THE FIRST CHARACTERIZATION OF THE WASH COMPLEX IN *C. ELEGANS* ENDOCYTIC RECYCLING

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ABSTRACT OF THE THESIS

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The quantity and distribution of proteins in the plasma membrane play an essential role in regulating a cell's response to its environment and resulting physiology. This cell surface protein mosaic is largely influenced by the endocytic recycling pathway, misregulation of which has been implicated in a variety of human conditions, including neurodegenerative diseases and cancer. Endocytosis and subsequent intracellular movement of cargo requires both membrane fission and vesicle movement, with the force for these processes provided by a spatially and temporally regulated dynamic branched actin network, generated by Arp2/3-mediated actin polymerization. While it has been determined that endocytosis requires the nucleation promoting factors WASP and WAVE at the plasma membrane, a role for the structurally-related WASH complex for this process in *C. elegans* remains more elusive. In mammalian systems, the WASH complex seems to act at early endosomes, facilitating retrograde trafficking via its FAM21 subunit interacting with retromer. However, a homologous protein to FAM21 in the *Caenorhabditis elegans* WASH complex has yet to be identified. This work provides the first characterization of the *C. elegans* WASH complex, detailing its function on RAB-5-, PI(3)P-positive early endosomes to ensure proper sorting during endocytic recycling, likely through retrograde trafficking to the Golgi apparatus. Through RNAis and genetic

crosses, these experiments reveal a functionally important role for the WASH complex in this pathway that is distinct from other nucleation promoting factors, including the WAVE complex, despite similarities between the two. Furthermore, proper recycling may be mediated in part by the protein C05G5.2, as these data suggest this could be the previously unidentified *C. elegans* FAM21 homolog.

Acknowledgement

I would like to thank Dr. Martha Soto for accepting me into her lab with no reservations, despite my non-traditional path as a Master's student. I would also like to thank my lab members Luigy Cordova Burgos, Sofya Borinskaya, and Denver Baptise, whose assistance and advice allowed this project to continue moving forward. Finally, this work would not be possible without the support of my family, friends, and coworkers, whose encouragement and positivity helped remind me of the value of hard work and my love of learning.

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Introduction

Actin is the most abundant protein in most eukaryotic cells, as it is involved in a wide variety of cellular processes[1]. The actin cytoskeleton consists of filamentous (F)-actin, assembled from 43-kDa globular (G)-actin monomers, which are present at high levels in the cytoplasm, as more than half of all cellular actin is found in the monomeric form. Upon polymerization, either linear or branched actin molecules are created, which are asymmetric, with a fast growing barbed end and a slower growing pointed end[2]. This polymerization process requires ATP hydrolysis, driven by association with interacting proteins, and enables a variety of cellular functions, including motility, organizing the contents of the cell, cytokinesis, cell migration, establishment of polarity during developmental morphogenesis, and, importantly, endocytosis[3]. In an effort to examine these processes, the model organism *Caenorhabditis elegans* was used in this study. This organism presents the advantages of being transparent, with a high fecundity in a short time, and has well characterized developmental stages, making it an ideal eukaryotic model organism in which to investigate cellular changes.

As assembly and disassembly of filaments are integral for actin function during such a wide variety of processes, these dynamic changes are under tight regulatory control by an assortment of actin-binding proteins[2]. More specifically, polymerization of actin does not occur spontaneously, as it is not kinetically favorable, meaning the initiation of new filament formation (referred to as nucleation) requires additional factors. The linear or branched polymeric structure determines which factors will be needed, with linear actin being nucleated by a group of proteins called the formins, while branched actin nucleation is driven by the 7-subunit actin-related protein-2/3 (Arp2/3) complex

(*Figure 1*)[2]. Once filament formation is initiated, the polymer extends rapidly until it is actively stopped[1]. This allows the branched actin networks in cells to change rapidly, undergoing nucleation and forming y-shaped anisotropic branches at a 70-degree angle from the initial actin fiber, referred to as the mother filament. After nucleation, elongation occurs at the fast-growing barbed end, or plus end[4]. The resulting branched actin networks can be rather extensive, in part due to the presence of actin capping protein, which restricts elongation by binding to barbed ends, allowing a vast network of short filaments with high branch density that provides enough stiffness to generate force[5].

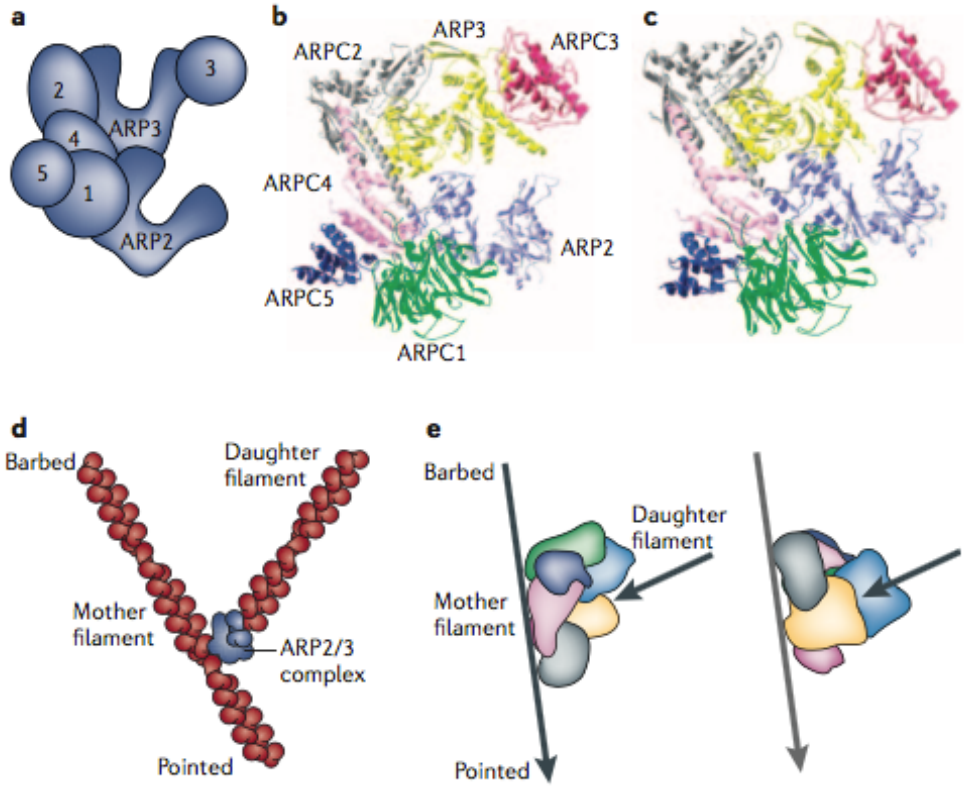


Figure 1: The Arp2/3 complex[2]. *A-C: Structure of the 7-subunit Arp2/3 complex. D, E: Actin nucleation of branched actin from mother filaments.*

However, the Arp2/3 complex has little biochemical activity on its own, requiring activation and a significant conformational change in order to induce actin polymerization. The proteins that activate the Arp2/3 complex are called type I nucleation promoting factors (NPFs)[2,6]. Type I NPFs work by binding to both the Arp2/3 complex and monomeric actin to initiate nucleation and polymerization. The first eukaryotic type I NPF to be identified was the Wiskott-Aldrich syndrome protein (WASP), named for the rare X-linked immunodeficiency disease in which it was discovered[7].

Following initial exploration of the WASP complex, five additional subfamilies of WASP proteins were identified across many eukaryotic organisms: neuronal-WASP (N-WASP), WASP and SCAR homolog (WASH), WASP family verprolin homolog (WAVE) also known as suppressor of cyclic AMP repressor (SCAR), junction-mediating regulatory protein (JMY), and WASP homolog associated with actin, membranes, and microtubules (WHAMM). These five WASP family proteins are structurally diverse, with unique domains that allow for their differing subcellular locations and interactions with proteins that can regulate their activity. However, they all share a conserved verprolin-connecting-and acidic (VCA) domain at their C-termini, (*Figure 2*) which allows for their actin nucleating abilities[7,8]. More specifically, the WASP homology 2 (WH2) domains near the C terminus bind monomeric actin, followed by the central/acidic region, which interacts with the Arp2/3 complex. Moreover, all NPFs contain a series of polyproline repeats, where Src-homology 3 (SH3)-domain-containing proteins can bind[1]. Contrarily, the N-termini of the five NPFs will vary, such that they contain domains that connect with different regulatory proteins. For example, WASP

proteins contain a WASP homology 1 (WH1) domain and a CRIB domain, while SCAR/WAVE has a SCAR homology domain (SHD), a WASH homology domain (WAHD), and a tubulin-binding region (TBR)[1,9]. Of the five NPFs identified, only three have been observed in *C. elegans*—WASP, WAVE, and WASH.

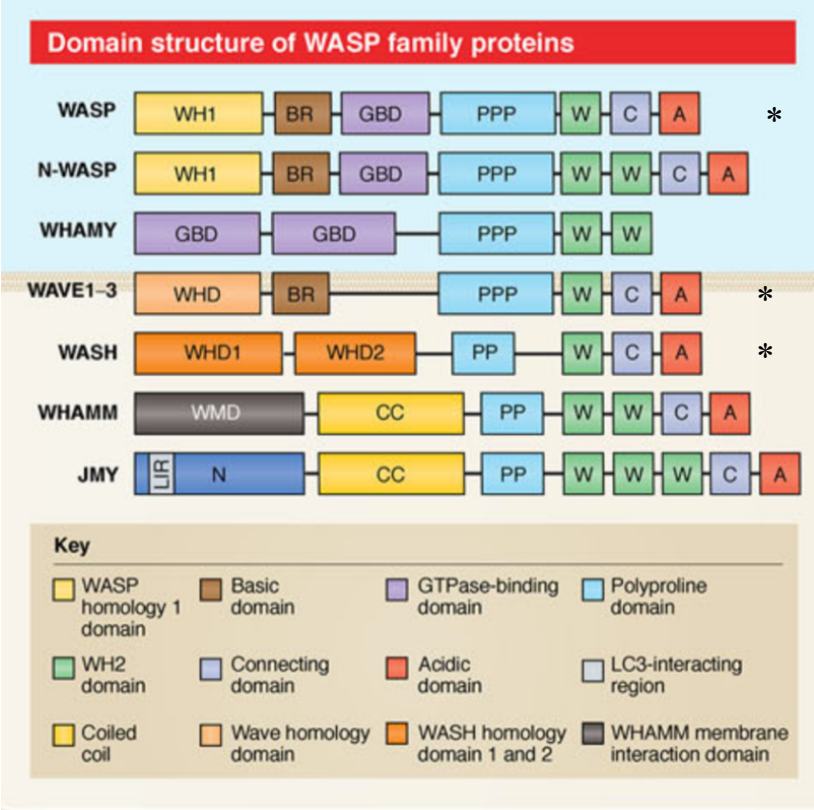


Figure 2: WASP family protein domains[7]*. All WASP family proteins share a similar C-terminus, containing polyproline repeats and a VCA region, but their N-termini will vary. Those marked with "*" above have been identified in C. elegans, with only one WAVE homolog, WAVE-1, existing in these worms.*

While most NPFs act independently, one surprising feature discovered about the WAVE complex was its ability to associate with other proteins to form a functional complex. WAVE is one part of a pentameric complex, with the five subunits being WAVE/SCAR, Specifically Rac-associated 1 (Sra1/Gex-2/CYFIP), NCK-associated proteins (NAP1/GEX-3), Ableson interacting protein (ABI-1/ABI) and Hematopoietic stem progenitor cell 300 (HSPC300/NUO-3)[10]. Similarly, it was recently discovered that WASH also exists in a pentameric complex, with a strong structural similarity to the WAVE complex (Figure 3)[11], suggesting an evolutionary relationship between the two. The WASH complex consists of WASH, FAM21, Strumpellin (KIAA0196), SWIP (Strumpellin and WASH-interacting protein)/KIAA1033, and Ccdc53 (coiled-coil domain-containing protein)[6]. For both the WAVE and WASH complex, knockdown of one component destabilizes the others, leading to the degradation of the complex as a whole[8,12].

Figure 3: Structural relationship between the WASH and WAVE complexes[11]*. Both WASH and WAVE assemble into pentameric complexes by interacting with other proteins.*

Furthermore, the Arp2/3 complex and these NPFs are part of larger signaling pathways, consisting of multiple steps that provide many opportunities for regulation,

allowing cells to exercise an even greater degree of spatial and temporal control over processes involving actin polymerization (Figure 4)[13]. These pathways involve both Rho-family GTPases and lipid second messengers[2]. First, GTPases are molecular switches, which cycle between GDP- and GTP-bound states. To help modulate this cycle, guanine-nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs) catalyze nucleotide exchange and hydrolysis, respectively[14]. This means that GEFs activate small GTPases by catalyzing the exchange of GDP to GTP, while GAPs inactivate small GTPases by enabling their GTPase activity (Figure 5)[15]. These enzymes are plentiful and essential for a variety of processes, such that in the human genome, up to 82 GEFs and 67 GAPs have been identified[16]. Second, in some of these NPFs, including WASP, the shape of the protein is such that the VCA domain is autoinhibited due to its interaction with the N-terminus. To release this interaction, the small GTPase Cdc42 (Cell division control protein 42) will bind with the help of the phospholipid phosphatidylinositol-4,5-bisphosphate ($PI(4,5)P_2$), allowing a conformational change in the NPF itself[17]. This exposes the autoinhibited VCA region so the verprolin-homology domain binds to G-actin, while the central and acidic domains mediate binding to Arp2/3[1,18]. However, the other NPFs are not autoinhibited, including WAVE and WASH, with less information known about how these two are regulated[18].

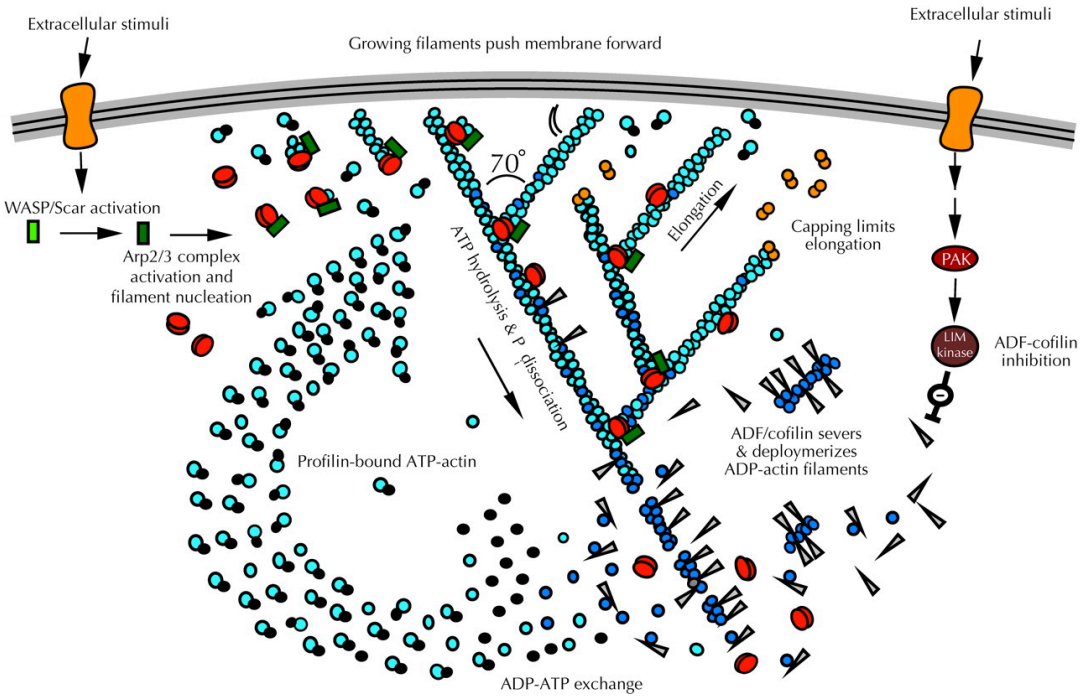


Figure 4: Branched actin assembly[13]*. Arp2/3 activation by the NPFs leads to branched actin networks, as part of a larger regulatory pathway*

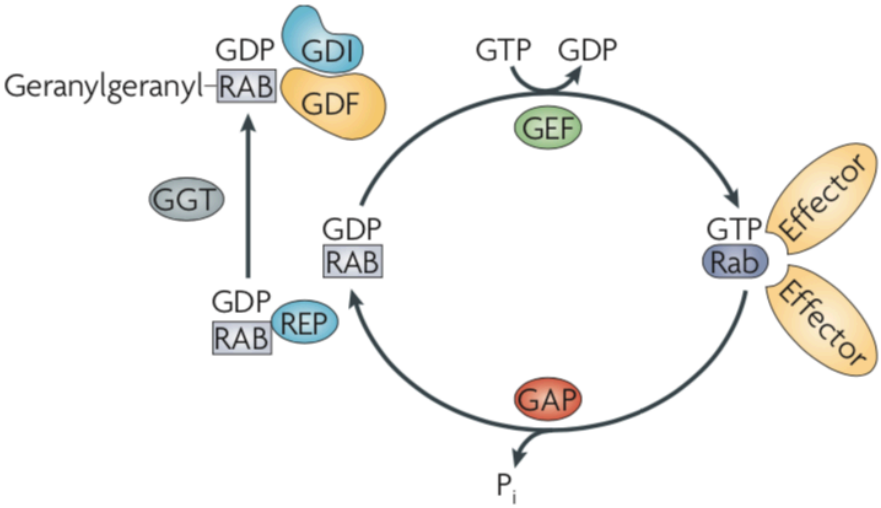


Figure 5: Activation of GEFs and GAPs[15]*. GEFs can activate small GTPases, while GAPs inactivate them, through the exchange of GDP to GTP and vice versa, respectively.*

Recently, an additional degree of regulation in these NPFs has come to light. It has been shown that phosphorylation is important for activating these proteins, with phosphorylation of serine and threonine amino acids in particular working to stimulate actin polymerization[1]. For example, tyrosine phosphorylation of WASP at Y293 increases its ability to bind to Arp2/3 without the need for Cdc42 or PI(4,5)P_2, perhaps by disrupting the autoinhibited conformation. Likewise, mutations that prevent Y293 phosphorylation result in complete deficiency of active WASP. This indicates that tyrosine phosphorylation is essential for WASP activation[19,20]. Similarly, the WAVE complex is phosphorylated at Y125 of the WAVE1 subunit by the Src non-receptor tyrosine kinase to release the VCA region, as well as at Ser343, Thr346, and Ser351 of WAVE2[21]. However, phosphorylation of the WASH complex is less straightforward. In mouse natural killer cells, for example, Y141 is a major site of phosphorylation that allows for their cytotoxic abilities. In other organisms, the WASH homology domain 2 (WHD2) contains a conserved ANDLQ/MY motif, with Y273, Y262, and Y261 being phosphorylated in *Drosophila,* humans, and mice, respectively. This allows for regulation in WASH activity and actin polymerization[22]. Despite the importance of conserved phosphorylation sites in these NPFs, the role of phosphorylation in *C. elegans* WASH complex activation has yet to be examined.

Functionally, the regulation of actin polymerization by Arp2/3 and its NPFs plays an integral role in two essential processes of developmental biology in *C. elegans*: morphogenesis and endocytic recycling. *C. elegans* nematodes have five actin genes, with ACT-1, -2, and -3 serving redundant roles during embryonic development[3]. It is already well understood that actin is essential for various steps in the process of

morphogenesis in these embryos, defined as the development of form, tissues, organs, and organisms[23,24]. For instance, actin establishes polarity in a one-cell embryo, a step essential for correct development moving forward, allowing the embryo to divide asymmetrically and defining the anterior and posterior domains[3]. The formation of this body axis prior to the first embryonic cleavage is attributed to the Partitioning-defective (*par)* genes, which are required for setting up the anterior-posterior axis[16]. After the ninth round of embryonic cell divisions, morphogenesis is largely controlled by actin in the epidermis, a single epithelial layer surrounding the animal[24]. Epidermal cells then undergo dorsal intercalation[24], whereby two rows of cells form a single row across the dorsal midline, followed by ventral enclosure, in which the ventral epidermal cells migrate towards the ventral midline, encasing underlying cells. Finally, the embryo undergoes elongation along the anterior-posterior axis, allowing progression from the comma stage to the two-fold stage and beyond (*Figure 6*)[24].

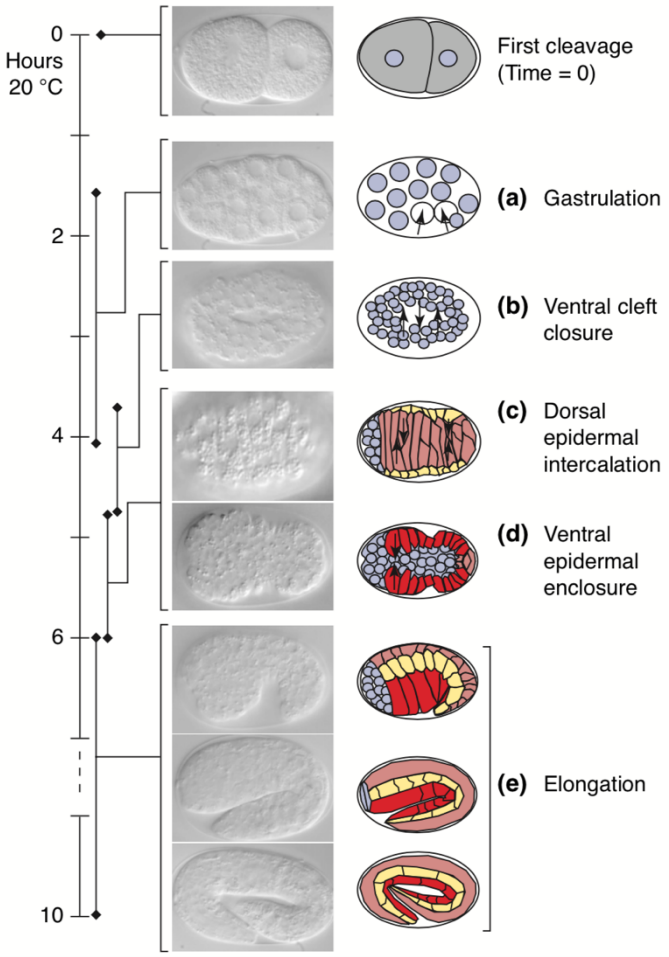


Figure 6: C. elegans embryonic morphogenesis[24]*. A well understood process requiring actin polymerization, morphogenesis in C. elegans consists of three main steps: dorsal intercalation, ventral enclosure, and elongation.*

In addition to its importance in embryonic morphogenesis, the branched actin polymerization pathway including Arp2/3 and the NPFs is also involved in maintaining protein composition at the plasma membrane**.** The plasma membrane is frequently described as a "fluid mosaic model", a simple yet accurate characterization that highlights its dynamic nature and diverse phospholipid-protein milieu, both of which are

essential for maintaining a cell's internal physiology. These various proteins and phospholipids must constantly change in their abundance and spatial distribution in order for the cell to maintain homeostasis and respond to changes in the extracellular environment[25]. For instance, alterations in these transmembrane proteins allow cells to regulate their ability to obtain materials and energy through ion and nutrient channels, as well as to interact with the environment through receptors for cellular signaling or antigen presentation[25].

One important process that occurs at the cell membrane that affects this 'mosaic' is endocytosis, in which materials smaller than 0.5um in diameter are brought into the cell following the membrane's invagination[26,27]. There are two main types of endocytosis—clathrin-mediated endocytosis, which requires the formation of clathrin-coated pits, and caveolae uptake[14]. Clathrin-mediated endocytosis occurs through a series of steps, including the formation of clathrin-coated pits, followed by their growth and maturation, and then subsequent scission, release, and uncoating of clathrin-coated vesicles (*Figure 7*)[28]. Contrarily, caveolae-mediated endocytosis requires the formation of caveolae instead, which are plasma membrane invaginations with a diameter of 50-100nm. Although there are other, more recently identified pathways, collectively referred to as clathrin-independent endocytosis, they are not thought to be main mechanisms of endocytosis and will not be addressed here[14].

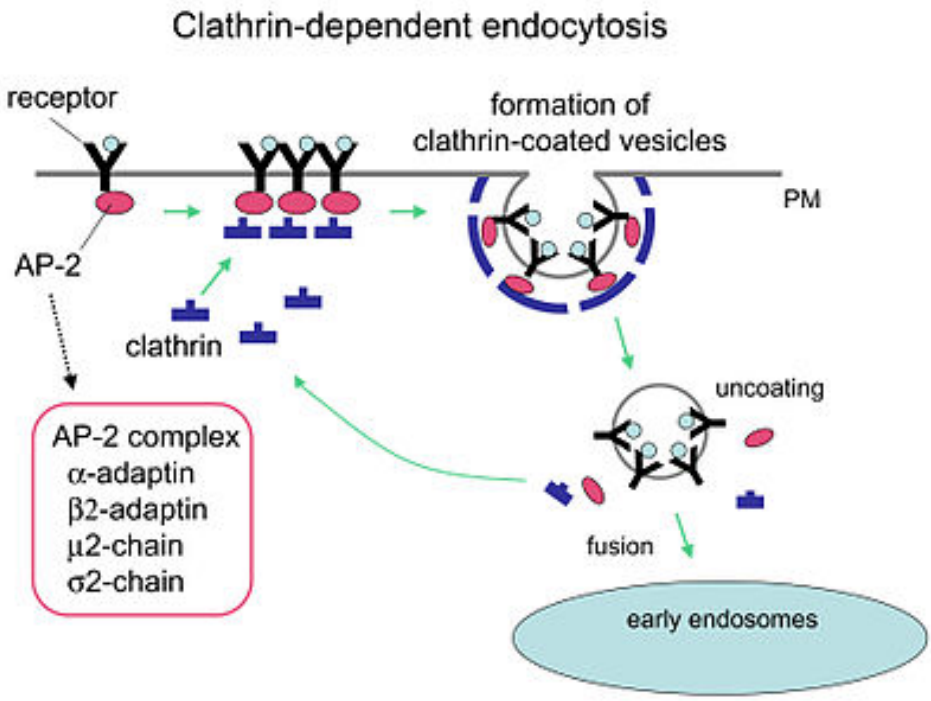


Figure 7: Clathrin-mediated endocytosis[28]*. The most well understood mechanism of endocytosis, requiring the formation of clathrin-coated pits to bring in cargo molecules.*

Regardless of the specific mechanism of uptake, the importance of conducting endocytosis while also maintaining the quantity and distribution of transmembrane proteins at the surface presents the cell with an interesting conundrum, in that it must bring in materials from the environment, but in doing so, will also internalize its own transmembrane proteins, disrupting their concentration. As such, the cell must determine the fate of these transmembrane proteins, hereby referred to as "cargo", once they enter the cell. Simply put, there are two main options—these cargoes can be returned to the cell membrane by a variety of recycling pathways, or can be sent to the lysosome to be degraded. It is essential that important cargo can return to the cell surface, rather than being missorted to lysosomes, to maintain the density of necessary transmembrane

proteins, including nutrient channels and signaling receptors. Sorting cargo to these multiple destinations requires the ability to recognize cargo proteins, partition them into discrete compartments, and ensure they are delivered to the appropriate destination to maintain the cell's physiology[12]. This movement of cargo from the membrane through various endosomes and back is referred to as endocytic recycling.

The process of endocytic recycling in eukaryotic cells occurs through several different but highly conserved pathways. Following internalization via endocytosis, molecules are trafficked and sorted by a series of tubulovesicular compartments referred to as endosomes[29]. The role of endosomes is to collect and sort internalized cargo, ensuring that they make it to their final destination[14]. Cargo first arrives at the early endosome, the main hub where initial sorting begins[30]. Early endosomes are approximately 100-500nm in diameter and contain inwardly budding intraluminal vesicles (ILVs), where cargo destined for the lysosomes are placed. These early endosomes then transition into late endosomes, which are much larger at 250-1000nm in diameter, with numerous ILVs called multivesicular bodies (MVBs). Cargo that needs to be recycled must remain excluded from ILVs and MVBs to avoid trafficking to lysosomes. From early and late endosomes, cargo will either travel to the lysosome for degradation, be sent to the trans-Golgi network (TGN) via retrograde trafficking, or be recycled directly back to the plasma membrane through either fast recycling or slow recycling by the endocytic recycling compartment (Figure 8)[32,44].

The pH of the endocytic compartments also helps ensure that proper trafficking of cargo will occur. As endosomes mature, their lumens become more acidic, with early

endosomes having a pH of 6.5, while late endosomes and the trans-Golgi network are at 5.5. The lysosome, where degradation of cargo occurs, has an even more acidic pH of 4.5[14]. This helps regulate sorting and allows for differential recycling, as receptors internalized with their ligands attached have different pH sensitivities for dissociation, meaning some dissociate and are recycled quickly while others remain bound to be degraded by lysosomes. Lysosomal sorting is mediated by ubiquitylated proteins interacting with the endosomal sorting complex required for transport (ESCRT) machinery, whereby ubiquitin-labeled cargo is sorted into vesicles to be sent to the lysosome[32]. While the degradative pathway via ubiquitylation and lysosomes is well understood, the mechanisms involved in the recycling pathway are not. However, it has been shown the invagination of the cell membrane, pinching off of vesicles, driving them from the plasma membrane into the cytoplasm, and changing one type of endosome into the next all require actin polymerization[2].

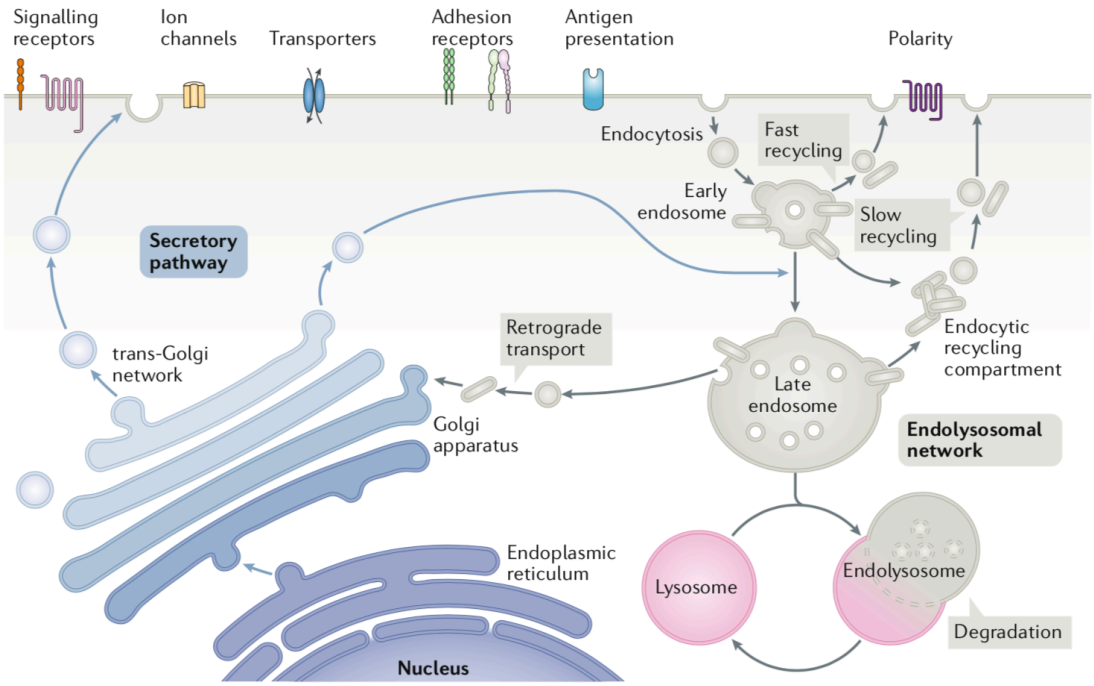


Figure 8: Endocytic recycling[44]*. Transmembrane cargo can have multiple fates upon arriving at early endosomes, the main sorting hub for trafficking.*

As actin polymerization is important for endocytic recycling, the role of the three *C. elegans* NPFs in this process should be examined. Although WASP and WAVE have been thoroughly studied in endocytic recycling, the role that WASH plays in this process is not as well understood. It does seem that WASH has a spatially and temporally distinct role in recycling compared to that of WAVE and WASP[6]. In mammalian systems, WASP localizes with clathrin at the plasma membrane, and WAVE has been shown to stimulate actin branching at the membrane as well[10,18]. Contrarily, WASH localizes to early endosomes to regulate the architecture of these endosomes and prevent missorting by sparing cargo from lysosomal degradation[9,35]. WASH allows the formation of branched actin filaments at this location, providing actin patches that are a platform for signaling

and can generate local force for production and scission of vesicles as cargo moves throughout the recycling pathway[12].

Despite this evidence, the mechanisms of WASH recruitment and activation at early endosomes are only partially understood. Thus far, research has shown that WASH plays an important role in retrograde trafficking through the Golgi apparatus by interacting with the retromer complex at early endosomes to allow for subsequent recycling[9]. More specifically, the FAM21 subunit of WASH interacts with retromer to be recruited onto endosomes, with phospholipid binding potentially playing a role in membrane targeting[33]. Retromer is an evolutionarily conserved protein complex that assembles on endosomes to mediate the transport of receptors to the trans-Golgi network (TGN) or the plasma membrane[36]. It consists of a core heterotrimer of VPS35, VPS26, and VPS29 subunits, collectively referred to as the cargo-selective complex (CSC)[8]. WASH is recruited to early endosomes by an interaction of the C-terminal tail of FAM21 with the VPS35 subunit of retromer[18], which then allows for actin nucleation and endosome budding (Figure 9)[35].

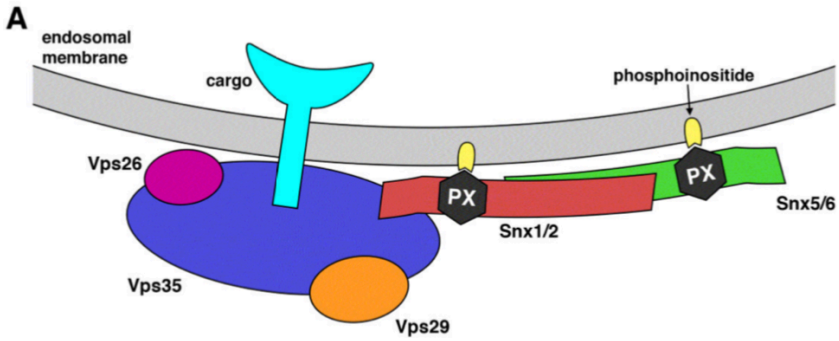


Figure 9: Retromer structure[35]. *C. elegans retromer consists of a core VPS35, VPS25, VPS29 heterotrimer and associates with a sorting nexin dimer.*

Additionally, retromer associates with an interchangeable sorting nexin (SNX) dimer[31], characterized by a highly conserved phox-homology (PX) domain, which can interact with various phosphotidylinositides, including phosphatidylinositol 3-phosphate (PI(3)P)[37]. In retromer, SNX1/2 and SNX5/6, which also include bin-amphiphysin-Rsy (BAR) domains and are collectively referred to as the SNX-BAR coat complex, can work to introduce membrane curvature and shape compartments (Figure 10)[8]. More specifically, theses SNXs generate a tubular subdomain of early endosomes, and then the coat complex serves as a docking site for the VPS26-VPS29-VPS35 cargo-selective subcomplex[31]. There is also variation in each sorting nexin's phosphatidylinositide binding abilities, allowing for targeting to distinct endosomal subdomains. For example, other sorting nexin proteins, such as SNX3, can interact with the cargo selection portion of the complex to help recruit the retromer to endosomal membranes. SNX3 is thus referred to as a retromer adaptor, as it can directly associate with certain cargo and help sort it to the Golgi, saving it from the degradative pathway[38]. As such, sorting nexins play an important role in coordinating membrane tubulation and cargo sorting[31].

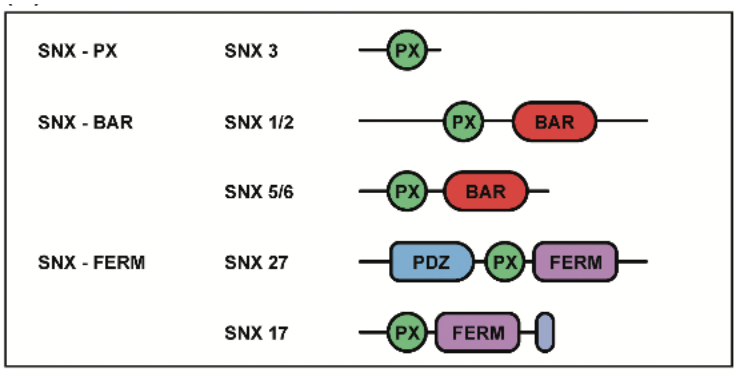


Figure 10: Sorting nexin domains[8]. *Sorting nexins present in the retromer complex contain phox-homology (PX) and bin-amphiphysin-Rys (BAR) domains. SNX-3, a retromer adaptor, only has a PX domain.*

In order for the pentameric WASH complex to interact with retromer, enabling recruitment to early endosomes and subsequent steps of recycling, the FAM21 subunit is required, making it arguably the most important component of the complex for trafficking[39]. Despite this, FAM21 is the most divergent of all of the subunits of the WASH complex across species[12]. Structurally, FAM21 is the largest member of the WASH complex, containing a 220-amino acid head domain that is necessary for WASH complex assembly and ensures the stability of the complex by binding to the WASH and SWIP subunits. At its C-terminus, FAM21 has a long, unstructured 1,100 amino acid tail, containing 21 repeats of a novel acidic motif, termed the LFa motif for its leucine, phenylalanine, acidic amino acid sequence: L-F-$[D/E]_{3-10}$-L-F[40,41]. In these repeats, the leucine residue can be sometimes replaced with another hydrophobic amino acid, such as isoleucine, valine, or methionine[40]. The LFa repeat motifs are essential for endosomal signaling, as they help to recruit a variety of proteins, including CapZ, RME-8, and the cargo selection complex of retromer. More specifically, the repeats in this tail bind to VPS35 of retromer (Figure 11)[42], and with lack of binding comes improper sorting of cargo[43,44]. For example, in the absence of FAM21 in *Dictyostelium* mutants, WASH can still be recruited to macropinosomes but retromer sequestration and retrieval could not occur[8]. These LFa motifs have different affinities for the CSC, with some interacting strongly and others weakly, if at all[40]. Of these repeats, those with a hydrophobic residue at the sixth position seem to bond the most strongly, suggesting this feature may allow for increased affinity for VPS35. As such, the last two repeats are most important for interactions with endosomes in mammalian cells, as deleting these motifs causes FAM21

to be localized only in the cytoplasm[40]. These findings suggest that FAM21 is of the utmost importance for proper recruitment of WASH and trafficking of cargo.

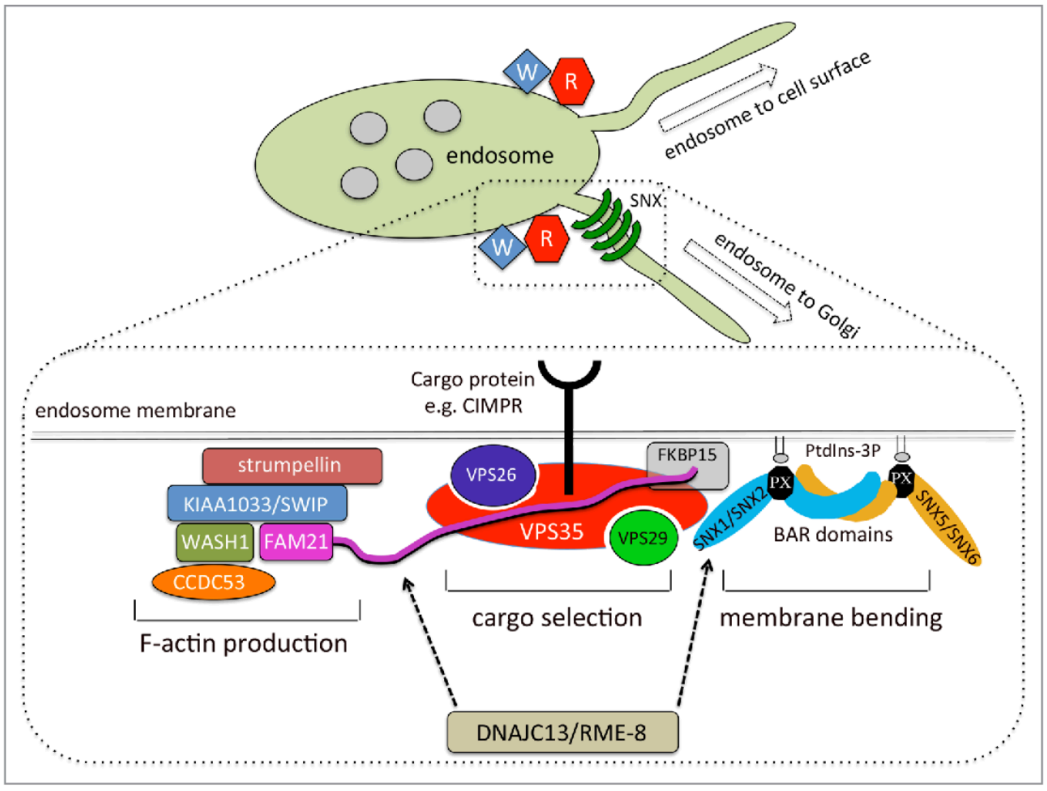


Figure 11: FAM21 of the WASH complex interacts with retromer[42]. *The FAM21 subunit has a long tail with LFa repeat motifs, which interacts with the VPS35 subunit of the cargo selection complex of retromer.*

Interestingly, however, the *C. elegans* WASH complex has only four subunits—WASH(ddl-2), Ccdc53 (ddl-1), Strumpellin, and SWIP, meaning it lacks a homologous FAM21 protein. In fact, there is very little information known about the *C. elegans* WASH complex, as WASH was only recently discovered and most research thus far has been conducted in mammalian systems. As such, there is very limited knowledge of other

organisms that could be used as a model system to reveal hitherto unknown information about WASH and recycling.

In summary, the three nucleation promoting factors present in *C. elegans* are part of larger metabolic pathways, controlled by direct phosphorylation, as well as GEF and GAP proteins, to allow for spatial and temporal regulation of branched actin networks created by Arp2/3. These networks contribute to driving the process of endocytic recycling, allowing engulfed cargo to return to the cell membrane in a direct or more circuitous route, as well as provide a force for changes during embryonic morphogenesis. Although the mechanisms underlying the WASH complex's role in these processes are not well understood, the complex and related molecules, such as Arp2/3, have been implicated in several human diseases. For instance, WASH has been linked to certain types of cancer, with an upregulation of this complex and other NPFs allowing tumors to metastasize and invade healthy tissues. This is partially because cancer cell invasion requires actin-rich structures, such as podosomes and invadopodia, that have adhesive and protrusive activities and allow for degradation of the extracellular matrix[2]. FAM21 has also been shown to contribute to resistance to chemotherapy drugs in some cancers, particularly pancreatic cancer, as it can regulate target genes in the nucleus[41]. Furthermore, the process of endocytic recycling has been connected to neurodegenerative disorders[45], including: an autosomal dominant mutation in VPS35 (D620N) associated with early onset Parkinson's disease, due to a destabilization of association between WASH and retromer[7,42,46]; SWIP mutations associated with late-onset Alzheimer's disease; and autosomal dominant Strumpellin mutations leading to hereditary spastic paraplegia (HSP)[41]. Additionally, interactome studies have identified an interaction

between the histone deacetylase Hdac4, a protein that contributes to the progression of Huntington's disease, and the WASH complex[47]. This suggests a potential role for Hdac4 in intracellular transport in neurons. More recently, the WASH complex has been connected to trafficking of Glut2 in pancreatic β-cells in a mouse model for type 2 diabetes, with pancreas-specific WASH deletions leading to impaired blood glucose clearance and reduction of insulin release due to Glut2 being aberrantly trafficked to lysosomes for degradation[48]. Finally, bacterial pathogens can take advantage of this system as well. For example, *Salmonella* injects its own GEF for Cdc42/Rac, called SopE, into host cells to induce actin cytoskeletal rearrangement and membrane ruffling, allowing the bacterium to be internalized[17].

As such, elucidating the molecular role of the WASH complex in endocytic recycling has vast implications for a wide range of human diseases, underpinning the importance of studying this NPF. In this paper, the role of the *C. elegans* WASH complex in recycling was investigated in the intestine to determine where it acts in the endocytic recycling pathway and how it can be recruited despite an apparent lack of the FAM21 subunit. It was concluded that, as in mammalian systems, WASH acts at early endosomes, serving an important role in permitting transmembrane proteins to return to the cell membrane following retrograde trafficking. Furthermore, it may do this through association with a previously unidentified protein, C05G5.2, that could be the missing FAM21 homolog in *C. elegans.*

Methodology:

Strains

All strains were cultured and maintained on LB media seeded with *Escherichia coli* OP50, kept at 20°C unless otherwise noted[49]. Strains were obtained from the Caenorhabditis Genetics Center (CGC) at the University of Minnesota, funded by the National Institutes of Health and National Bio-Resource project of the MEXT Japan, or were gifted from individual labs as specified below (Table 1). Strains created in this study were built through genetic crosses.

Table 1: Strains used in this study

Strain name	**Genotype**	**Source**
N2	Wild type	CGC
OX421	*aqp-1p::aqp-1::gfp; rol-6*	Falashruti Patel
OX441	*aqp-4p::aqp-4::gfp; rol-6*	Falashruti Patel
NG324	*wsp-1 (gm324)*	CGC
RB2380	*Y48E1B.1 (ddl-2; ok3235)*	CGC
OX898	*aqp-1::gfp; wsp-1(gm324)*	This study
OX899	*aqp-4::gfp; wsp-1(gm324)*	This study
OX940	*aqp-1::gfp; ddl-2(ok3235)*	This study
OX942	*aqp-4::gfp; ddl-2(ok3235)*	This study
OX943	*erm-1::gfp; ddl-2 (ok3235)*	This study
OX944	*erm-1::gfp; wsp-1 (ok3235)*	This study
VJ718	*pErm-1::erm-1::mCherry unc119*	Verena Gobel
RT1619	*pwIs890 Pvha-6::Akt-PH::GFP*	Barth Grant
RT1120	*pwIs446 Pvha-6::PH::GFP*	Barth Grant
RT348	*pwIs140 Pvha-6::GFP::2xFYVE*	Barth Grant
RT2293	*vha-6p::tagrfp::rab5*	Barth Grant
RT2619	*pwIs956 Pvha-6::tagRFP::RAB-10*	Barth Grant
RT2296	*pwIs849 v6-tagRFP-rab-7-Cbunc119*	Barth Grant
RT311	*Vha-6::gfp::rab-11*	CGC
RT1315	*pwIs503 [vha-6p::mans::GFP + Cbr-unc-119(+)]*	CGC
OX939	*snx-3 (tm1595)*	Barth Grant
GS2813	*cup-5 (ar465)*	Barth Grant
RT774	*act-5::gfp*	Barth Grant
OX784	*Pglo-1-LifeAct::mCherry*	Sofya Borinskaya
RT2071	*MIG14::GFP*	Barth Grant

RNAi by feeding

Bacteria expressing double-stranded RNA homologous to the gene of interest were used to knockdown gene expression. RNAi samples were obtained from the Driscoll lab library at Rutgers University. RNAi feeding was carried out at either 20°C for 72 hours or 23°C for 48 hours and worms were imaged as described below. N2 wild type embryos were scored for lethality to confirm successful treatment.

Genetic Crosses

New strains were built using principles of genetics to cross two existing strains into one another. The general steps were as follows: six N2 males were crossed into a hermaphrodite for one strain of interest, typically one that had an observable phenotype, such as being a roller or fluorescently tagged. After three days, 5 males were selected and crossed to the second strain of interest. Both of these steps were carried out on mating plates, seeded with less food to encourage reproduction. From there, hermaphrodites were singled out and screened until homozygosity for both traits was achieved.

Building a new ddl-2 deletion via CRISPR

A new deletion in the ddl-2 subunit of WASH was built using clustered regularly interspaced short palindromic repeat (CRISPR) techniques. A PCR-based amplification and annealing technique, adapted from the Mello lab[50], was used with some modifications. Briefly, protospacer adjacent motifs (PAM) sites were identified by finding "NGG" or "CCN" sequences on the "+" DNA strand, as close to the start and stop codon as possible. The efficiency of editing at this PAM site, including likelihood of off-site targeting, was verified by checking the sequence on the ChopChop website from Harvard University (https://chopchop.cbu.uib.no/). CRISPR RNA (crRNA) sequences

were designed for these PAM sites, with oligonucleotides containing 20 nucleotides upstream of (and not including) the PAM site, followed by 22 nucleotides corresponding to universal sequence that interacts with the tracrRNA (GUUUUAGAGCUAUGCUGUUUUG)[51]. Reverse complement sequences were used for 'CCN' PAM sites, while strands with 'NGG' PAM sites were used as is. Lastly, regardless of the sequence, the first base for each crRNA was changed to a guanine to increase cutting efficiency. This leads to blunt end DNA double stranded breaks between the two PAM sites, cleaving out the majority of the gene[52].

Table 2: crRNA sequences for ddl-2

Oligonucleotide description	**Sequence**
MSo1767: 5' crRNA	ggcgtggaatcagcggaaca
MSo1764: 3' crRNA	gaattttgatgatgaggaat

Samples were amplified via nested polymerase chain reaction (PCR) and short bands of 200 (for inside primers) or 400 (for outside primers) base pairs indicated successful deletions. Bands were excised and gel purified following the Qiagen QIAquick Gel Extraction Kit protocol and sent for sequencing to confirm the deletions.

Table 3: Primers for Nested PCR to confirm deletions

Primer name and description	**Primer sequence**
MSo1788: 5' forward outside primer	gtaccaatctaagcttttccc
MSo1790: 5' forward inside primer	tttccaactgttctagccag
MSo1789: 3' reverse outside primer	ccgtttttttacagaaatttctcg
MSo1791: 3' reverse inside primer	gtgaattcccatagtttttaacgg

Live imaging of adult intestine

Adult intestines were imaged in animals at the L4 stage of development, determined based on the appearance of the vulva, as shown below (Figure 12). Images were taken at 63x magnification, focusing on the region of the intestine behind the pair of leading cells, at both apical and basolateral views. Animals were placed on 10% agarose pads, paralyzed with levamisole, and imaged within ten minutes of completing the slide preparation. Intestines were imaged on a laser spinning disk confocal microscope with Yokogawa scan head on a Zeiss Axiolmager Z1m microscope, using 300 exposure and 30% power. Images were captured on a Photometrics Evolve 512 EMCCD camera using Metamorph software.

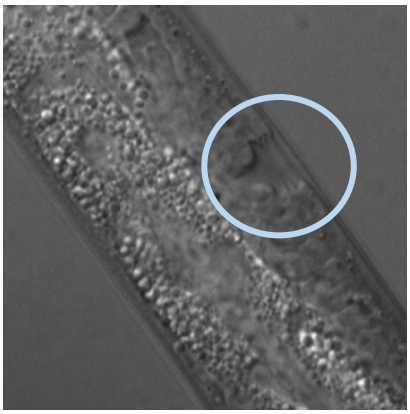


Figure 12: L4 stage vulva*. The shape of the vulva, circled, was used to determine if worms were at the appropriate stage to be imaged.*

Quantification of immunofluorescence and statistical analysis

Images were analyzed in ImageJ and statistical analysis performed using GraphPad Prism software. Intensity across the intestine, from the basal to apical surface, was measured using the line tool and average intensity values were obtained, as shown

with the yellow shapes on the example worm below (Figure 13). Vesicles were counted in a 50x50 pixel square region and size was determined using the oval tracing tool to determine total area. All data was collected for at least five locations in the *C. elegans* intestine and on at least five worms, allowing a minimum of 25 data points to be utilized per treatment. Data are expressed as mean ± standard error. A two-sided t-test with unequal variance and Welch's correction was used to determine significance of differences observed between wild type worms and one experimental group, with $p<0.05$ denoting statistical significance. Similarly, a one-way analysis of variance, followed by Tukey's post-hoc test, were performed to compare differences between more than two groups.

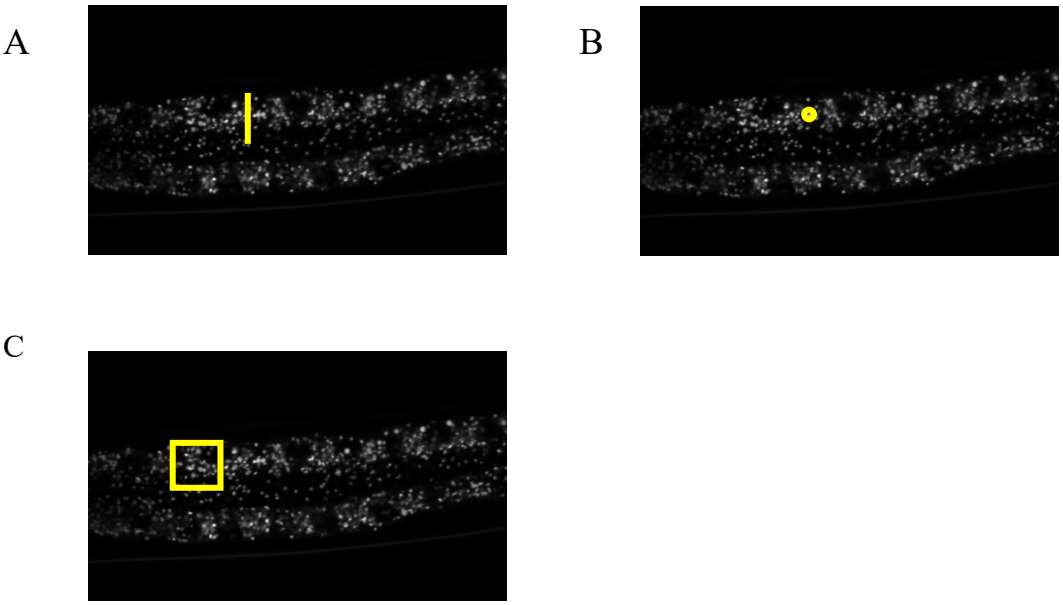


Figure 13: Measurement methods for figures. *(A): Line scan tool measured from basal to apical region, in 5 places on 5 worms, to find average intensity. (B): Ellipse tool encircled vesicles to find area. (C): 50x50 pixel square area was drawn and number of vesicles counted inside that region.*

Results:

The WASH complex acts by nucleating actin at early endosomes

The process of endocytic recycling requires internalized cargo to be properly sorted to the appropriate destination, ultimately allowing it to return to the plasma membrane. WASP and WAVE have been implicated early in this process, in allowing cargo to first enter cells and travel to early endosomes, the initial sorting hub of the pathway. However, the role that WASH plays in this process is not well understood, particularly in polarized epithelial cells, despite the prevalence of these cells in many organisms, including humans[53].

To determine this role, the *C. elegans* intestine was examined. The intestine is one of the largest organs in C. elegans, and the site of frequent trafficking, as digestion and macromolecule storage require transport between the intestine and peripheral tissues[54]. This organ is a tube consisting of 20 epithelial cells that display apicobasal polarity (Figure 14), with the apical membrane facing the intestinal lumen, the lateral membrane allowing for cell-cell adhesion[16,55], and the basal membrane facing the body cavity[54].

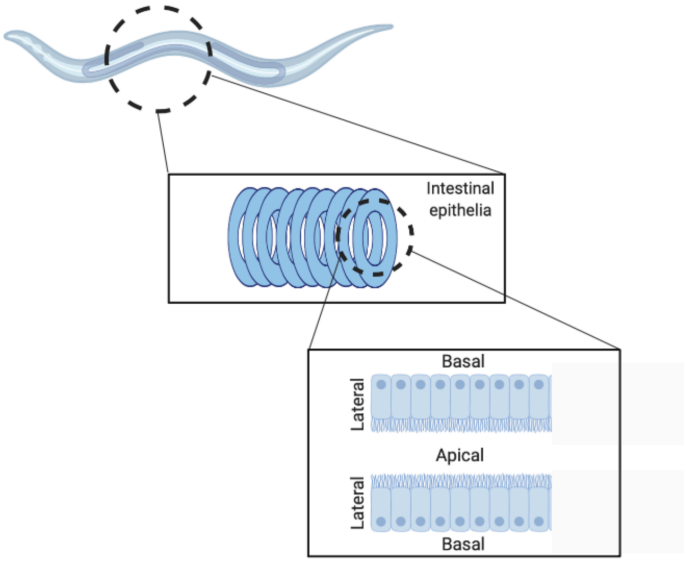


Figure 14: Structure of C. elegans intestine. *The intestine consists of polarized epithelial cells, with certain proteins found enriched on the apical surface and others on the basolateral surface.*

To examine the role of the WASH complex in *C. elegans* endocytic recycling, important endosomes in this pathway were examined. Each compartment of the endolysosomal system is characterized by a particular distribution of effectors and phospholipids, with these identities being dynamic and fluid, changing as the endosomes mature and cargo moves through the pathway[14]. More specifically, different compartments are characterized by specific Rab GTPases, highly conserved small monomeric GTPases that alternate between two states—the GTP-bound "on" form and the GDP-bound "off" form[56]. When active, they can recruit effector proteins to the surfaces of compartments, as well as control vesicle formation, targeting, and fusion to ensure directionality of transport[15,57]. The following Rab GTPases characterize specific compartments in the endocytic recycling pathway: RAB-5 at early endosomes, RAB-7 at late endosomes, RAB-10 at basolateral recycling endosomes, and RAB-11 at apical

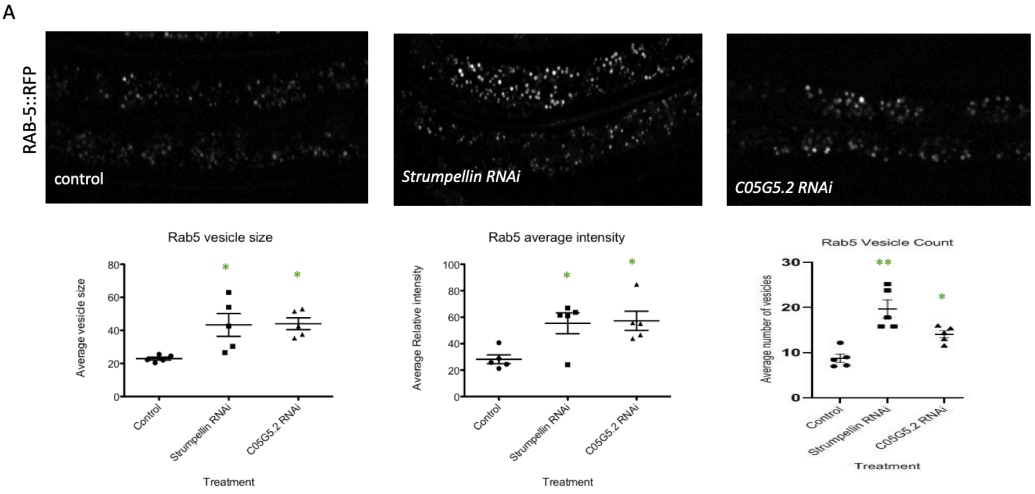
recycling endosomes[28,58]. To convert compartments from one type to the next, a 'Rab cascade' must occur, in which an upstream RAB recruits a GEF to activate and recruit a downstream Rab, and also recruits a GAP for the upstream RAB[14]. For example, RAB-5 at early endosomes serves to activate RAB-7, until a threshold level of GTP-bound RAB-7 is reached, at which time a negative feedback loop will inactivate RAB-5[15]. As such, using fluorescent tags on each of these RAB proteins can demonstrate their quantity and distribution in the *C. elegans* intestine.

First, these RAB-labeled endosomes, as well as the Mannosidase-labeled (AMAN-2) Golgi apparatus, were examined in wild type worms and then compared to worms depleted of the Strumpellin subunit of WASH through RNAi. As knockdown of Strumpellin can potentially destabilize the entire complex, this is an effective way to suppress WASH activity. Furthermore, of the four *C. elegans* subunits, Strumpellin RNAi is the most consistent and lethal. Following WASH complex knockdown, endosomes were measured in three ways—average size, average relative intensity, and average number of endosomes in a particular area. As shown in the methodology, intensity was measured using a line scan from the basal surface to the apical surface and then taking an average, size was measured using the oval tracing tool and finding the area of the vesicle, and number was found by drawing a 50x50 pixel square area and counting the number of vesicles inside.

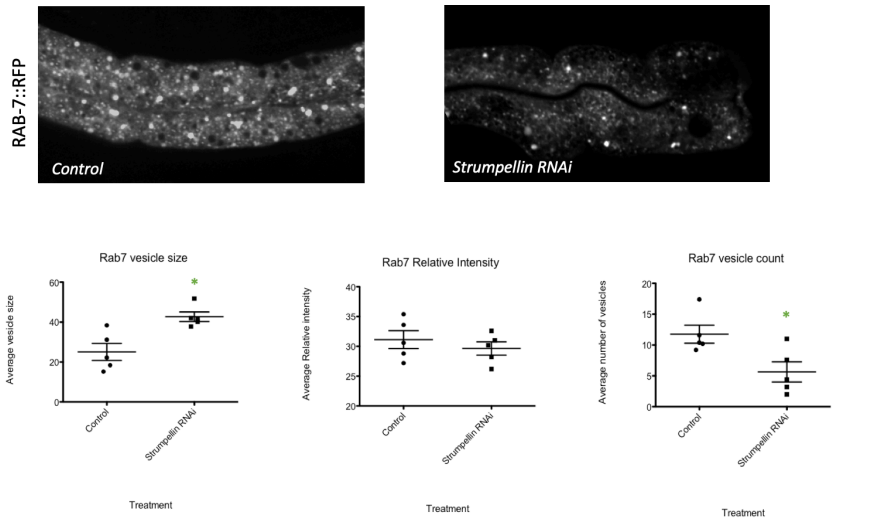
The WASH complex is expected to influence multiple trafficking pathways, as WASH-mediated actin assembly mediates early sorting due to its role on early endosomes, controlling membrane remodeling during endosome biogenesis[6]. Results

from this study were consistent with this hypothesis, as loss of the WASH complex leads to an increase in RAB-5-positive early endosomes, but a decrease in RAB-7 late endosomes, RAB-10 and RAB-11 recycling endosomes, and AMAN-2, a Golgi marker (Figure 15). These RAB-5::RFP endosomes were also larger in size than control worms. This indicates that cargo is being endocytosed and arriving at early endosomes, but then remaining there rather than proceeding to subsequent recycling steps, suggesting that WASH acts at early endosomes.

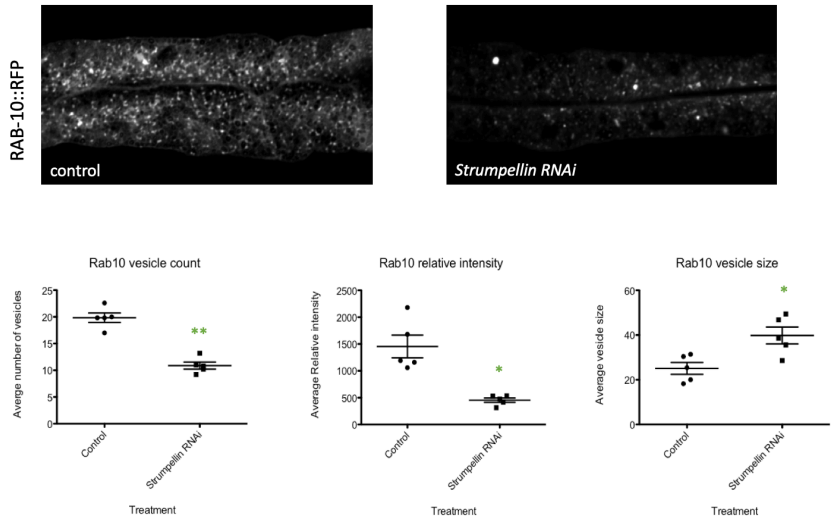
A


RAB-5::RFP
control
Strumpellin RNAi
C05G5.2 RNAi
Rab5 vesicle size
Average vesicle size
Control
Strumpellin RNAi
C05G5.2 RNAi
Treatment
Rab5 average intensity
Average Relative intensity
Rab5 Vesicle Count
Average number of vesicles

B


RAB-7::RFP
Control
Strumpellin RNAi
Rab7 vesicle size
Average vesicle size
Rab7 Relative Intensity
Average Relative intensity
Rab7 vesicle count
Average number of vesicles
Treatment

C


RAB-10::RFP
control
Strumpellin RNAi
Rab10 vesicle count
Avergе number of vesicles
Rab10 relative intensity
Average Relative intensity
Rab10 vesicle size
Average vesicle size
Treatment

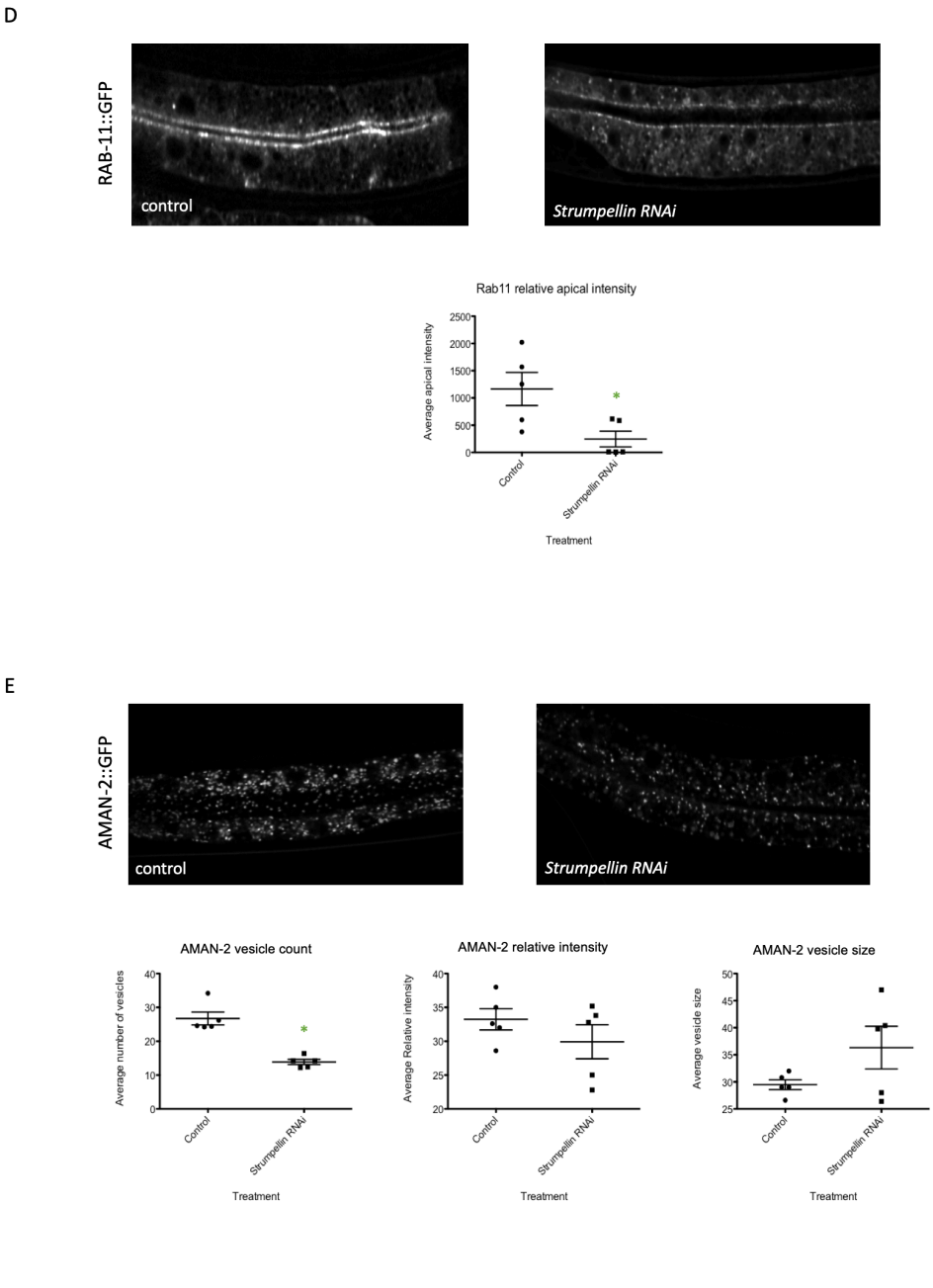


Figure 15: RAB-labeled endosomes following loss of WASH*. Strumpellin RNAi leads to an increase in early endosomes, followed by decrease in subsequent endosomes involved in recycling and retrograde trafficking. All worms shown are L4 stage, with a focus on the intestinal cells immediately following the first region after the pharynx. n=5, with 5 measurements per worm. *=p<0.05; **=p<0.01, based on one-way ANOVA and Tukey's post-hoc in (A) and Student's T test with Welch's correction in (B-E).*

Additionally, certain compartments in the endocytic pathway can be identified by their phosphatidylinositol phospholipid (PIP) composition. These lipids can recruit different protein complexes to membranes, cooperating with Rab GTPases to define membrane identity[15], as well as binding to proteins containing Pleckstrin homology (PH) domains with high affinity and specificity[58]. These lipids are phosphorylated phosphatidylinositol derivatives, synthesized in the endoplasmic reticulum and delivered to their respective compartments. Their phosphorylation at the 3, 4, and 5 positions of the inositol ring is reversible, mediated by kinases and phosphatases[59]. In endocytic recycling, phosphatidylinositol-4,5-bisphosphate ($PI(4,5)P_2$) and phosphatidylinositol-3,4,5-triphosphate ($PI(3,4,5)P_3$) are enriched at the plasma membrane, while phosphatidylinositol 3-monophosphate (PI(3)P) is enriched at early endosomes and their intraluminal vesicles[60, 61]. These PIPs can recruit GEFs and GAPs to target membranes, and their kinases and phosphatases are often Rab effectors. For instance, RAB-5 recruits PI3 kinase to produce PI(3)P on early endosomes, further characterizing these compartments.

To confirm the results observed above with the RAB-labeled endosomes, the same experiment was repeated using Strumpellin RNAi but examining GFP-labeled PIP distribution instead of Rab protein distribution. Consistent with the RAB-labeled endosome results, the phosphotidylinositides enriched at the cell membrane remained unchanged on Strumpellin RNAi (Figure 16A, B), while levels of PI(3)P-enriched early endosomes increased (Figure 16C). This again supports that the WASH complex acts at early endosomes. Furthermore, this role differs from that of the WAVE and WASP complex, as reported by Rotter[6], Patel & Soto[10], and Bai & Grant[53]. WAVE and WASP

act early in endocytic recycling, playing a role at the cell surface rather than at early endosomes.

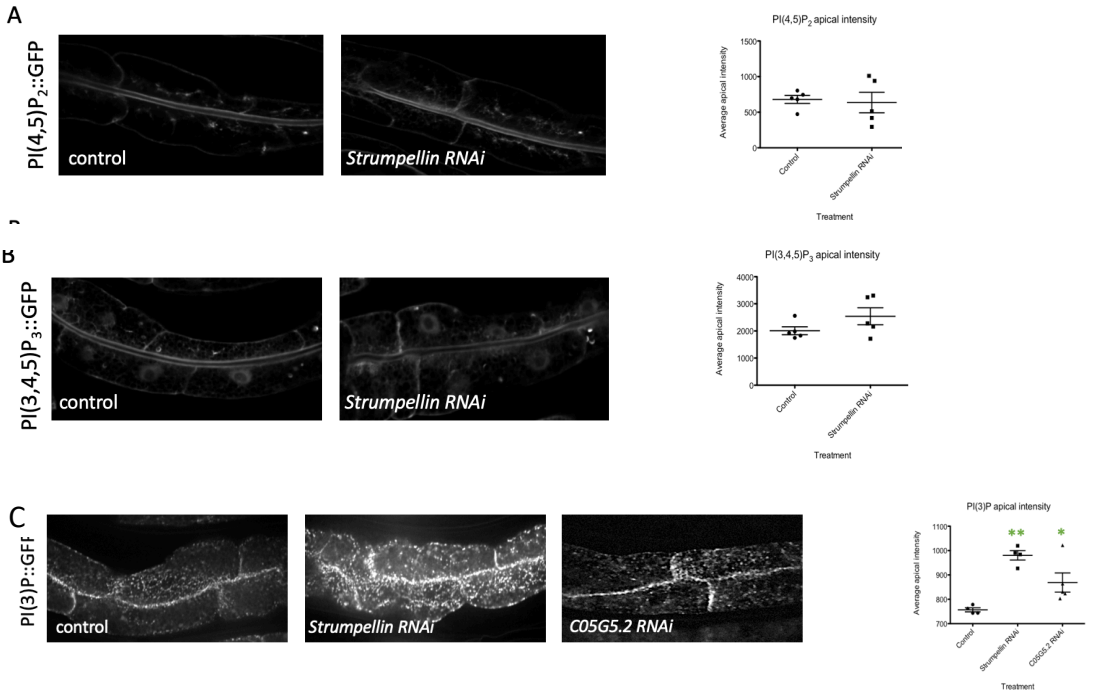


Figure 16: PIP intensity following loss of WASH. *(A) Strumpellin RNAi leads to an increase in PI(3)P found on early endosomes, but (B) no change in PI(4,5)P_2 or PI(3,4,5)P_3 on the plasma membrane. n=5, with 5 measurements per worm. *=p<0.05; **=p<0.01, based on Student T test with Welch's correction.*

Taken together, these results suggest that the WASH complex is playing a role at early endosomes, presumably to induce actin nucleation that provides a force for transitioning cargo to subsequent steps in the recycling pathway. This is similar to the role identified for mammalian WASH, which has been shown to colocalize with markers of early and slow-recycling endosomes, and to a lesser extent with fast-recycling and late endosomes[9, 19,35].

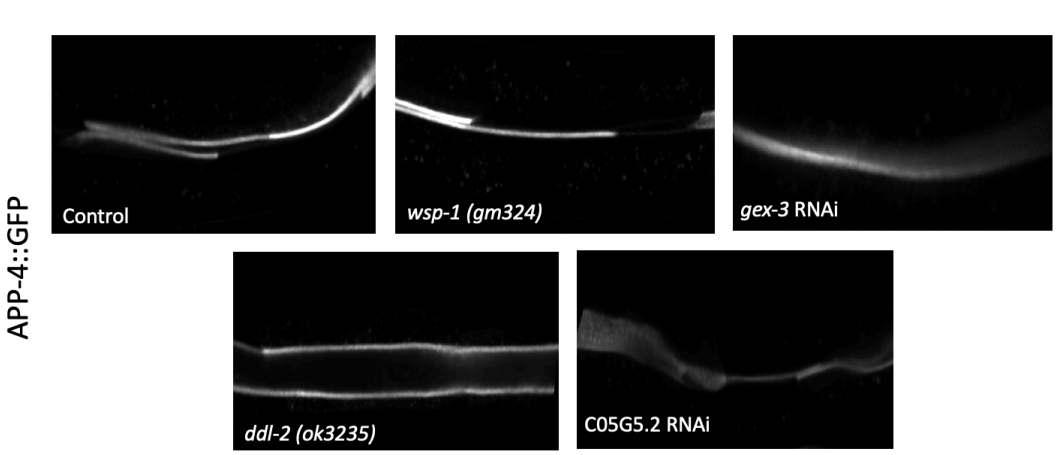
The WASH complex plays a functionally important role in recycling cargo

Although WASH seems to act at early endosomes, there are other nucleation promoting factors in *C. elegans* that could work to mediate recycling. These factors may work in conjunction with WASH, meaning that all three are required, or one may be able to compensate to allow recycling in the absence of WASH. As such, to determine if the WASH complex has an indispensable role in recycling, its ability to return key apical and basolateral cargo to the intestinal membrane was examined. While previous trafficking studies have examined the transport of human proteins in the *C. elegans* intestine, few investigate endogenous transmembrane proteins specific to these worms. As such, the distribution of several common apical and basolateral membrane-associated proteins was analyzed in wild type worms, then compared to that of worms crossed with mutant WASH1, or *ddl-2(ok3235)*. All the proteins used here have human homologs, ensuring that the data obtained represents actual *C. elegans* trafficking pathways, while still having implications for mammalian systems. Additionally, proteins enriched on both the apical and basolateral surfaces of the intestine were included, with a wide range of functions, to reflect the great metabolic diversity of this organ[54]. On the apical surface, aquaporin-4 (AQP-4::GFP) and Ezrin, Radixin, and Moesin-1 protein (ERM-1::GFP) were used, each tagged with GFP to visualize their distribution and intensity. AQP-4 is a channel protein responsible for transporting water[62,63], while ERM-1 is required for apical membrane morphogenesis, attaching to the cell membrane and actin to support epithelial integrity, polarity, junction formation, and tubulogenesis[64]. It is worth noting that this protein is membrane-associated, rather than a transmembrane protein; however, it does associate with other transmembrane proteins, including junctional proteins[23], so may be trafficked

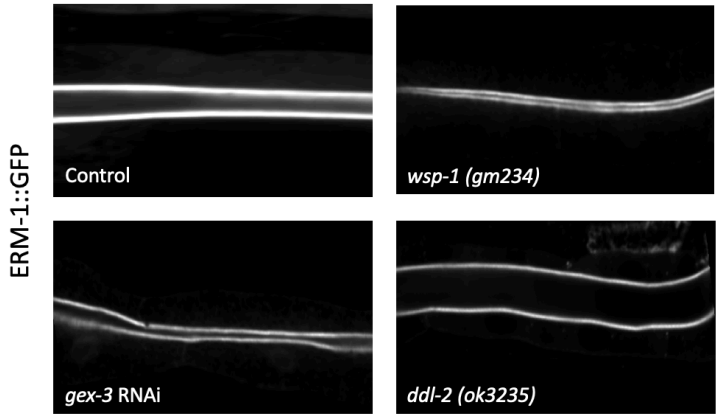
alongside this molecule. On the basolateral surface, aquaporin-1 (AQP-1::GFP) was examined, which transports water, ammonia, and monovalent cations[62,63], as well as the Wnt-binding protein MIG14::GFP, which undergoes retrograde trafficking and binds to Wnt proteins at the Golgi apparatus to recycle them back to the plasma membrane[65].

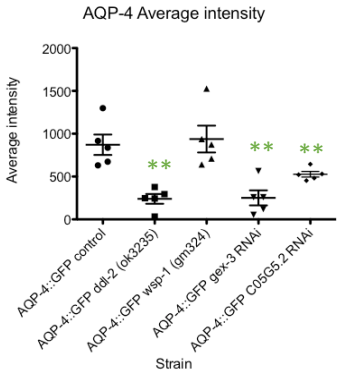
For all of the transmembrane proteins examined in mutant *ddl-2(ok3235)* worms, loss of WASH impaired recycling, as indicated by a reduction in all four proteins at the apical and basolateral surfaces compared to the control (Figure 17). Strumpellin RNAi gave similar results to crossing the *ddl-2(ok3235)* mutants (data not shown), so mutants were used here as they are a more reliable and consistent method of protein knock down. These data suggest that WASH plays a functionally important role in recycling, one that cannot be replaced by the other nucleation promoting factors.

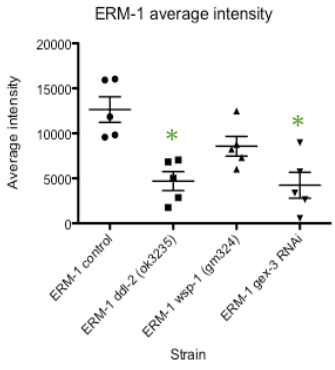
A


APP-4::GFP
Control
wsp-1 (gm324)
gex-3 RNAi
ddl-2 (ok3235)
C05G5.2 RNAi

B


ERM-1::GFP
Control
wsp-1 (gm234)
gex-3 RNAi
ddl-2 (ok3235)


AQP-4 Average intensity
Average intensity
2000
1500
1000
500
0
**
**
**
AQP-4::GFP control
AQP-4::GFP ddl-2 (ok3235)
AQP-4::GFP wsp-1 (gm324)
AQP-4::GFP gex-3 RNAi
AQP-4::GFP C05G5.2 RNAi
Strain


ERM-1 average intensity
Average intensity
20000
15000
10000
5000
0
*
*
ERM-1 control
ERM-1 ddl-2 (ok3235)
ERM-1 wsp-1 (gm324)
ERM-1 gex-3 RNAi
Strain

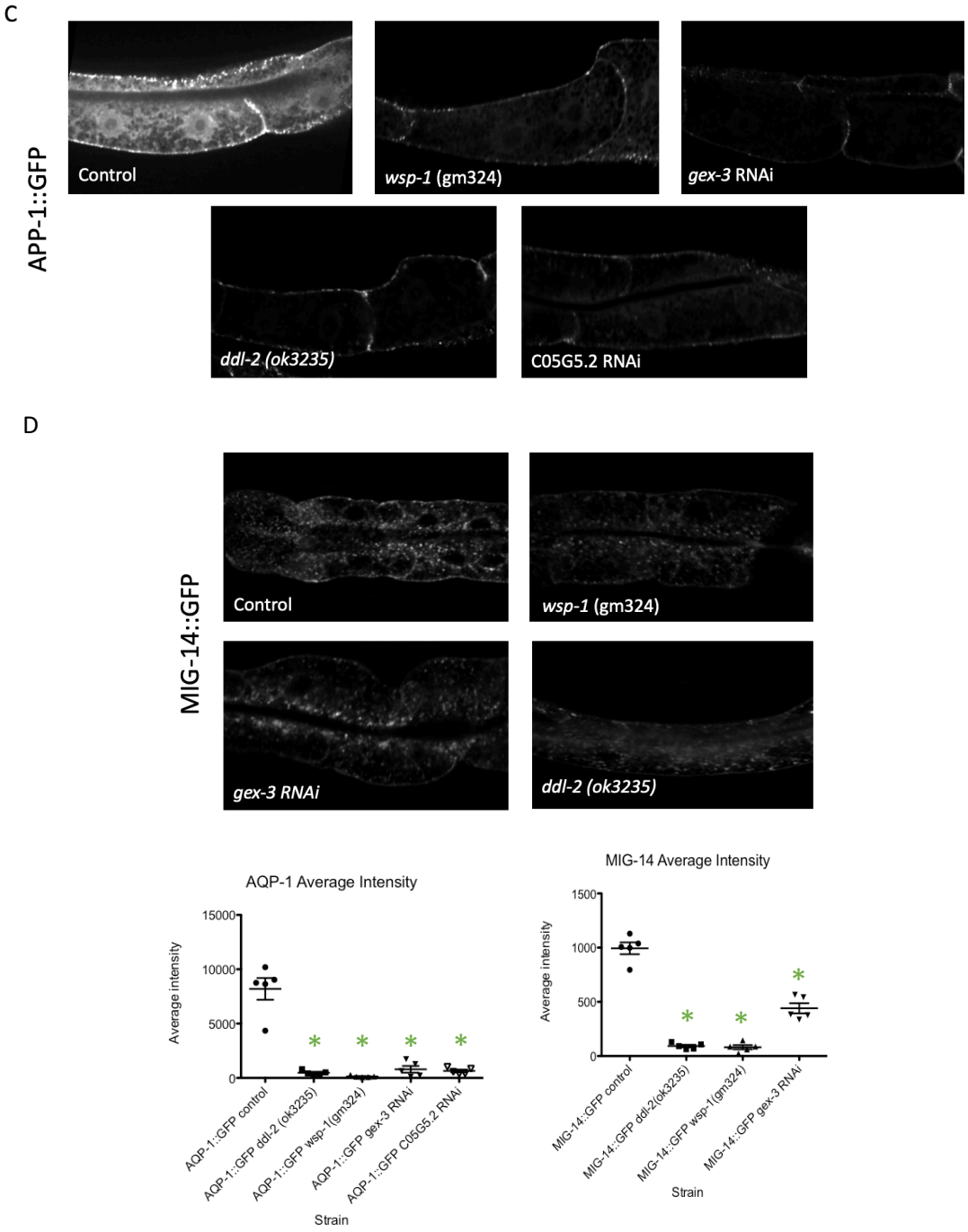
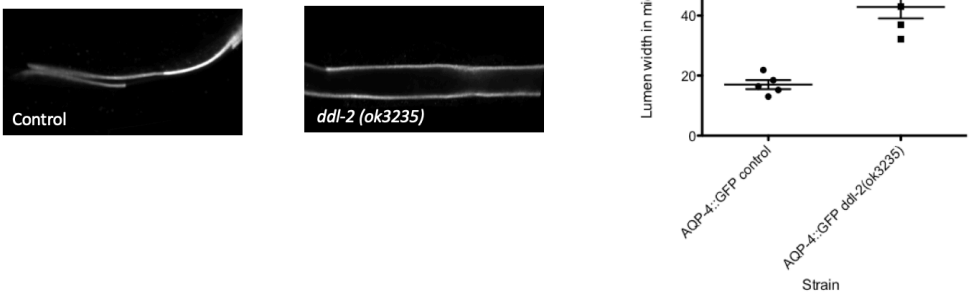


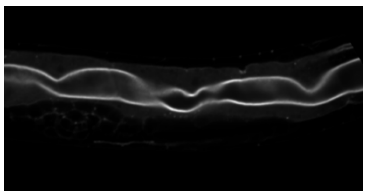
Figure 17: Transmembrane protein intensity. *Loss of WASH impairs the return of both apical and basolateral proteins to the plasma membrane. Contrarily, loss of WASP or WAVE does not uniformly reduce all proteins at the cell surface. A, B: Apical transmembrane proteins AQP-4::GFP and ERM-1::GFP. C, D: Basolateral transmembrane proteins AQP-1::GFP and MIG-14::GFP. n=5, with 5 measurements per worm. **=p<0.01, based on one-way ANOVA and Tukey's post-hoc.*

In addition to affecting the ability to recycle transmembrane proteins effectively, loss of WASH also alters the morphology of the intestinal lumen itself. This can be best visualized by examining the transmembrane protein AQP-4::GFP, which marks the apical surface of the intestine. Measuring from one apical surface across to the other, worms with loss of WASH via *ddl-2(ok3235)* mutants had a significantly increased width compared to that of other NPF mutants and N2 worms (Figure 18A). Furthermore, these worms also had an increased incidence of defective lumen shape, with a ruffled lumen characterized by increased bends and curvature, such as that depicted in Figure 18B. This suggests that mutations to the WASH complex, more so than WAVE and WASP, alter the shape of the lumen. However, it should be noted that WAVE knockdown was achieved through RNAi here, so it is difficult to compare this to the other NPFs, for which mutants were used.

A



B



Strain Name	% Worms with Defective Lumen
AQP-4::GFP	16%
AQP-4::GFP *ddl-2 (ok3235)*	35%
AQP-4::GFP on Strumpellin RNAi	17%
AQP-4::GFP on gex-3 RNAi	18%
AQP-4::GFP *wsp-1 (gm324)*	10%

Figure 18: Intestinal lumen morphology. *Loss of WASH through the mutant ddl-2(ok3235) results in increased lumen width, as well as increased prevalence of ruffled lumen shape. **=p<0.01 based on Student's T test with Welch's correction, n=15 worms per group.*

WASH, WAVE, and WASP play functionally distinct roles in recycling despite being NPFs

One striking similarity between the WASH and WAVE complexes is their pentameric structure, with each protein in the WAVE complex having a corresponding homolog in the WASH complex, suggesting a similar role for the two. As both are nucleation promoting factors, they can nucleate branched actin through activating Arp2/3. However, mammalian studies suggest that these two complexes, as well as the WASP complex, act at different places in endocytic recycling and play important and distinct roles. To examine the role of WASP and WAVE in trafficking, their ability to return the same apical and basolateral intestine proteins described to the plasma membrane was studied. As above, WASP mutants *wsp-1*(*gm324)* were crossed into each of these transmembrane proteins, fluorescently labeled, to monitor changes compared to wild type worms. As WAVE mutants are highly lethal in adult worms, however, RNAi of one of the WAVE subunits, *gex-3*, was used instead for this NPF.

Similar to the WASH mutants, *gex-3* RNAi decreased the presence of all transmembrane proteins examined (Fig 17 above). However, this was not the case with *wsp-1(gm324)* mutants, in which some cargo, including AQP-4::GFP and ERM-1::GFP, could still be recycled effectively. Similarly, with WASH, the reduction of the basolateral proteins was more substantial than of the apical proteins (Figure 17). This may have important implications for trafficking in polarized cells in adults, suggesting some cargo selection ability, perhaps through retrograde transport, which requires retromer and the cargo selection complex.

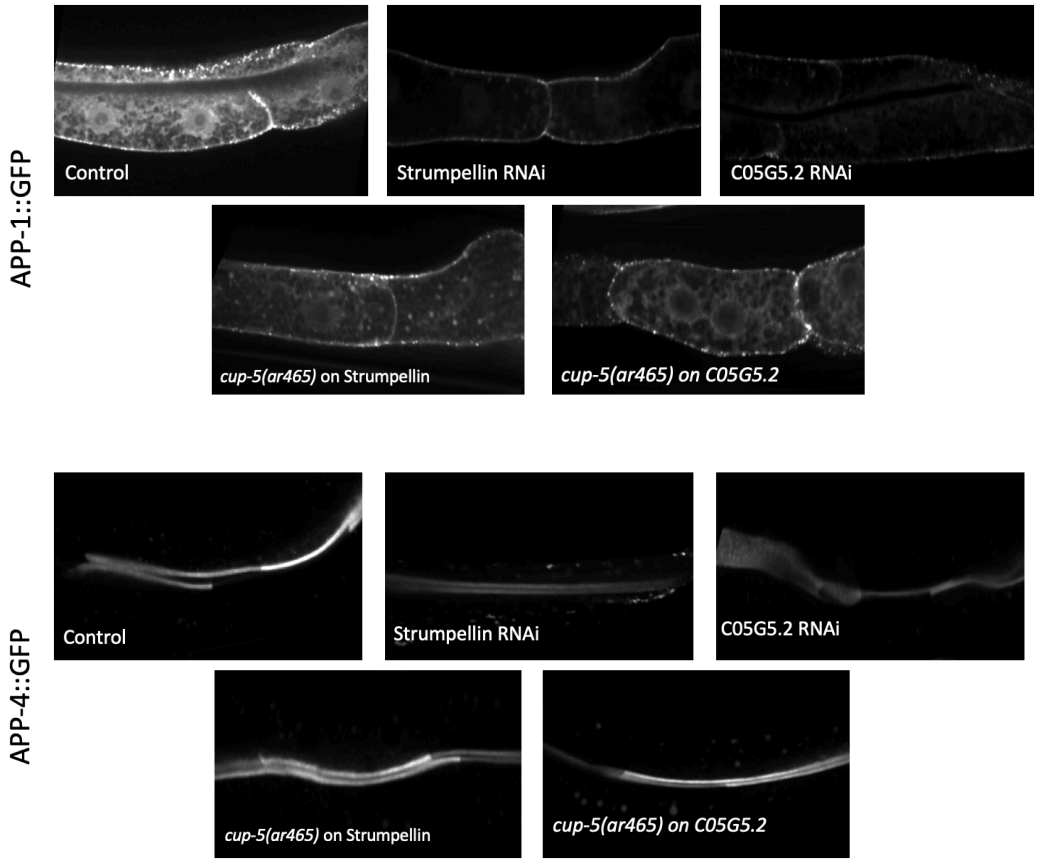
Loss of WASH causes cargo to be mistrafficked to lysosomes, rather than being transported via retrograde recycling

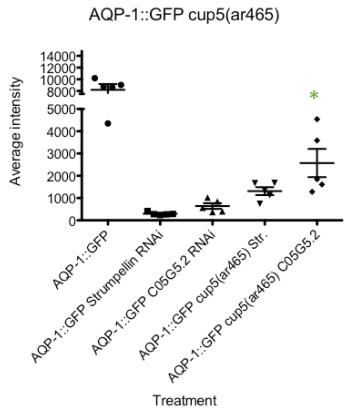
As WASH serves an important role in retrograde trafficking via the interaction between FAM21 and retromer in mammals, it was hypothesized that without WASH, cargo could not be recycled because it is instead being misdirected to lysosomes. This would explain the reduction in transmembrane cargo at the plasma membrane in *ddl-2(ok3235)* mutants. To determine if this is the case, two of the transmembrane proteins examined above were crossed with *cup-5(ar465)* mutants, which have endocytic trafficking defects due to issues with lysosomal trafficking. While these mutants have no defects in viability, they are characterized by an accumulation of refractile bodies that resemble cell corpses[66]. If cargo is being mistrafficked to lysosomes without WASH, these *cup-5(ar465)* mutants with lysosomal trafficking defects should be able to rescue the loss of cargo seen without WASH, increasing cargo quantities as less will be aberrantly trafficked to lysosomes.

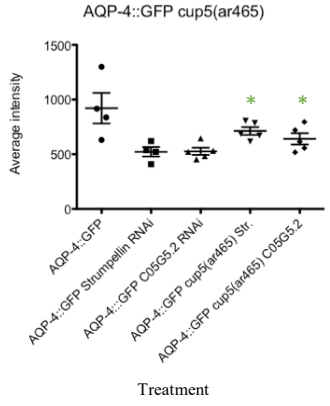
As shown in Figure 19, the presence of *cup-5(ar465)* is able to partially recover the presence of apical and basolateral transmembrane proteins. Levels of AQP-1::GFP and AQP-4::GFP increased almost 4.5-fold and 1.5-fold, respectively, when crossed with *cup-5(ar465)* and placed on Strumpellin RNAi. As such, altering lysosomal function can partially mitigate the effects of eliminating the WASH complex, suggesting that the observed decrease in transmembrane proteins without WASH is at least partially due to misdirection of cargo to lysosomes to be degraded.

To ascertain that WASH is involved in retrograde trafficking, AQP-1::GFP and AQP-4::GFP were next crossed into *snx-3(tm1595)* worms. SNX-3 is a retromer adaptor, as it can directly associate with cargo and help sort it to the Golgi[31]. As such, these mutants have defects in retrograde trafficking, meaning they cannot effectively recycle proteins back to the plasma membrane. As shown in Figure 19B, *snx-3(tm1595)* mutants cause similar drops in AQP-1::GFP and AQP-4::GFP, as seen with Strumpellin RNAi. This further supports that the WASH complex is involved in trafficking to the Golgi apparatus.

A


APP-1::GFP
Control
Strumpellin RNAi
C05G5.2 RNAi
cup-5(ar465) on Strumpellin
cup-5(ar465) on C05G5.2
APP-4::GFP
Control
Strumpellin RNAi
C05G5.2 RNAi
cup-5(ar465) on Strumpellin
cup-5(ar465) on C05G5.2


AQP-1::GFP cup5(ar465)
Average intensity
14000
12000
10000
8000
5000
4000
3000
2000
1000
0
AQP-1::GFP
AQP-1::GFP Strumpellin RNAi
AQP-1::GFP C05G5.2 RNAi
AQP-1::GFP cup5(ar465) Str.
AQP-1::GFP cup5(ar465) C05G5.2
Treatment


AQP-4::GFP cup5(ar465)
Average intensity
1500
1000
500
0
AQP-4::GFP
AQP-4::GFP Strumpellin RNAi
AQP-4::GFP C05G5.2 RNAi
AQP-4::GFP cup5(ar465) Str.
AQP-4::GFP cup5(ar465) C05G5.2
Treatment

B

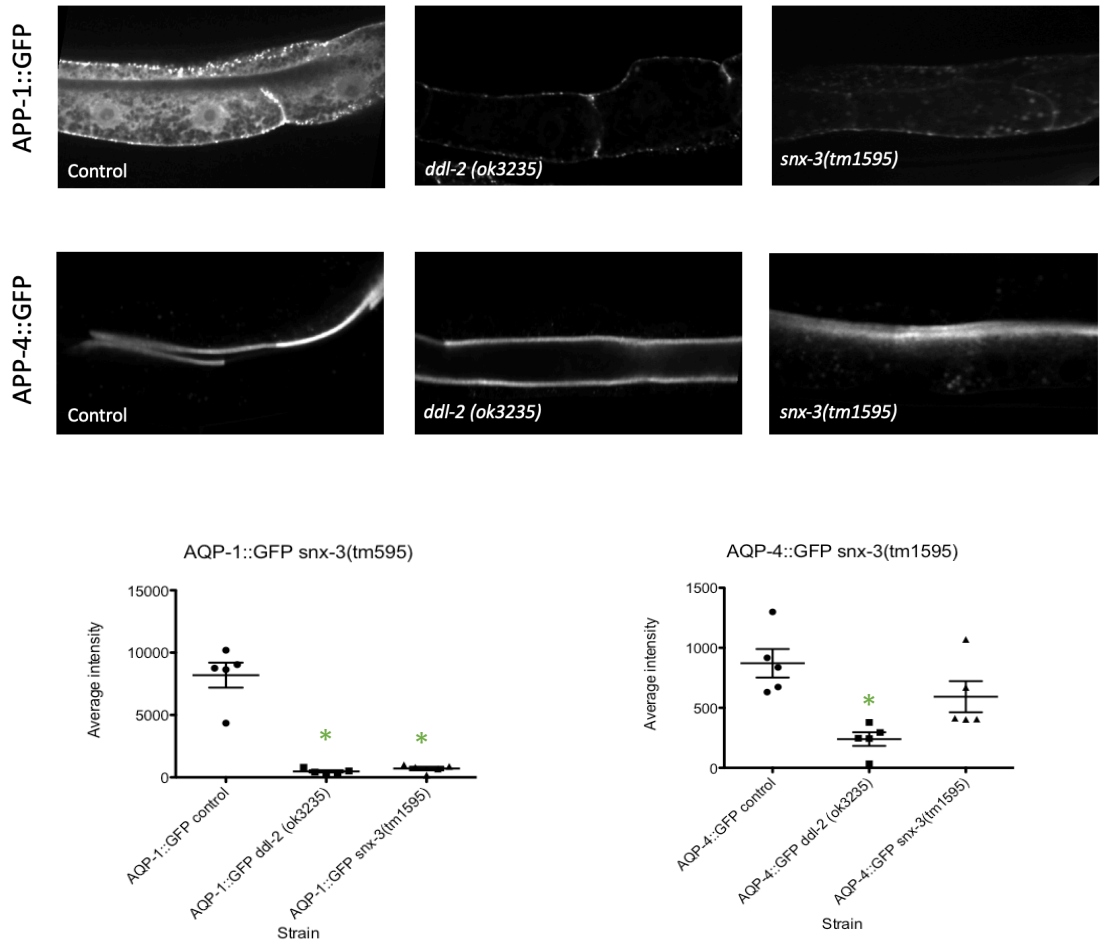


Figure 19: Loss of WASH mistrafficks cargo to lysosomes to be degraded. *cup-5(ar465) mutants are able to rescue the loss of AQP1::GFP and AQP-4::GFP with ddl-2(ok3235) mutants. Similarly, snx-3(tm1595) mutants give the same loss in transmembrane proteins seen in the ddl-2(ok3235) mutants. n=5, with 5 measurements per worm. *=p<0.05, **=p<0.01 based on one-way ANOVA and Tukey's post-hoc.*

The C. elegans WASH complex lacks a conserved tyrosine phosphorylation site

Although these results demonstrate that the WASH is acting on early endosomes to stimulate Arp2/3-mediated actin polymerization, it remains unclear how this complex is activated and recruited. For WASP and WAVE, activation can be achieved by phosphorylation of conserved residues. These complexes are phosphorylated by Src family kinases[67] at particular sites, such as the residue Y293 or 291 of the WASP1 in mice and humans, respectively[19], and the Y125 residue of WAVE1[21], which releases the VCA region. Similarly, a comparison of the WASHC1 protein of several organisms in previous work identified a conserved residue, ANDLQ/MY, in which the final tyrosine is phosphorylated. This residue is well conserved amongst humans, mice, and fruit flies, found in their WHD2 domains[22]. As such, these sequences were aligned to the *C. elegans* ddl-2 subunit to determine if a similar tyrosine phosphorylation site could be identified. Despite similarities between all of the other organisms shown, *C. elegans* seems to lack this site (boxed region, Figure 20). As such, is seems that *C. elegans* WASH is not activated by phosphorylation at this same conserved motif. This may mean it is being recruited to early endosomes in a different manner or via phosphorylation at another location.

Figure 20. C. elegans WASH lacks a conserved phosphorylation site. *The WASH subunit has a conserved phosphorylation site across different species, shown in the boxed region of the sequence with the tyrosine residue to be phosphorylated highlighted. Notably, the C. elegans WASH lacks this site.*

The protein C05G5.2 is the potential FAM21 homolog in C. elegans

Most studies involving the WASH complex have been conducted in mammals, with WASH recruitment dependent upon the FAM21 subunit binding to phospholipids, although little is known about this specific interaction and how it is recruited only to certain endosomes[6]. Upon being recruited, the FAM21 subunit then plays an indispensable role in facilitating recycling to the Golgi apparatus via an interaction with the VPS35 subunit of retromer. Despite strong structural similarities between the mammalian and *C. elegans* WASH complex, however, *C. elegans* seems to lack a FAM21 subunit. Based on the essential role of this subunit in mammals, as well as the fact that it is located in the middle of the complex and links WASH to all of the important players in recycling, it is more likely that a homologous protein exists that has not yet been found.

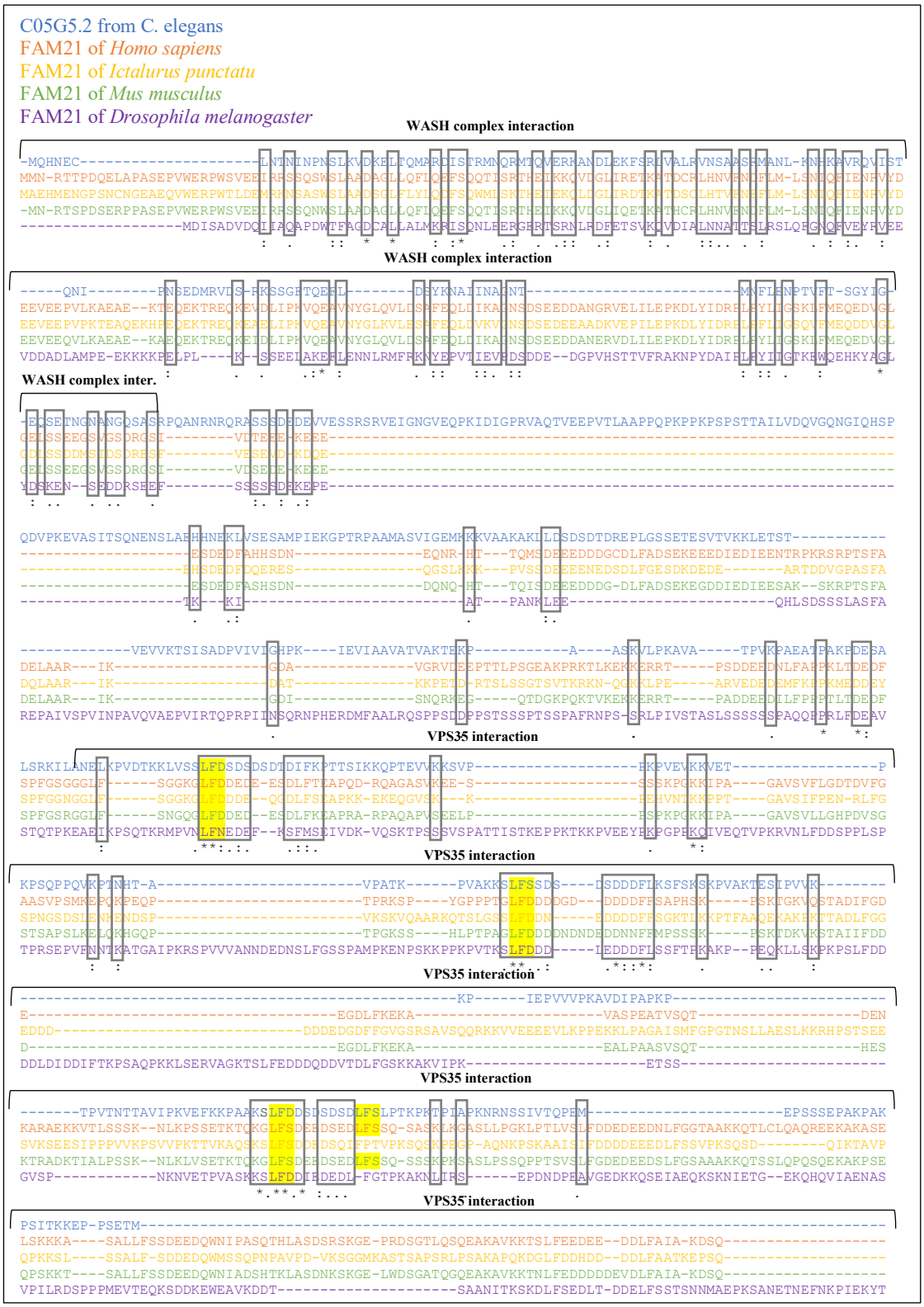
In an effort to identify this FAM21 homolog, several BLAST searches were conducted, as summarized in Table 4. To start with the most broad approach, the FAM21 sequence from a variety of model organisms was searched against the entire *C. elegans* genome and, unsurprisingly, no significant matches were obtained. Next, to approach this in a different manner, smaller portions of these FAM21 sequences were used, such as the region that interacts with the retromer subunit VPS35, as well as the portion that interacts with the WASH subunit. Again, few significant matches were observed. Finally, particular motifs were searched, such as the motif that has been shown to interact with capping protein[68], as well as the characteristic leucine-phenylalanine-acidic repeats found in FAM21, with no significant results.

Table 4: FAM21 BLAST searches

BLAST searches performed in *Caenorhabditis elegans*	**Results**
FAM21, whole sequence, from human, *Drosophila,* mouse, and hamster	No matches of significant similarity
Capping protein motif (*LXHXTXXRPK(X)$_6$P)*	No matches
FAM21 region that interacts with retromer VPS35	Matches of low identity (<20%)
FAM21 region that interacts with WASH subunit	No matches
LFa repeat motif	No matches

Table 4: FAM21 BLAST search summary. *Minimal matches were returned in searching for a potential C. elegans FAM21 homolog using the NCBI BLAST program. BLAST searches using both the entire sequence and particular motifs yielded no significant matches.*

After these initial discouraging results, mention of a protein in a paper by Jia, Billadeau, et. al.[8] shed some new light on the search for a FAM21 homolog. This unnamed protein, referred to as C05G5.2, was suggested to be a FAM21 homolog on the basis of its three LFa repeats[8]. To examine the relationship between C05G5.2 and the known FAM21 subunits from various other organisms, a sequence alignment was performed (Figure 21). Notably, the C05G5.2 sequence aligns to other FAM21 sequences from its N terminus to C terminus, although the *C. elegans* homolog is considerably shorter, and contains four LFa motifs in the region known to interact with VPS35 of retromer. There is also significant alignment in the beginning portion of the sequence, which is known to interact with the rest of the WASH complex, but not as much alignment in the region at the end of the sequence that interacts with CapZα[11,40].


C05G5.2 from C. elegans
FAM21 of Homo sapiens
FAM21 of Ictalurus punctatu
FAM21 of Mus musculus
FAM21 of Drosophila melanogaster
WASH complex interaction
WASH complex interaction
WASH complex inter.
VPS35 interaction
VPS35 interaction
VPS35 interaction
VPS35 interaction
VPS35 interaction
VPS35 interaction

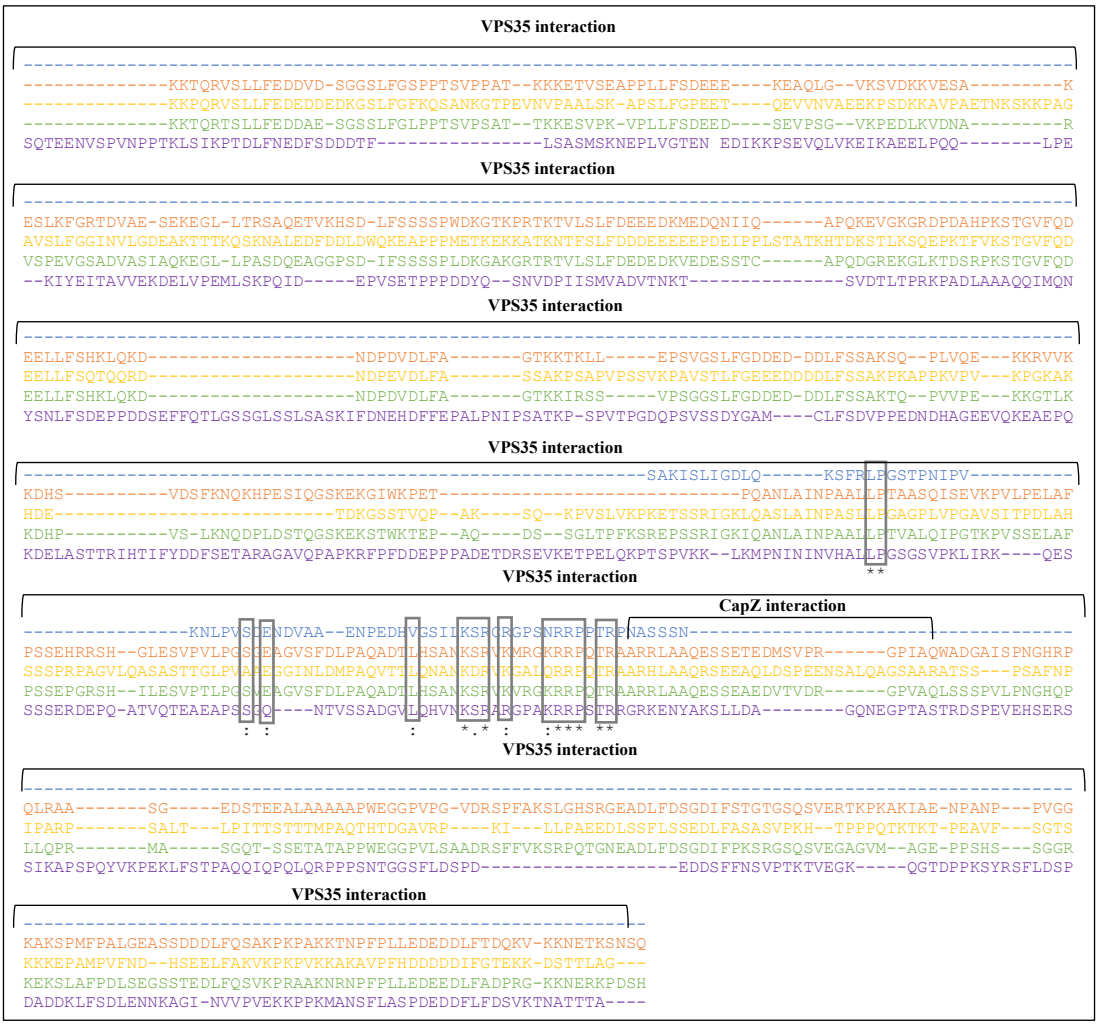
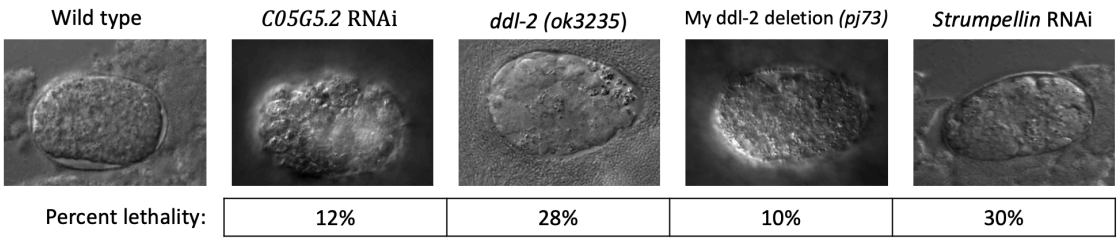


Figure 21: FAM21 sequence alignments. *Alignment of the FAM21 subunits of human, catfish, mouse, and fruit fly compared to the C. elegans C05G5.2 protein shows significant regions of similarity from N terminus to C terminus. LFa repeat motifs in C05G5.2 are highlighted. Boxes are used to highlight related regions, with symbols below indicating the specific relationship: dashes are used indicate gaps, asterisks for identical residues, double dots for strong similarity, and single dots for weak similarity. Regions of interest with important binding partners are noted above the sequence, with the H. sapiens sequence amino acids: 1024-1047 interacting with CapZ, 1-200 interacting with the WASH complex, and 357-1318 containing LFa repeats that interact with retromer* [11,40].

After observing the sequence homology between the *C. elegans* protein C05G5.2 and FAM21 subunits from four different species, further evidence was needed to support that this protein might be the FAM21 homolog. As such, worms were put on C05G5.2 RNAi to see if eliminating this protein would cause any lethality in the embryos. This led to about 12% lethality (Figure 22) due to morphogenesis defects. This is similar to defects that cause lethality in *ddl-2(ok3235)* and *ddl-2(pj73)* mutants, as well as in *Strumpellin* RNAi worms.

A



B

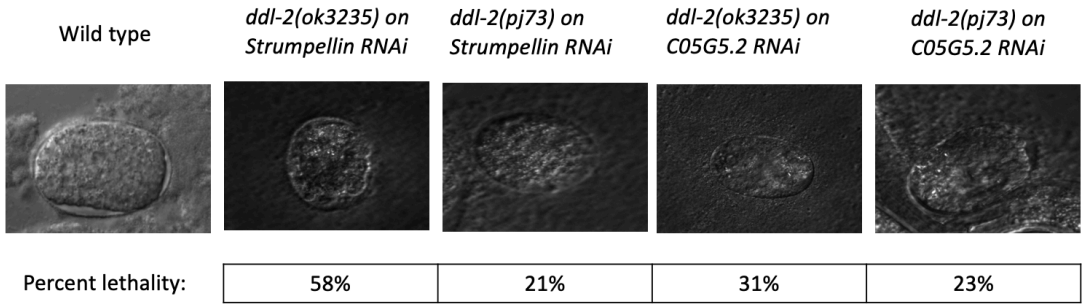


Figure 22: C05G5.2 RNAi causes embryonic lethality due to morphogenesis defects. *(A) Similar to the defects seen in WASH and Strumpellin, C05G5.2 RNAi leads to about 12% embryonic lethality. (B) ddl-2(ok3235) mutants placed on Strumpellin RNAi and C05G5.2 RNAi lead to substantial increases in lethality, causing about double the number of embryos to experience morphogenic defects. n>200 embryos.*

After this phenotype was observed in embryos, the RAB-5::RFP and PI(3)P::GFP worms, which mark early endosomes, were placed on C05G5.2 RNAi, grown to L4 stage and analyzed. If this protein is indeed a FAM21 homolog, it was expected that the same results would be observed as were seen with loss of the WASH complex through Strumpellin RNAi. This was indeed the case, with C05G5.2 RNAi demonstrating an increase in RAB-5- and PI(3)P-positive early endosomes (Figures 15A and 16C). It was also hypothesized that putting one apical and one basolateral transmembrane protein strain on C05G5.2 RNAi would lead to a decrease in apical and basolateral transmembrane proteins, similar to that observed with loss of the WASH complex through *ddl-2(ok3235)* mutants and Strumpellin RNAi. Indeed, a substantial reduction in both AQP-1::GFP and AQP-4::GFP was observed (Figure 17), suggesting that this protein is involved in trafficking and providing further evidence that it could be the FAM21 homolog. Furthermore, crossing these worms with *cup-5(ar465)* mutants and then putting them on C05G5.2 RNAi was also able to partially rescue levels of AQP-1::GFP and AQP-4::GFP, similar to that seen on Strumpellin RNAi (Figure 19A). This again suggests that this protein is involved in trafficking in a role similar to, or in cooperation with, the WASH complex.

Furthermore, if this protein cooperates with the WASH complex to mediate recycling, then it may also be involved in generating branched actin networks that provide a force for moving cargo through the endocytic recycling pathway. To determine if this is the case, the *C. elegans* ACT-5::GFP protein levels were examined. This actin protein is expressed specifically in the intestine, and localizes to the apical surface while also showing expression in puncta[54]. As expected, loss of WASH via Strumpellin RNAi

leads to overall decrease in actin levels. A similar decrease was also observed with C05G5.2 RNAi (Figure 23), suggesting that both the WASH complex and C05G5.2 are involved in actin branching through Arp2/3, allowing recycling to occur in the intestinal epithelium. However, it should be noted that the apical signal for ACT-5::GFP was not as strong as expected even in the control worms, so this experiment should be repeated using another actin marker, such as pGlo-LifeAct::mCherry, to confirm these results.

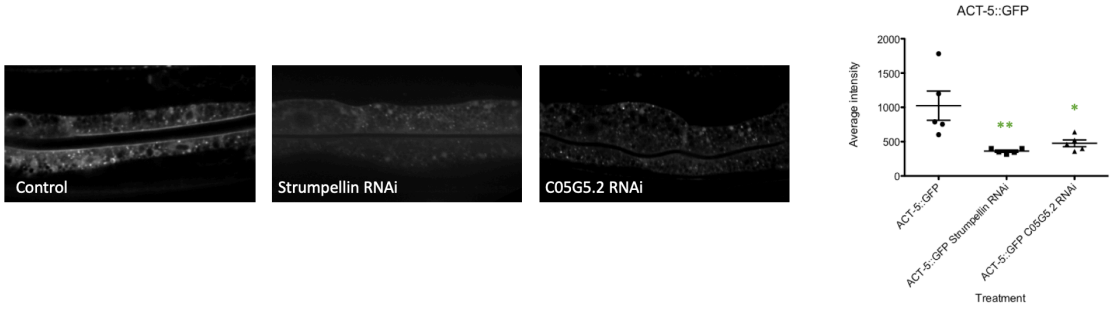


Figure 23: Actin intensity following loss of WASH and C05G5.2*. Loss of the protein C05G5.2 through RNAi leads to loss of intestinal actin, similar to that observed with loss of WASH via Strumpellin RNAi. n=5, with 5 measurements per worm. *=p<0.05; **=p<0.01 based on one-way ANOVA and Tukey's post-hoc.*

WASH interacts with protein C05G5.2 to mediate recycling from early endosomes to the Golgi apparatus

To further determine the role of C05G5.2, *ddl-2(ok3235)* and *ddl-2(pj73)* mutants were placed onto C05G5.2 RNAi. If these two complexes both act in the same linear pathway, then this should lead to no changes embryonic lethality. As shown in Figure 22, however, lethality counts of both *ddl-2(ok3235)* and *ddl-2(pj73)* on Strumpellin RNAi increased from 28% and 10% to 58% and 21%, respectively. Similarly, both mutants on C05G5.2 RNAi saw increased lethality, from 28% to 31% for *ddl-2(ok3235)* and from 10% to 23% for *ddl-2(pj73).* Again, these defects were due to issues with morphogenesis.

Finally, if the C05G5.2 protein is indeed interacting with WASH to support retrograde trafficking, then the *cup-5(ar465)* mutants crossed with AQP-1::GFP and AQP-4:GFP should be able to partially rescue the drop in these proteins after being placed on C05G5.2 RNAi. As seen in Figure 19A, these proteins are elevated compared to those without *cup-5(ar465)*, suggesting that defects in C05G5.2 causes missorting to lysosomes, as seen in WASH knockouts through Strumpellin RNAi.

Discussion:

Branched actin polymerization, initiated by Arp2/3-mediated actin nucleation, provides a force for many key processes, including developmental morphogenesis and endocytosis. Although more is known about the earliest nucleation promoting factors to be discovered, including WASP, little information exists characterizing the WASH complex. As such, this study seeks to provide the first characterization of this complex in *C. elegans*, a useful model organism for studying various cellular processes.

The results shown here demonstrate that the WASH complex plays a key role in embryonic morphogenesis. Worms lacking the DDL-2 subunit of the WASH complex, through an existing strain (*ddl-2(ok3235)*) and a new deletion created here via CRISPR, which removed almost the entirety of the protein (*ddl-2 pj73)*, had embryonic lethality of 28% and 10%, respectively. While the newly created allele has a smaller lethality, this could be due to previously reported issues with the *ddl-2(ok3235)* allele. Additionally, removal of the Strumpellin subunit, through RNAi, caused 30% lethality. In all cases, lethality was due to a defect in the process of morphogenesis, as shown in the images of embryos with clear physical cellular abnormalities, and worsened by combining these two methods of removing the WASH complex (Figure 22). This is similar to observations of development upon removal of the other two branched actin nucleators, WASP and WAVE. Additionally, loss of WASH through the mutant *ddl-2(ok3235)* caused increased apical lumen width and higher frequency of abnormal ruffled lumen shape of the intestine. This could suggest issues with the morphology of the intestine when it is created during development, although early lumen formation of polarized epithelial cells would have to be examined to ascertain this. To further characterize the role that WASH

plays in this process, we can next examine which stages of morphogenesis are affected, to see if the defects occur during dorsal intercalation, ventral enclosure, or elongation (Figure 6)[24]. This can be compared to the stages most impacted by lack of WASP and WAVE, to determine if each of these nucleators acts at distinct times during development or if they are all necessary at all stages.

In addition to its role in embryonic morphogenesis, actin nucleation plays a key role in intracellular trafficking of molecules. To maintain the quantity and distribution of essential proteins at the plasma membrane, a cell must be able to recycle these cargo following endocytosis. The progression of these proteins through the endolysosomal system requires a force for the scission of vesicles and subsequent movement through the cytoplasm. The fate of these proteins must be correctly determined early on, at early endosomes, to ensure that cargoes, such as ion and nutrient channels, signaling receptors, and adhesion molecules, can be maintained in the proper amount at the cell surface.

This work demonstrates that WASH would act at early endosomes, as has been observed in mammalian systems, where WASH overexpression increased actin assembly in the perinuclear region of the cell, rich in early endosomes, but depletion of WASH diminished Arp2/3 localization to this region[6]. As shown through RAB-5::RFP and PI(3)P::GFP-labeled early endosome levels, loss of WASH increases these endosomes while decreasing those in later recycling steps, including late endosomes and recycling endosomes. However, levels of $PI(4,5)P_2$ and $PI(3,4,5)P_3$ are unaffected by loss of WASH. Taking together, these data suggest that WASH acts at early endosomes, in contrast to WAVE and WASP, which act at the plasma membrane. However, eliminating

all WASH complex molecules through mutations or RNAi only captures changes that occur in the beginning of the pathway, as there is no WASH present to allow molecules to progress past early endosomes to observe defects in later recycling steps. For example, previous studies have indicated that WASH may also play smaller role at late endosomes[12,18,39]. Although a decrease in RAB-7::RFP endosomes was observed here with loss of WASH, these compartments were also increased in size (Figure 15). This could imply that some cargo was still able to get to this step, but then is being held up at this stage as well, or more simply that increased cargo reaches late endosomes when recycling fails. To observe this further, CRISPR can be used to tag the ddl-2 subunit, so that its fluorescent pattern can be overlaid with each of the RAB-labeled endosomes to determine precisely where WASH colocalizes. Additionally, if the WASH complex is working to activate Arp2/3 at early endosomes, actin levels at this specific location should be examined. To do this, ACT-5::GFP worms can be crossed into RAB-5::RFP worms, and colocalization of these two proteins with and without WASH can be determined.

In addition to demonstrating that WASH is primarily acting at early endosomes, this study also indicates that this role is of functional importance in the proper recycling of cargo, as eliminating WASH decreases levels of several different endogenous *C. elegans* intestinal proteins at both the apical and basolateral surfaces. These include the apical proteins AQP-4::GFP and ERM-1::GFP (although this protein is likely trafficked with other cargo, such as cell adhesion molecules, as it is membrane-associated), as well as the basolateral proteins AQP-1::GFP and MIG14::GFP. WASH was eliminated here through use of the existing *ddl-2(ok3235)* allele, however it would also be useful to

repeat these experiments by crossing in the new allele created here using CRISPR, *ddl-2(pj73),* as this deleted nearly the entire sequence of this protein.

Next, these experiments reveal a role for WASH that differs from that of WASP and WAVE, as levels of all proteins did not uniformly drop for all three nucleation promoting factors, demonstrating again that these NPFs act differently in trafficking and suggesting a cargo selection ability. More specifically, apical proteins were not decreased as substantially as basolateral proteins, implying that these factors are acting differently in polarized cells to traffic cargo to each surface. This could provide insight as to why morphogenesis defects were observed—perhaps the ability to establish polarity in cells throughout development, as well as traffic materials to the correct place, is being impacted. This effect on apical proteins was especially true for WASP mutants, as levels of AQP-4::GFP and ERM-1::GFP did not drop as drastically in *wsp-1(gm324)* worms. This could mean that WASP is not necessary for recycling of these apical proteins, or perhaps that the other NFP enriched at the plasma membrane, WAVE, can compensate for loss of WASP during endocytosis.

The decreases observed in these intestinal proteins are likely due to the fact that they are being missorted, with their fates being improperly determined without WASH at early endosomes. WASH typically interacts with retromer at early endosomes in mammalian systems[9,43,44], suggesting that it plays a key role in retrograde trafficking through the Golgi apparatus. This was supported by data demonstrating that *snx-3(tm1595)* mutants, a protein that interacts with retromer, led to similar defects in recycling of AQP-1::GFP and AQP-4::GFP (Figure 19B). To confirm that WASH is

important solely for retrograde trafficking, the ability to recycle cargo that do not follow the retromer pathway could be examined, such as DAF-4. Similarly, loss of WASH leads to a decrease in effective recycling, as evident in a reduction in cargo at the membrane, because these cargoes are misdirected to lysosomes for degradation. This was demonstrated through crossing AQP-1::GFP and AQP-4::GFP with *cup-5(ar465)* worms, which have defective lysosomal trafficking. Altering the ability of molecules to travel to lysosomes was able to partially recover levels of these proteins (Figure 19A), suggesting that the trafficking defects observed without WASH are at least partially due to cargo being missorted to lysosomes. To confirm that this reduction is actually due to endocytic recycling pathway defects, rather than the secretory pathway, which also moves material to the lysosome, endocytosis could be blocked using mutants of APA-2, a protein important for clathrin-dependent endocytosis (although clathrin-dependent cargo would need to be used here).

It is also possible that the other nucleation promoting factors can compensate for the loss of WASH. However, observations that these complexes act at different steps of recycling suggest that WASH plays a distinct role. As WASP and WAVE both act at the plasma membrane, it would be interesting to see if both are necessary and sufficient for proper trafficking. To do this, double mutant strains could be created that lack both complexes. As these worms would likely be rather sick, it would be useful to use CRISPR and the auxin-inducible degron system to knock down both complexes only at particular locations and times. RNAi could also be used, with mutants for one of the NPFs placed on to RNAi for the other.

Finally, this study provides insight as to how the WASH complex gets activated and recruited to early endosomes to mediate retrograde trafficking. WASH activation in mammals is achieved through phosphorylation at conserved tyrosine sites, similar to those that are phosphorylated to activate WASP and WAVE. However, alignment of the WASH1 subunits from several species to *C. elegans* revealed that *C. elegans* lacks this conserved site, although closer examination of the ddl-2 subunit reveals that it does contain five tyrosine amino acids that could serve as potential sites for phosphorylation. To determine their importance in WASH activation, point mutations could be made to each of these amino acids and the resulting ability to recruit WASH to early endosomes and activate it to turn on Arp2/3 could be examined. It is also possible that other amino acid residues, such as serine or threonine, could be phosphorylated to activate WASH. To determine this, the Src family tyrosine kinases could be knocked out and subsequent ability to activate WASH could be determined. If WASH can still be activated without this, then amino acids aside from tyrosine might be more relevant to examine.

Lastly, this work suggests that the protein C05G5.2 provides a strong candidate for the FAM21 homolog in *C. elegans.* Despite the necessity of this subunit in everything from the protist *Dictyostelium* to humans[69], no similar protein had been identified in *C. elegans* prior to this study. First, a sequence comparison of C05G5.2 with the FAM21 subunit of four different organisms demonstrates that this protein aligns from its N-terminus to C-terminus, with regions of significant sequence similarity. These regions include two LFa repeat motifs, which uniquely characterize the FAM21 subunit and allow it to interact with the VPS35 subunit of retromer. Comparison of the phylogeny of these different organisms might provide insight as to why C05G5.2 in *C. elegans* is much

shorter than FAM21, as well as why it only has two LFa repeats. Overall, the percent identity between C05G5.2 and the four other FAM21 subunits is only 4%, while the percent similarity is 16%, which can explain why BLAST searches did not generate any significant matches. Next, RNAi of C05G5.2 demonstrated that it is similarly important in morphogenesis, with embryonic lethality caused by morphogenesis defects, like those seen in WASH mutants. Surprisingly, this lethality is more substantial when *ddl-2(ok3235)* and *ddl-2(pj73)* mutants were placed on C05G5.2 RNAi, as was the case when these mutants were on Strumpellin RNAi. As it was hypothesized that all of this molecules act in one pathway, it was expected that lethality counts would remain unchanged when more than one component was eliminated. However, it is possible that individual RNAi experiments did not fully deplete all the proteins of interest, making that lethality count lower than expected, so that mutants placed onto RNAi appeared to increase the lethality. Additionally, it is possible that the subunits of the WASH complex also play separate roles in other processes in the cell. This is not unreasonable to consider, as this has already been shown for the structurally-related WAVE complex.[70] Because of the limitations of RNAi, to be more certain of these results, CRISPR could also be used to generate a knock out of this gene, to see if the same morphogenesis and trafficking defects are observed. Furthermore, loss of this protein through RNAi caused an increase in RAB-5::RFP and PI(3)P::GFP early endosome markers, similar to results obtained with Strumpellin RNAi. It also causes a similar decrease in ACT-5::GFP levels, again as seen with Strumpellin RNAi, although the weak apical signal for these control worms suggests this experiment should be repeated using another actin marker, such as pGlo Life-Act::mCherry. Finally, this protein is functionally important in trafficking, as

C05G5.2 RNAi led to similar decreases in AQP-1::GFP and AQP-4::GFP levels, with *cup-5(ar465)* being able to partially rescue these levels. To further ascertain that this protein can associate with the WASH complex, this protein could be tagged via CRISPR to determine if it can colocalize with the WASH complex at early endosomes and can be immunoprecipitated with the WASH complex *in vitro*. Similarly, pull-down assays could be used to confirm that this protein binds to the VPS35 subunit of retromer, or to RME-8, both of which bind to FAM21[71]. This would provide more concrete evidence that C05G5.2 is the previously unidentified FAM21 subunit of the *C. elegans* WASH complex. It is essential to confirm this, as the FAM21 subunit of the WASH complex in other organisms is the largest and arguably most important portion of the complex for endocytic recycling, allowing WASH to be recruited to early endosomes and to interact with retromer to mediate retrograde trafficking.

Overall, this study has elucidated a role for the *C. elegans* WASH complex in endocytic recycling. While the complex seems to act at RAB-5-, PI(3)P-positive early endosomes, how it is recruited to this specific endosome population remains unclear. Despite these ambiguities, it is of functional importance, allowing for proper recycling of cargo that is not misdirected to lysosomes. Furthermore, this role is distinct from that of the other nucleation promoting factors, WASP and WAVE, which seem to act at the plasma membrane instead of early endosomes. A model summarizing this transport process is shown in Figure 24. Most importantly, this study suggests that WASH seems to cooperate with the newly identified C05G5.2 protein, as evidence indicates this may be the essential FAM21 homolog in *C. elegans.* In addition to providing another model organism in which to study a plethora of human diseases related to actin nucleators and

trafficking, such as cancer, Parkinson's disease, and diabetes, characterizing the *C. elegans* WASH complex can provide a new perspective on this protein that may be overlooked in mammalian systems, including novel proteins that may interact with the complex and are evolutionarily conserved.

In summary, this study provides the first characterization of the recently identified and poorly understood WASH complex in *C. elegans*, demonstrating that it plays an essential role in two processes involving actin nucleation—morphogenesis and intracellular protein trafficking.

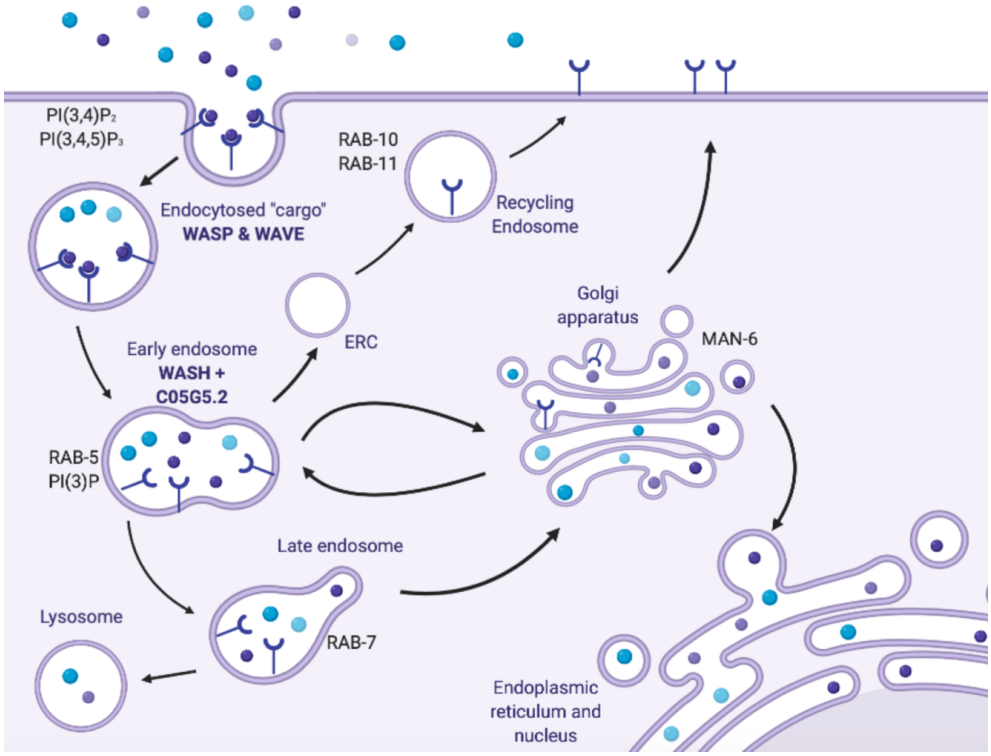


Figure 24: A model for endocytic recycling in C. elegans. *Data from this study supports that the WASH complex acts at RAB-5, PI(3)P-positive early endosomes, similar to its role in mammalian systems. Furthermore, it may interact with C05G5.2, a potential homolog for the FAM21 subunit.*

References:

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