-GFP plasmids into wild-type, *apl5Δ*, and *vps4Δ* strains. An AP-3 large subunit knockout disrupts the AP-3 pathway, creating deficiencies in the cargo sorting (Fig. 1) (Plemel et al., 2021). Apl5 is one of these large subunits, without it we see a disruption in the AP-3 pathway function. A Vps4 knockout disrupts the VPS pathway and cargo gets trapped in the endosomes on the way to the vacuole (Babst, 1997). A comparison of the two deficient pathways indicates which pathway Vph1 is likely to go through to reach the vacuole. Vph1-GFP in wild-type and *apl5Δ* looked similar, with a ring around the vacuole (Fig. 8). Vph1-GFP in *vps4Δ* contains a faint ring and a large accumulation of GFP next to the vacuole. This result is consistent with a change in endosome morphology called a class E compartment, due to the protein getting trapped in the endosome on its way to the vacuole (Russell et al., 2012). The class E compartment characteristics of Vph1-GFP indicated that Vph1 goes through the VPS pathway. This is consistent with what we expected because the literature indicates the V-ATPase is transported through the VPS Pathway, including Vph1 (Tomisha et al.,

2014). A low-copy plasmid of Stv1-GFP was unsuccessful in visualizing Stv1, and an overexpression plasmid was used instead. In wild-type, *apl5Δ*, and *vps4Δ* strains, Stv1-GFP is seen throughout the cell, and appears to be localizing to the ER and other organelles, like the vacuole and nucleus, which is inconsistent with Stv1-GFP localization (Kawasaki-Nishi et al., 2001). Due to the mislocalized signal of Stv1 to the ER, I cannot make a definitive conclusion of the effects of Apl5 or Vps4 mutants on Stv1 localization. A drawback to using plasmids is that a wild-type copy of the gene is present in the cells and is unlabeled, and thus cannot be visualized. This experiment should be repeated with a genomic knockout of Vph1 and Stv1 or a genomic integration of GFP in those genes.

To identify if there is AP-3 large subunit dependence on Stv1 and Vph1, I examined their expression in yeast by western blot. In Apl5, the wild-type strain appeared to have the most protein, indicated by the white blot which is consistent with overexposure. The Vph1 knockout showed less expression than the wild-type, however, it appeared to have more expression than the Stv1 knockout. Although we do see some effect of *vph1Δ* and *stv1Δ* on Apl5, we cannot conclude this because the western blot must be repeated, and imaged without overexposure. Por1 is a control to ensure that the loading of the lanes and concentration of the samples are consistent. Por1 is 30 kDa, and the proteins were at the right size, but the abundance was inconsistent. Apl6 appears to be consistent in wild-type, *vph1Δ,* and *stv1Δ*. If the effect of the V-ATPase subunits of Apl5 is real, this could indicate that the V-ATPase is involved in AP-3 subunit assembly. Apl5 binding to Vph1, Stv1, Vma10 or other V-ATPase subunits should be tested by mass spectrometry and the impacts of Apl5 analyzed by western blot.

As mentioned, AP-1 and AP-2 are homologous to AP-3. Recently, the assembly of AP-1 and AP-2 has been investigated. It was determined that the AP-1 and AP-2 γ/α and σ subunits,

which are homologs of Apl5 and Aps3, are the first proteins to join the complex(Wan et al., 2021). The next subunits to join together are the β and μ subunits, which are Apl6 and Apm3 homologs. To test if AP-3 had similar subunit assembly to AP-1 and AP-2, I performed a western blot to examine the subunit dependance of Apl5 and Apl6 on assembly. I blotted for Apl5-GFP and Apl6-GFP in subunit knockout strains, *aps3Δ, apm3Δ,* and *apl5Δ/apl6Δ*. The results revealed that Apl5-GFP in wild-type had the most abundance, whereas, the *apl6Δ, apm3Δ*, and *aps3Δ* knockouts did not have significant protein abundance compared to wild-type (Fig. 10A). Apl6-GFP all of the knockout strains were consistent with the band seen in wild-type (Fig. 10B). Apl5 protein abundance in wild type indicates that the other subunits in the complex are required for Apl5 presence in the cell. Without the other subunits, it appears that Apl5 is degraded as shown by the faint bands that vary in size. This could indicate that Apl5 assembles late in complex formation, and without the other subunits it is degraded. In Apl6-GFP, in all mutant strains, and in wild-type, the bands remain constant. This could indicate that Apl6 is subunit independent, and could assemble first in the complex. The stability of the protein could indicate that it is necessary in the initiation of AP-3 formation. In the future, Apm3-GFP and Aps3-GFP in subunit knockout strains should be tested to identify if there is subunit dependence for all subunits. It would be interesting to see if AP-3 assembly continues the same pattern of a two-step assembly and should be further investigated by western blot.

Future Directions

BiFC is a great way to test protein interactions, however, it is not always accurate because the protein of interest could interact with surrounding proteins, or an intermediate of its interactor, revealing a false-positive BiFC interaction. The positive BiFC interactors should be further tested, along with the V-ATPase subunits, to confirm an AP-3 interaction. This can be done through mass spectrometry, protein purifications, pulldowns, or potentially a virtual analysis. To further investigate the validity of the result, a western blot of the large AP-3 subunits and knockouts of the 17 interactors should be performed to identify if these proteins have an effect on the protein abundance. This could give insight into their interactions and determine whether the interaction is important to the AP-3 complex or the AP-3 pathway. Additionally, another BiFC screening with the VN library with Apl5-VC could yield more proteins and narrow down the list of candidate interactors, or potentially uncover new candidates. Although Apl5 and Apl6 are in the same complex, it could be beneficial to test if there is a difference between the two large subunits because Apl5 is known to directly bind other proteins and there could be some variation in the BiFC result (Angers and Merz, 2009).

A knockout of the V-ATPase genes disrupts the formation of the V-ATPase, with the exception of Vph1/Stv1 which only exhibits a phenotype if both are knocked out (Reviewed in: Kane, 2006). In the future, it could be beneficial to test how complete disruption of V-ATPase (e.g., *vma3*Δ) affects Apl6 interaction with Vma10 and Vma13 by BiFC. This result can help determine if the V-ATPase has an effect on AP-3. The effect on AP-3 can be tested through western blot, to look at the large subunit protein abundance, or GNSS, to identify if disrupted V-ATPase leads to AP-3 pathway dysfunction. Vma3 subunit of the V-ATPase and it part of the V0 integral membrane domain. The BiFC interactions of other V-ATPase subunits, like Vma3,

and Apl6 should be tested to determine if the interaction is domain-specific. This result could help identify a pattern of AP-3 and V-ATPase interaction, and reveal the reason why Vma13 did not interact. In addition to these BiFC interactions, an integrated GFP in the V-ATPase subunits, specifically Vph1 and Stv1, should be performed to show the pathway that Stv1 goes through. Additionally, Vph1-GFP and Stv1-GFP are incorporated into the cell via plasmid. There are wild-type copies of Vph1 and Stv1 in the cell, and with a genomic integration it will provide more accurate results.

In this study, we saw that Apl5 is subunit-dependent and Apl6 is subunit-independent. In the future, Apm3-GFP and Aps3-GFP in Apl5, Apl6, Apm3/Aps3 knockout strains should be investigated. The subunit assembly of AP-1 and AP-2 are shown to have large subunits coupled up with medium and small subunit subunits. A western blot of Apm3-GFP and Aps3-GFP should be performed to determine the subunit dependance of the medium and small subunits, and could reveal more about the subunit assembly of AP-3. Additionally, the western blot of Apl5-GFP and Apl6-GFP should be repeated to confirm the accuracy of the result.

Materials and Methods

Yeast Cell Culture and Transformation

Yeast cells were cultured overnight in a growth medium, YPD(1% Yeast Extract, 2% Peptone, 2% Dextrose), or selective growth media YNB(2% Glucose, 0.67% Yeast Nitrogen Base w/o Amino Acids, 0.067% amino acid drop-out mix). Cells were diluted to 0.2 ODU/mL and incubated until mid-logarithmic stage (0.5 – 0.8 ODU/mL). Cells were centrifuged at ~5,000 x g for 5 minutes at room temperature (RT). Supernatant was aspirated, and the pellet was resuspended in 1 mL of sterile water, and centrifuged at ~10,000 x g for 5 minutes - repeated. Cells were resuspended in 1mL of 0.1M LiOAc/TE (0.1 M lithium acetate, 10 mM Tris pH 7.5, 1 mM EDTA pH 8) and rotated at RT for 10 minutes. Sample was centrifuged at ~10,000 x g for 5 minutes and the pellet was resuspended in 50 µL 0.1 M LiOAc/TE, 10 µL of 10 mg/mL Salmon Sperm DNA(Invitrogen), DNA (1 µg plasmid DNA) to be transformed and 700 µL of 40% PEG-4000(in 0.1 M LiOAc/TE). Cells were rotated for 30 minutes at RT, and heat shocked for 20 minutes at 42℃. Cells were centrifuged at ~10,000 x g for 5 minutes, resuspended in sterile water, and spread onto an agar medium. Plates were incubated at 26℃.

CMAC Staining and Imaging

Cells were grown to mid-logarithmic stage (0.5 – 0.8 ODU/mL) and centrifuged at ~5,000 x g for 5 minutes at RT. Supernatant was aspirated and pellet was resuspended in 1mL of sterile water. Sample was centrifuged again, and resuspended in 1mL of blank YNB media (2% Glucose, 0.67% Yeast Nitrogen Base w/o Amino Acids). CellTracker CMAC Blue Stain (Invitrogen) (100µM) was added and rotated for 15-30 minutes at RT. Cells were rinsed with sterile water, resuspended in YNB media, and placed on a ConA/PolyK pre-treated glass slide and cover slip. 10% laser power was used at 50ms exposure, low gain. Live yeast cells were then

observed at RT with an inverted fluorescence microscope (Ti2 2E PSF; Nikon) equipped with a Yokogawa CSU-X1 spinning disk confocal system and a 100 (1.45 numerical aperture) oil objective (Plan Apo λ; Nikon). Images taken with an Andor iXON Ultra 512x512 EMCCD camera were acquired with Micromanager version 2.0 software and analyzed with ImageJ software (NIH).

Western Blot

Cells were grown to mid-logarithmic stage (0.5 – 0.8 ODU/mL) and centrifuged at ~5,000 x g for 5 minutes at RT. Cells were resuspended in 900 µL of sterile water and 100 µL of 100% trichloroacetic acid, vortexed, and incubated on ice for ≥ 20 minutes. Samples were centrifuged at 13,000 x g for 5 minutes at 4℃, and resuspended in 1mL of -20℃ acetone by water-bath sonication. Samples were incubated on ice for ≥ 20 minutes and the acetone wash was repeated. Samples were centrifuged 13,000 x g for 5 minutes and the supernatant was aspirated. Cell pellet was dried by speed-vac for 15 minutes. 1X Laemmli SDS sample buffer (Diluted from 4X: 250mM Tris-Base/Tris-HCl, 8% SDS, 40% Glycerol, 8% SDS, 0.05% Bromophenol blue, 6% 2-mercaptoethanol) (ThermoScientific) diluted the pellet to 5 ODU equivalent. Sample pH was adjusted by 6.8 pH Tris Buffer (12.11% Tris base, HCl to pH 6.8). Samples were vortexed with glass beads (Sigma) for 15 minutes. Samples were heated at 90-100℃ for 5 minutes.

20 µL aliquots of the samples were added to a precast polyacrylamide gel (BIO-RAD Mini-PROTEAN TGX) and run at 200V for 40 minutes. Gel was transferred to a nitrocellulose membrane run at 90V for 90 minutes at 4℃. Membranes were blocked in 5% milk in TBST((TBS + 0.05% Tween-20)) for 1 hour at RT. Primary antibodies in a new 5% milk in TBST solution were added to the membrane overnight. Membrane was washed in TBST four

times, with two 5 minute washes, and a secondary antibody in a 5% milk in TBST solution was added to the membrane for an hour. Membrane was washed five times, with two 5 minutes washes, and one wash in TBS(10mM Tris pH 8, 100mM NaCl,), and was imaged with SuperSignal Pico West PLUS Chemiluminescent Substrate (ThermoScientific).
Antibodies: α-GFP (Roche, 1:1000), α-Por1 (Sigma, 1:3000), α-Mouse (Sigma, 1:3000)

Electrophoresis was used to separate proteins based on their size. Proteins, or protein containing a tag (like GFP), were blotted for using a primary antibody. Antibodies were used to recognize and bind their target protein, particle, or antibody of interest (Pillai-Kastoori et al., 2020). Secondary antibodies were used to bind the primary antibody for visualization. Secondary antibodies contained HRP(horseradish peroxidase)-conjugated and was detected with chemiluminescence, SuperSignal Pico (HRP-Conjugated Secondary Antibody, n.d.). Apl5 has a molecular weight of ~107 kDa, and Apl6 has a molecular weight of ~91.6 kDa (*Saccharomyces Genome Database*, n.d.). GFP tag that was added to visualize the protein via western blot is around 27 kDa and is from a plasmid that contained the large subunits fused to the GFP (Urkert and Günzburg, 2000). Apl5-GFP fusion contains 6 GFP tags fused (269 kDa) and Apl6-GFP is fused to 3 GFP tags (172 kDa). Por1 was blotted for as a loading control (30 kDa).

Mating and Sporulation

Haploid strains with MATa and MATalpha mating type were mixed on a plate. Strain mixture was plated on two selective plates. Diploids were identified by a mating type check and left in a shaker for 1 week in Sporulation Media (1% potassium acetate, 0.1% Bacto yeast extract, 0.05% glucose). Cell suspension (1X10^8 cells) was added to a polypropylene tube containing 180µL of sterile water. Cells were washed 2x before being suspended in 180µL of sterile water. 20µL of 5mg/ml solution of Zymolyase 20T in ZL buffer (0.1 M NaPO4, pH 6.5,

1.2 M Sorbital, and 40% Glycerol) was added to the cells and was incubated at 30℃ for an hour. Cells were centrifuged at 13,000 x g and washed with 1mL sterile water. Cells were centrifuged at 13,000 x g and resuspended in 100µL of sterile water, and vortexed on high for 2 minutes. Supernatant was poured out and rinsed twice with sterile water, supernatant is poured out each time. 1.0ml sterile 0.01% NP-40 detergent was added to the tube and sonicated for 1 minute. 1:10 serial dilutions of the sonicated mixture were made and 200µL of the solution was plated on YPD or YNB selective plates.

Bimolecular Fluorescence Complementation Approach

VN fragments of Venus GFP were fused to the 102 proteins identified. VN-fused proteins were checked for by selectable markers. Selection plates were used to select strains that contained the cassette, with the VN/VC fragments integrated in the genomic DNA. These cassettes were DNA sequences that contained a selective marker. Selective markers enabled cells to survive in an environment deficient in an essential amino acid or nucleotide or provided resistance in the presence of a fungicide (Bähler et al., 1998). VN strains were mating type a (MAT a). VC strains were fused to the Apl6, AP-3 large subunit, and was mating type alpha (MAT alpha). MAT a and MAT alpha strains containing the fragments were mated to create a diploid that contained the VN and VC fragments. Selective plates, -URA(VN) and +KAN(VC) were used to identify the diploid. Diploid cells were heterozygous for the wild-type gene and VN/VC fused genes.

Diploids were sporulated back into haploids with sporulation media. Sporulation media created an inhabitable environment through potassium acetate. Acetate raised the pH of the sporulation media to initiate yeast sporulation (Dickenson et al., 1983; Honigberg and Purnapatre, 2003). Selection plates were used to isolate haploids that contained both VN and VC

fragments. Sporulation created haploid cells homozygous for VN and VC fragments. Vacuole and dead cells were stained with CellTracker CMAC Blue Stain (Invitrogen). CMAC is a hydrophobic compound that crosses the plasma membrane into the cytoplasm and accumulates in the vacuolar lumen, presumably by pumps on the vacuolar membrane (Shoji et al., 2006). In dead cells, the pump will no longer function and the entire cell will fluoresce with CMAC.

Confocal fluorescence microscopy of the 102 candidates identified 17 positive AP-3 interactors. Images were normalized to a known AP-3 interactor to ensure signal accuracy using ImageJ. Protein interactions were not guaranteed because of possible interaction between the protein of interest interacting with another protein near AP-3, or they interacted transiently, due to the strong and irreversible BiFC interaction (Wong and O'Bryan, 2011). AP-3 interaction with a protein transiently pulls the protein away from its original location because of the strong VN-VC connection. BiFC is an efficient screening method and is an indicator that the proteins were in close proximity to interact, and should further be pursued as potential AP-3 interactors.

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