Function and regulation of the WASH complex in the endocytic cycle

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A thesis submitted to the University of Glasgow for the degree of Doctor of Philosophy

October 2012

Abstract

The WASH complex is highly conserved and consists of the actin nucleation promoter, WASH, and several regulatory subunits; Strumpellin, SWIP, ccdc53 and FAM21. Previously, it has been shown that WASH directs construction of actin coats on lysosomes. This actin coat is required for removal of V-ATPase complexes from lysosomal membranes, allowing neutralization and maturation to post-lysosomes. WASH null cells are blocked at the acidic lysosome stage and are thus unable to perform exocytosis.

We now show that FAM21 acts at a different step in the same pathway. FAM21 nulls are still blocked in exocytosis, but the remaining complex is functional in removal of V-ATPase, allowing progression to post-lysosome. We hypothesize that the role of FAM21 is to release the WASH complex from post-lysosome membranes in order to allow recycling back to newly formed acidic lysosomes. We have also shown that capping protein interacts with the WASH complex through FAM21, and this interaction is essential for progression to exocytosis, likely contributing to the mechanism by which FAM21 regulates and releases the WASH complex from post-lysosomal membranes.

Table of Contents

List of Figures

List of Tables

Acknowledgements

A huge thanks to everyone who has contributed to this thesis and helped me through my PhD. Thank you to my supervisor, Robert Insall, who has helped me develop the project and kept me motivated throughout my time here. Pete Thomason, Jason King and Douwe Veltman all have my gratitude for answering my endless questions and giving me valuable advice and ideas. I also thank Tobias Zech, especially for his help with the mammalian work, and contributing a great deal of time and effort into making the project a success. Also, thank you to Michael Carnell, who supplied a sound foundation on which to build my project and taught me many of the experimental procedures. Finally, thanks to everyone else in the Insall and Machesky labs for all their kindness and friendship over the last four years.

Author's Declaration

I declare that, except where explicit reference is made to the contribution of others, that this dissertation is the result of my own work and has not been submitted for any other degree at the University of Glasgow or any other institution.

Signature:

Printed Name:

Abbreviations

cAMP	cyclic adenosine monophosphate
ATP	adenosine triphosphate
DNA	deoxyribonucleic acid
F-actin	filamentous actin
G-actin	globular actin
ER	endoplasmic reticulum
MLCK	myosin light chain kinase
ROCK	Rho kinase
Arp2/3	Actin-related protein 2/3
ARPC1-5	Actin-related protein 2/3 complex 1-5
WASP	Wiskott Aldrich syndrome protein
N-WASP	neuronal WASP
Scar/WAVE	suppressor of cAR/WASP family verprolin homologous protein
WHAMM	WASP homologue associated with actin, membranes, and microtubules
JMY	junction mediating and regulatory protein
WASH	WASP and Scar homologue
FH	formin homology
Cobl	Cordon-bleu
WH1/2	WASP homology 1/2
Abp1	actin binding protein 1
NPF	nucleation promoting factor
N terminus	amino terminus
C terminus	carboxyl terminus
GBD	GTP-ase binding domain
WHD1/2	WASH homology domain 1/2
ActA	actin assembly-inducing protein
SRA1	specifically Rac1-associated protein 1
HSPC300	haematopoieitc stem/progenitor cell protein
NAP1	nucleosome assembly protein 1
ABI	abelson tyrosine kinase interactor
Rac1	Ras-related C3 botulinum toxin substrate 1

IRSp53	insulin receptor substrate protein of 53 kDa
mRNA	messenger ribonucleic acid
WIP	WASP interacting protein
Cdc42	cell division control protein 42
GTPase	guanosine triphosphatase
V-ATPase	vacuolar adenosine triphosphatase
Capu	cappuccino
TAP	tandem affinity purification
EGF	epithelial growth factor
Dia1	diaphanous 1
Tf	transferrin
EGFR	epidermal growth factor receptor
SNX	sorting nexin
VSP	vacuolar protein sorting-associated protein

CHAPTER 1
INTRODUCTION

1 Introduction

1.1 Dictyostelium as a model organism

Dictyostelium discoideum is an amoeba which naturally lives in the soil (Raper, 1935) and has the ability to sense and move towards bacteria, its natural food source. Under normal conditions, *D. discoideum* exists as a single cell, however starvation sets into motion a system whereby each cell signals to neighbouring cells by secreting pulses of cyclic adenosine monophosphate (cAMP; Gerisch and Wick, 1975). These pulses of cAMP create a gradient up which the cells move (Tomchik and Devreotes, 1981) where they eventually aggregate and begin differentiation in order to form a multicellular structure known as a fruiting body (Olive and Stoianovitch, 1975). This is composed of a stalk and spore head, containing spores which can be released when conditions are suitable to ensure survival of the cell population.

The *D. discoideum* genome is composed of 6 chromosomes (Cox et al., 1990; Loomis, 1998) which have been fully sequenced (Eichinger et al., 2005). Cells are haploid making it relatively easy to carry out manipulations to the cells and their DNA such as knocking out a gene, a process which is far more complex and time consuming in diploid mammalian cells. Specific strains of *D. discoideum* were created by selection and mutagenesis of the wild type strain NC4, to allow them to live off a synthetic, nutrient rich, liquid medium (Loomis, 1971; Sussman and Sussman, 1967; Watts and Ashworth, 1970). These cells, known as axenic cells, can be cultured aseptically to allow study in a sterile environment.

Dictyostelium is a eukaryotic organism therefore many of the mechanisms which occur within the cells are parallel to those in mammalian systems, making it an ideal model in which to study many aspects of cell function. Motility can be induced in cells by starvation or addition of cAMP (Gerisch and Wick, 1975) therefore cells are often used to investigate cell movement and chemotaxis (Devreotes and Zigmond, 1988). The actin cytoskeleton is also studied extensively in *Dictyostelium* (de Hostos et al., 1993; Niewohner et al., 1997), as well as phagocytosis (Vogel et al., 1980) and many other processes.

1.2 The Actin Cytoskeleton

1.2.1 Actin

Actin is one of the most highly conserved, abundant proteins in the eukaryotic kingdom (Korn, 1982; Vandekerckhove and Weber, 1978). It is essential for numerous processes such as cytokinesis (Maupin and Pollard, 1986), endocytosis (Taunton et al., 2000) and exocytosis (Muallem et al., 1995), transport of vesicles within cells (Hirschberg et al., 1998; Valderrama et al., 2001), pathogen invasion (Dramsi and Cossart, 1998) and cell motility (Cramer et al., 1994). There are several different isoforms of actin in mammals, with six different types known in humans classed into three groups (Vandekerckhove and Weber, 1978). α-actin is involved in muscle contraction; there are three isoforms specific for each of skeletal, smooth, and cardiac muscle. There are two γ-actin isoforms; γ2-actin is also involved in smooth muscle contraction, whereas γ1-actin and ß-actin are both cytoplasmic forms, which constitute the cell cytoskeleton. γ1-actin and ß-actin are ubiquitously expressed in non-muscle mammalian cells and are involved in numerous cellular processes (Herman, 1993; Khaitlina, 2001).

1.2.2 Polymerization and polarization

Actin exists in pools of monomers known as G-actin (globular actin), which have no known biological function in this form (Korn et al., 1987), but can polymerize to form filaments known as F-actin (filamentous actin; Rich and Estes, 1976). Actin dimers are relatively unstable, therefore for a trimer must form a nucleus from which polymerization can begin (Pollard, 1986). The elongation of actin filaments is dependent on local concentration of G-actin (Oosawa and Asakura, 1975; Korn, 1982). At a high concentration, G-actin monomers bind both ends of the filament, elongating it in both directions. At a low concentration, G-actin monomers dissociate from both ends. At equilibrium, when there are equal concentrations of G-actin and F-actin, a constant exchange would take place between monomers at the ends and the filaments would remain in a 'steady state', however polarization favours elongation at the 'plus' end of filaments. G-actin monomers are bound to adenosine triphosphate (ATP), and once the actin monomer is incorporated into a filament, this is irreversibly hydrolysed to ADP-P_i (Carlier and Pantaloni, 1988; Kelleher et al., 1995). This hydrolysis of ATP causes polarization of the filaments, leading to a bias in addition of ATP-

bound G-actin monomers at the 'plus' end of the filament, and depolymerization of ADP-bound monomers at the 'minus' end (Kelleher et al., 1995; Wegner, 1976; Bonder et al., 1983). Even polarized filaments can be elongated or depolymerized from both ends if the concentration of G-actin is high or low enough, therefore a critical concentration of monomers is required which ensures addition of monomers only at the plus end, and depolymerization only at the minus end (Coue et al., 1987). The elongation of filaments in one direction can result in a process known as 'treadmilling' (Wegner, 1976). This occurs at sites of dynamic activity such as the leading edge of cells, whereby the actin filaments are constantly turned over (fig. 1.1).

Actin filaments create a huge variety of different structures in cells, and their polarity can be exploited in order to create force. In general, filaments are preferentially created in cells in the direction of the plus end extending towards the plasma membrane (Small et al., 1978). Bundles and fibres can be formed by the creation of long, parallel filaments all polarized in the same direction, and these are involved in making finger-like protrusions at the cell membrane called filopodia (Goldman and Knipe, 1973; Small and Celis, 1978). A network of shorter, more branched filaments can make mesh structures such as lamellipodia in many cell types (Abercrombie et al., 1970), which drive cell movement at the leading edge of the cell by providing the forward force on the plasma membrane (Miyata et al., 1999). As well as providing force to extend the plasma membrane, actin is also involved in many intracellular processes, usually in the form of a coat or ring. Both endocytosis and exocytosis require actin, as do endosomal vesicles within the cytoplasm (discussed in detail later). These actin coats contribute to movement and scission of vesicles, and they also play an important role for trafficking of vesicles at the Golgi (Egea et al., 2006).

1.2.3 Actin Binding Proteins

Actin must be highly regulated in cells to ensure that filaments are appropriately polymerized at the correct location and form the correct structure. Regulation of filaments by actin binding proteins (ABPs) allows control over their length and rates of elongation or depolymerization. Regulation of available G-actin is also important. Sequestration is required in order to prevent spontaneous nucleation within the cell (Fechheimer and Zigmond, 1993; Hartwig and Kwiatkowski,

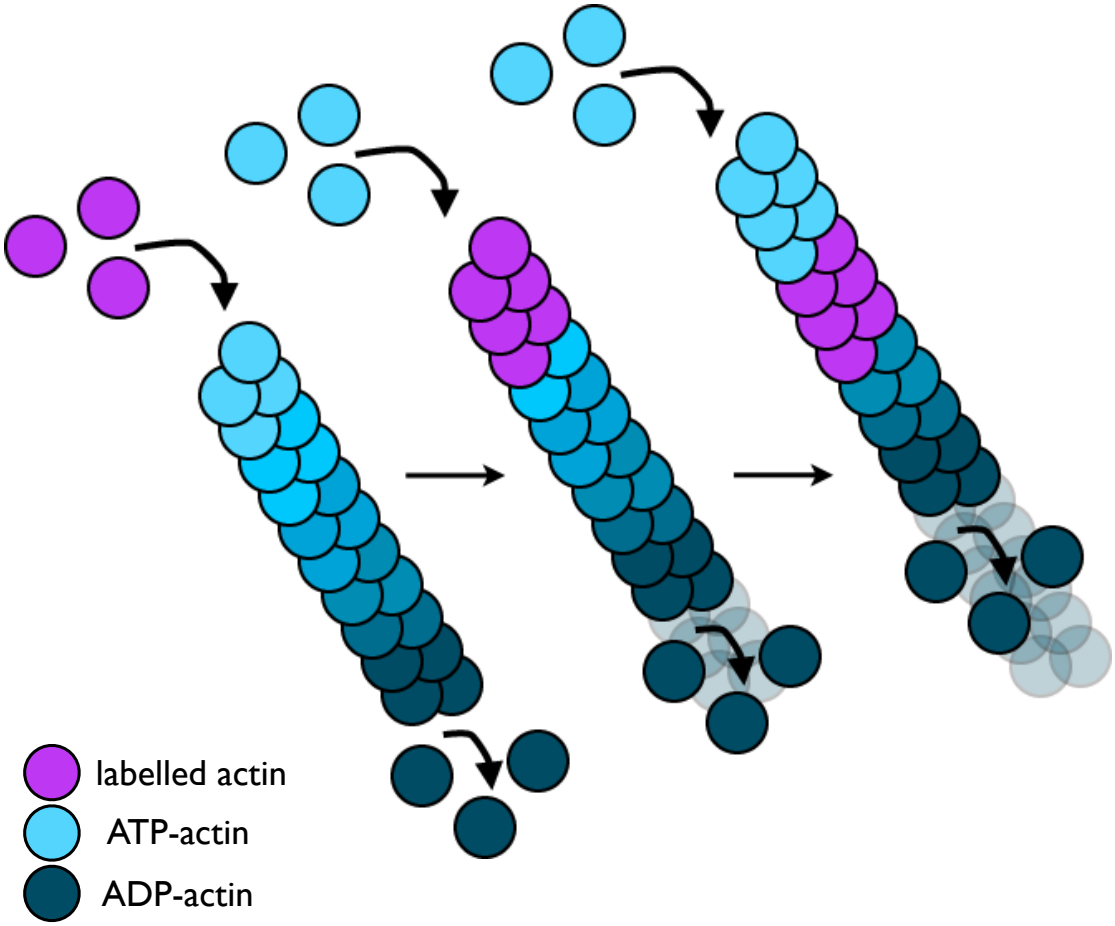

Figure 1.1 Actin treadmilling

ATP-G-actin monomers are added at the plus end of the filament, while ADP-actin is depolymerized from the minus end. This results in a 'treadmilling' action whereby the filaments grow in one direction.

1991). It is also important to control local concentrations of G-actin; structures such as lamellipodia have a filament turnover rate higher than that possible under normal physiological conditions (Wang, 1985). This is achieved by maintaining a high monomer concentration at the plus ends of filaments at the leading edge. Normally, this high concentration would prevent depolymerization from the minus end, therefore proteins which sequester monomers from the minus end to allow depolymerisation are vital.

Regulation of actin is achieved by an array of different ABPs which fall into three main groups; those that bind monomers of G-actin, those that bind the ends of filaments, and those that bind within actin filaments. A few examples of ABPs are discussed here, most of which are ubiquitous among eukaryotes and essential to the correct function and regulation of actin structures *in vivo*.

1.2.3.1 Capping proteins

Filament length can be regulated by proteins which cap the plus or minus ends. Capping of the end of a filament prevents both further elongation and dissociation of monomers (Casella et al., 1986). Capping of the plus end usually promotes disassembly of filaments, as fragmentation and depolymerization take place with no further elongation, whereas capping of the minus end, by proteins such as tropomodulin (Weber et al., 1994), is associated with stabilization of filaments by preventing depolymerization from the minus end while the filament continues to elongate at the plus end (Winder and Ayscough, 2005).

CapZ is the most abundant plus end capping protein in eukaryotes (Schafer et al., 1996). Originally identified in the Z-line of muscle, CapZ is a heterodimer which consists of an α- and ß- subunit (Casella et al., 1987). The *H. sapiens* genome contains four genes encoding the capping protein subunits; three genes encode CapZα1-3, and one gene encodes two different isoforms of CapZß (Cooper et al., 1991; Hartmann et al., 1990). Capping proteins are essential in regulation of filament elongation by restricting polymerization to selected plus ends, and this prevents the depletion of local G-actin (Pantaloni et al., 2001). For example, within lamellipodia, there is a highly branched network of short actin filaments and therefore potentially numerous free plus ends. Capping of filaments restricts the growing filaments to those at the very front of the leading

edge which allows for the high rate of turnover required for cell motility (Bailley et al., 1999). Filaments can also be uncapped to allow continuation of polymerization by a number of different methods. Severing of filaments is one way by which to free a plus end from a previously capped filament (see below). Filaments are also uncapped when they interact with specific polyphosphoinositides, for example actin filaments in platelets have been shown to be uncapped through an interaction with phosphatidylinositol 4,5-bisphosphate (PIP2; Hartwig et al., 1995).

Gelsolins are a family of proteins able to both cap plus ends and sever filaments (Cunningham et al., 1991), hence the name of the *D. discoideum* orthologue, severin (Robinson et al., 1999). Severing of filaments can result in a number of outcomes depending on its context. By severing a filament, gelsolin exposes a plus end for elongation, but severing can also contribute to disassembly of filaments (Hartwig et al., 1995; Sun et al., 1999). After severing a filament, gelsolin remains bound to the plus end as a cap, preventing the severed filaments from rejoining (Reichert et al., 1996).

1.2.3.2 ADF/Cofilin

One of the most studied families of depolymerizing factors is the ADF/cofilin group of proteins (Bamburg et al., 1980). This family incorporates a huge number of different proteins such as ADF (actin depolymerizing factor), cofilin, destrin, and coactosin. Only ADF and cofilin are found in vertebrates, and the major difference between the two are the tissues and stages of development at which they are expressed (Bamburg and Bray, 1987; Yonezawa et al., 1990).

ADF/cofilin plays a role in the disassembly of actin filaments (McGough et al., 1997). It binds to two actin monomers incorporated into a filament which causes the filament to twist. The strain put on the filament by this conformational change results in fragmentation. ADF/cofilin have a higher affinity for ADP-F-actin therefore promoting depolymerization towards the minus end of the filament (Carlier et al., 1997). Capping by gelsolin has been shown to alter the structure of filaments which enhances binding and depolymerization by ADF/cofilin (Ressad et al., 1998). In general, severing of capped filaments

results in their disassembly, whereas severing towards the minus end of a growing filament increases its turnover and treadmilling.

1.2.3.3 Monomer Binding Proteins

Profilins have a high affinity for actin monomers. They alter the conformation of monomers which opens the nucleotide binding cleft, and this enhances the exchange of ADP for ATP (Goldschmidt-Clermont et al., 1991; Pantaloni and Carlier 1993). Actin monomers have a higher affinity for ATP than ADP, and the high concentration of ATP in cell cytoplasm means exchange is likely to occur rapidly on free and profilin-bound ADP-G-actin (Wanger and Wegner, 1983).

B-thymosins, the most well known being thymosin ß4, are responsible for binding monomers to sequester the ADP-G-actin released from filaments. They do the opposite of profilin, by preventing the exchange of ADP for ATP in the bound monomer (Goldschmidt-Clermont et al., 1992). This contributes to preventing elongation from the minus ends of filaments, as well as spontaneous nucleation of actin within the cell. The affinity of thymosin ß4 for ATP-G-actin is actually much higher than that of ADP-G-actin (Carlier et al., 1993), therefore when localised at sites of polymerization, in concert with profilin it can actually work to enhance the exchange of ADP- for ATP-G-actin.

1.2.3.4 Other actin regulators

There are other proteins involved in the regulation of actin structures which work not to help extend or cap filaments, but to control the interaction between the filaments. Cross-linking proteins bundle filaments together or link them to create networks. Filamin forms a V-shaped dimer which loosely connects actin filaments to form a network (Gorlin et al., 1990; Weihing, 1988). It is required to stabilize networks such as those that form lamellipodia at the leading edge of cells and is essential for efficient cell movement. Fascin is another cross-linking protein which, as opposed to forming networks, packs filaments into tight bundles which can drive long, thin protrusions from the cell membrane involved in cell communication and movement (Otto, 1979). These cross-linking proteins often have the ability to orientate the filaments to ensure they are all parallel and polarized in the same direction (Loomis et al., 2003). This allows movement of motor proteins along the bundles, or ensures directional force for motility.

1.2.4 Myosin

Myosin proteins are involved in many cellular processes, such as motility, phagocytosis and cytokinesis, as well as muscle contraction in higher eukaryotes (Korn, 1978). Mammals have nine different classes of myosins encoded by 28 genes (Sellers, 2000). Myosin molecules are composed of a head, neck and tail region (Korn and Hammer, 1988). The head region of myosin has an ATP binding cleft and an actin binding site (Rayment et al., 1993). The tail region forms a coiled-coil structure which allows an interaction between myosin molecules to form dimers (Cheney and Mooseker, 1992). All myosins are composed of heavy and light chains. Myosin II is composed of two heavy chains, each consisting of a head and tail domain, and two light chains, which associate with the neck regions of the heavy chains (Korn and Hammer, 1988). Myosins are often regulated by phosphorylation of the heavy or light chains, but this varies greatly between types of myosin and species. Myosin II is regulated by light chain phosphorylation in vertebrates, which allows it to bind actin (Moussavi et al., 1993), and this is done by a number of kinases such as myosin light chain kinase (MLCK) and Rho-kinase (ROCK; Matsumura et al., 2001; Somlyo and Somlyo, 2003). In *Dictyostelium,* it is phosphorylation of the heavy chain that activates it for cytokinesis, rather than the light chain (De La Roche et al., 2002; Ostrow et al., 1994).

1.2.4.1 Myosin II

Myosin II is the only myosin which is able to assemble into filaments. It is found in muscle cells where bipolar myosin II filaments work in association with actin filaments to cause muscle contraction (Hartman and Spudich, 2012). During contraction, thick filaments composed of myosin II slide along thinner actin filaments, by repetitive interactions between the myosin head domains and the actin filament. ADP-myosin binds actin (Korn and Hammer, 1988; Warrick and Spudich, 1987). This ADP is then exchanged for ATP which causes a conformational change in the myosin molecule; the neck region rotates around the head region, detaching the head from the actin and moving it along the actin filament. This ATP is then hydrolysed to ADP and the head reattaches to the actin filament (Holmes, 1997; Houdusse et al., 1999). This cycle of binding

and release allows the myosin and actin filaments to slide past one another (Howard, 1997).

Myosin II is also important in non-muscle cells. It is involved in motility, allowing contraction of the rear of the cell in conjunction with the forward force created by actin at the leading edge (Iwadate and Yumura, 2008). It is also essential for cytokinesis (De Lozanne and Spudich, 1987; Mabuchi and Okuno, 1977) contributing to the formation of the actomyosin contractile ring required to separate the two daughter cells.

1.2.4.2 Myosin Motors

Other myosins are motor proteins, many of which form dimers. These motor myosins 'walk' along actin filaments using the same concept of binding and release of actin and ATP described above (De La Cruz and Ostap, 2004). Myosin V is a motor involved in trafficking of ER vesicles along actin filaments in cells towards the plus ends (Kuznetsov et al., 1994; Tabb et al., 1998). Myosins I and VI are also implicated in vesicle transport, from the Golgi to the plasma membrane (Buss et al., 1998; Fath et al., 1994; Montes de Oca et al., 1997). Myosin VI is thought to be specifically involved in transport to the minus ends of actin filaments, unlike most other myosin motors (Wells et al., 1999).

Myosin I does not form dimers but consists of a single head domain and a short tail (Korn et al., 1987; Maruta et al., 1979; Pollard and Korn, 1973). It encompasses the largest class of myosin motors involved in a huge range of cellular functions. For example, *Dictyostelium* myosin I is required for correct spindle formation during cytokinesis (Rump et al., 2011). Myosin I is also thought to be the main myosin motor associated with the endocytic pathway (Raposo et al., 1999).

1.3 Actin and the Endocytic Cycle

1.3.1 Types of endocytosis

There are several ways in which a cell can take up external constituents, including phagocytosis, macropinocytosis and micropinocytosis. While macropinocytosis is specifically for the bulk uptake of external fluid (Hacker et al., 1997), the role of micropinocytosis is to recycle membrane, and membrane

receptors. Micropinosomes (pinosomes smaller than 0.2μm diameter) have a very large surface area to volume ratio in order to maximize the amount of membrane (Swanson and Watts, 1995), whereas the opposite is true for macropinosomes (Racoosin and Swanson, 1992). Phagocytosis differs from pinocytosis because its purpose is to specifically engulf an object (Allen and Aderem, 1996). Phagocytic protrusions are guided protrusions which sense and reach around the edges of the object to be consumed, whereas for macropinosomes the membrane is randomly protruded in order to form a pinocytic cup, which then closes to form an internalized vesicle.

Many mammalian cell types use macropinocytosis, but those that constitutively perform macropinocytosis are cells such as dendritic cells and macrophages. These cells use macropinocytosis to sample their environment for antigens (Norbury, 2006) which, if detected, are then displayed for recognition by cytotoxic T cells (Sallusto et al., 1995). Many cell types can be induced to perform macropinocytosis by addition of growth factors. For example, epithelial cells can be stimulated to increase their rates of macropinocytosis with EGF (Epithelial Growth Factor; Sandvig and van Deurs, 1990). As described earlier, wild type strains of *Dictyostelium* survive by phagocytosing and digesting bacteria found in the soil, however laboratory strains known as axenic strains survive solely off a diet of liquid medium which they take up by macropinocytosis.

1.3.2 Mechanisms of Endocytosis

The process of endocytosis, both for particle (Maniak et al., 1995) and fluid uptake (Hacker et al., 1997), is highly regulated by many different pathways in metazoans, however the involvement of actin is universal in eukaryotes and this is discussed here. Endocytosis can be clathrin-dependent or –independent. The clathrin-dependent pathway is mostly important in the uptake of external ligands through membrane receptors. Clathrin coated pits are sites of endocytosis which form almost uniformly over the cell surface and are limited in size by the clathrin coat itself (Bretscher and Thomson, 1983). Clathrin-independent endocytosis occurs more randomly across the cell surface and there is no coat to restrict its size. Macropinocytosis by axenic *Dictyostelium* cells is

clathrin-independent, although *Dictyostelium* cells are also able to perform clathrin-mediated endocytosis (Hacker et al., 1997).

The involvement of actin in endocytosis depends not only on cell type, but also on the form of endocytosis taking place. The process of macropinocytosis requires actin filaments in order to create the protruding arms of membrane which close over to form a macropinosome (Racoosin and Swanson, 1992). In yeast, actin is not required for the initial recruitment of endocytic proteins to sites of endocytosis, such as adaptor proteins and actin nucleating machinery (Kaksonen et al., 2003). In contrast, in mammalian cells actin is required for the initial formation of clathrin-coated pits (Yarar et al., 2005). For clathrin-mediated endocytosis, actin has been shown to be essential for membrane invagination in all cells, however studies differ in concluding whether actin is responsible for creating the invaginations directly, or rather just acts to regulate the invagination by acting as a barrier (Rocca et al., 2008; Yarar et al., 2005). This is possibly dependent on cell type and context. During the process of invagination, the actin machinery remains at the plasma membrane and does not move down into the cell with the incoming vesicle (Kaksonen et al., 2003).

For most forms of endocytosis, actin plays a role in scission of the newly formed vesicle into the cytosol. Polymerization and contraction of a ring of actin at the neck of the budding vesicle occurs at clathrin-coated pits, where the actin works in concert with dynamin to resolve the vesicle form the plasma membrane (Takenawa and Suetsugu, 2007).

After endocytosis is complete, these actin structures dissociate from the vesicle within a minute (Konzok et al., 1999; Lee and Knecht, 2002; Maniak et al., 1995), however actin continues to be important in vesicle progression along the entire endocytic pathway and is recruited for several other functions during this transit. One of these is vesicle movement within the cytosol. There is evidence that after vesicle formation, actin comets then project the vesicle away from the cortex directly after being endocytosed (Clarke and Maddera, 2006; Lu and Clarke, 2005), and also propel the vesicle through the cell (Rauchenberger et al., 1997). Some pathogens such as *Listeria monocytogenes* high-jack the host actin machinery in order to propel them through the cytosol (Brieher et al.,

2004; Cameron et al., 2001; Theriot et al., 1992; Welch et al., 1997). In this case, the actin polymerization is restricted to the region of contact between the bacterium and the actin filaments, and is kept spatially separate from the remainder of the filaments where rapid depolymerisation occurs. This ensures a highly dynamic turnover of the actin filaments to maximize the rate of propulsion.

1.3.3 The Endocytic Pathways in Metazoans

The pathways a vesicle can follow in mammalian cells vary greatly, dependent on the type of cargo within the vesicle, and the cell type. The different possible routes are summarised in figure 1.2. The different compartments of the mammalian endosomal system can be identified by known markers, predominantly Rabs, a family of small GTPases, which coordinate much of the transit of vesicles. Rab4 is an early endosomal marker (van der Sluijs et al., 1991), whereas Rab7 associates with late endosomes (Chavrier et al., 1990). These associations are also conserved in *Dictyostelium* (Buczynski et al., 1997; Bush et al., 1994). Receptor-ligand complexes are often dissociated in the early or sorting endosome, and the receptor can then be recycled back to the plasma membrane whereas the ligand may follow a different route such as the degradative pathway into lysosomes.

1.3.4 The Endocytic Pathway in Dictyostelium

1.3.4.1 Early Endosomal Events

Axenic *Dictyostelium* cells use constitutive macropinocytosis to take up liquid media for nutrients (Hacker et al., 1997). After the initial uptake of a vesicle for either macropinocytosis or phagocytosis by the processes outlined above, acidification occurs within one minute (Hacker et al., 1997). This is achieved by addition of V-ATPase complexes to the membrane of the vesicle by fusion with a number of smaller, V-ATPase decorated vesicles (Clarke et al., 2002b; Hacker et al., 1997). Early endosomes undergo a number of fusion and fission events shortly after formation. Their shape becomes more elongated and tubular, typical of the endolysosomal networks in both *Dictyostelium* and mammalian cells (Clarke et al., 2002). Early endosomes fuse with other endosomes at the same stage of the cycle, which contributes to the addition of V-ATPase on the membrane, as well as beginning to concentrate and sort endosomal contents.

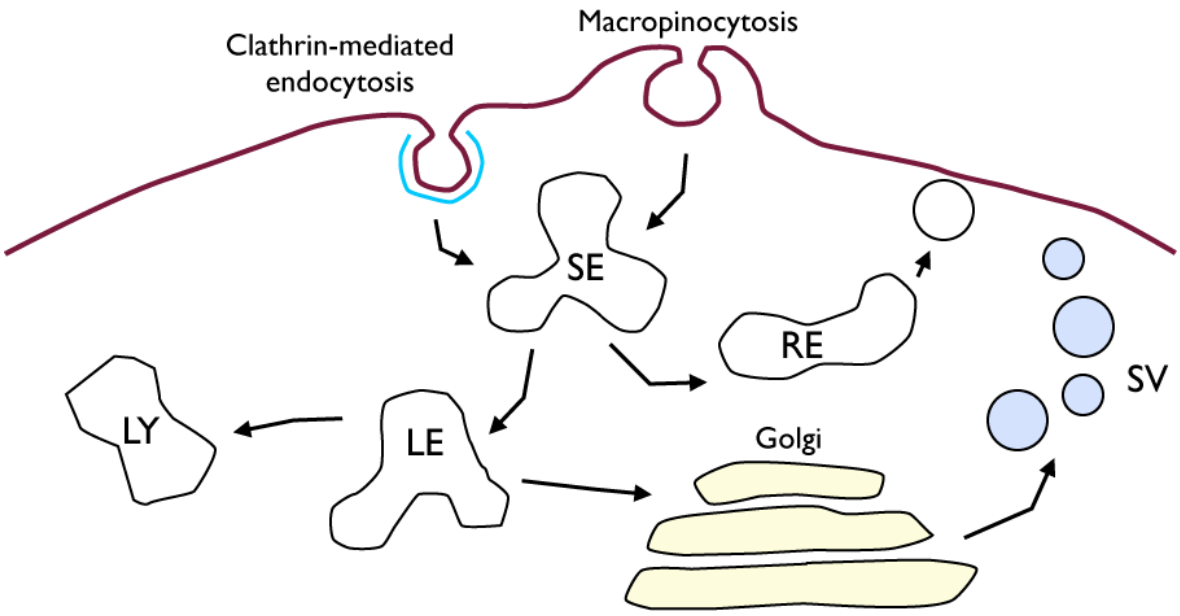

Figure 1.2 Endocytic routes in mammalian cells

Mammalian cells can take up membrane, receptors and external constituents by numerous different methods, two of the major mechanisms being clathrin-mediated endocytosis and macropinocytosis. After uptake, the cargo can follow a number of routes. Initially, cargo is sorted in the sorting/early endosome. From here, cargo such as receptors can be recycled to the cell surface via recycling endosome, whilst others can be transported through the degradative pathway, through the late endosome and into lysosomes. Some cargo follows the retrograde pathway, being trafficked to the Golgi body.

Figure derived from Marsh and Helenius, 2006.

1.3.4.2 Acidification and V-ATPase

V-ATPase (vacuolar-ATPase) is a cellular proton pump, specific to vacuolar compartments. In *Dictyostelium*, it is found on the membranes of endosomal vesicles (Adessi et al., 1995) and the contractile vacuole (Fok et al., 1993), an organelle responsible for regulation of cellular osmotic pressure (Heuser et al., 1993). Like other proton pumps, it uses the energy from the hydrolysis of ATP to actively pump protons across a membrane. These H^+ ions lower the pH of the lumen of vesicles, important for a number of purposes; dissociation of receptor-ligand complexes for receptor recycling (Geuze et al., 1983) and providing an optimum environment for the function of digestive enzymes (Kakinuma et al., 1981) are two examples of requirements for an acidic environment. The V-ATPase complex consists of two functional parts, the V_1 and V_0 domains, composed of eight and six different subunits, respectively. The V_1 domain binds and hydrolyses ATP (Arai et al., 1988; Ohira et al., 2006), while the V_0 domain spans the membrane and transports the proton across it (Arai et al., 1988; Hirata et al., 2003; Powell et al., 2000).

The addition of V-ATPase to newly formed endosomes results in a drop in pH to approximately 4.6 (Aubry et al., 1993). At these early stages, endosomal vesicles often also undergo homotypic fusion, seen by the mixing of fluorescent markers taken up by cells (Clarke et al., 2002). This fusion is regulated to ensure earlier compartments do not inappropriately fuse with later compartments, and this is controlled by proteins such as LvsB and vacuolin B, both negative regulators of vesicle fusion. Mutants lacking LvsB have been shown to develop enlarged, acidic endosomal compartments through excessive fusion, and this is related to the Chediak-Higashi syndrome in humans (Barbosa et al., 1996; Harris et al., 2002). Vacuolins A and B both mark late endosomes, and although loss of vacuolin A seems not to affect cells, the loss of vacuolin B results in a delay in exocytosis and enlargement of post-lysosomal compartments (Jenne et al., 1998).

1.3.4.3 Endosomal Progression

The acidic endosomes, known as lysosomes, remain acidic for approximately 30 minutes, during which time nutrients and fluid are extracted, and the contents become concentrated (Clarke et al., 2002; Neuhaus et al., 2002). Once the

vesicle is ready to mature, the V-ATPase molecules are removed from the vesicle membrane, allowing an efflux of H+ ions and neutralization of the lumen (Nolta et al., 1994; Rauchenberger et al., 1997). The vesicle is then transported back to the plasma membrane where the indigestible contents are expelled by exocytosis, a total of 1-2 hours after initial uptake (Clarke et al., 2002).

During progression through the endocytic cycle, actin coats have been observed on vesicles (Drengk et al., 2003; Insall et al., 2001; Maniak et al., 1995). Lee and Knecht (2002) reported seeing a weak actin coat which persisted on post-lysosomal vesicles. These intermediate actin coats have been suggested to play a role in transport of vesicles through the cell or control of fusion between different populations, keeping them spatially separate from one another (Lee and Knecht, 2002; Maniak, et al., 1995). This actin coat was shown to be insufficient for exocytosis (Lee and Knecth, 2002) for which further actin polymerization was required. Rauchenberger et al. (1997) also showed that this earlier actin coat must first dissociate from the vesicle to allow fusion with the plasma membrane for exocytosis to occur. This indicates that the vesicular actin coat seen on vesicles at this stage is separate from that seen on vesicles at exocytosis, described below. It has now been shown that these intermediate actin coats have a different purpose and are the product of WASH activation of the Arp2/3 complex, described in detail later.

1.3.5 Mechanisms of Exocytosis

In all cell types, actin plays an essential role in exocytosis. The first evidence of the involvement of actin in exocytosis was the build up of secretory vesicles in yeast cells with mutated actin (Novick and Botstein, 1985).

In mammals, cells such as immune cells and neurons perform exocytosis, and can do so by a 'kiss-and-run' method, where the intracellular vesicle briefly docks with the plasma membrane, releases its contents to the exterior, and then detaches from the membrane (Valtorta et al., 2001). Actin coating of the vesicular membrane in this case is often associated with stabilising the vesicle, to allow diffusion of the contents to the exterior. These cells can also perform exocytosis by complete vesicular fusion, where the vesicle membrane fuses with, and becomes incorporated into, the plasma membrane on release of its

contents. In this instance, the actin coat is often associated with myosin and uses a contracting force to expel the contents of the vesicle.

Dictyostelium uses the complete fusion mechanism to excrete indigestible contents from vesicles (Maniak, 2003). Actin strongly localises to vesicles as they dock with the plasma membrane, directly before the release of their contents (Lee and Knecht, 2002). The actin coat squeezes the vesicle, rapidly reducing its volume and forcing it into the plasma membrane. It quickly dissipates within a minute of release of the vesicular contents (Lee and Knecht, 2002).

1.4 Nucleation Promoting Factors

1.4.1 Actin Filament Nucleation and the Arp2/3 Complex

The initiation of polymerization from a pool of G-actin monomers is known as nucleation. This can either be *de novo*, or after a free plus end has been created by severing an existing filament (Condeelis, 1993, Zigmond, 1996). Nucleation requires the initial formation of a trimer of G-actin monomers (Pollard, 1986). Two of the major components which are able to nucleate filaments are the Arp2/3 complex and formins.

The Arp2/3 complex is the major nucleation machinery in many cells; it is a seven subunit complex consisting of Arp2, Arp3 and ARPC1-5, all of which are highly conserved throughout the eukaryotic kingdom (Machesky et al., 1994; Mullins et al., 1997). When creating branched filaments, it binds existing actin filaments and is able to initiate polymerization of a branch at an angle of 70^{o} to the mother filament (Mullins et al., 1998); this is known as the dendritic model of actin filament formation. It is thought that ARPC2 and ARPC4 are important in binding the mother filament (Rouiller, 2008) while Arp2 and Arp3 mimic an actin dimer (Kelleher et al., 1995; Machesky et al., 1994; Mullins et al., 1997). To begin nucleation, the addition of a 'third' G-actin monomer is required to form a trimer. Arp2/3 alone is inefficient at binding the monomer, as the Arp2 and Arp3 subunits are not suitably aligned, however nucleation promoting factors (NPFs) such as Wiskott Aldrich Syndrome Protein (WASP) family proteins bind the Arp2/3 complex and cause a conformational change, bringing Arp2 and Arp3 into close proximity, and also bringing them into contact with an actin

monomer to form the trimer required to initiate nucleation (Robinson et al., 2001).

Other proteins can also nucleate actin filaments, such as Spire (Quinlan et al., 2005), Cordon-bleu (Cobl; Ahuja et al., 2007) and formins (see below). Spire and Cobl contain multiple WASP homology 2 domains (WH2; discussed in detail later; Ahuja et al., 2007; Otto et al., 2000; Wellington et al., 1999), which bind multiple actin monomers and allow these proteins to initiate *de novo* actin filament nucleation (Ahuja et al., 2007; Quinlan et al., 2005). Spire is also able to sever and cap filaments, and sequester actin monomers, making it a multi-tasking regulator of actin structures (Bosch et al., 2007).

Formins work as dimers (Moseley et al., 2004) which nucleate and elongate actin filaments (Pruyne et al., 2002). Unlike the Arp2/3 complex which remains at the base of the new filament, formins travel with the extending end (Campellone and Welch, 2010; Chesarone et al., 2010). They are defined by the presence of formin homology (FH) 1 and 2 domains (Castrillon and Wasserman, 1994). The FH2 domain binds the plus ends of actin filaments to promote elongation (Pruyne et al., 2002; Sagot et al., 2002) whilst the FH1 domain acts by increasing the amount of local G-actin monomers to stimulate elongation (Gould et al., 2011). It is also suggested that profilin binds the FH1 domain of formins, in order to deliver the actin monomer efficiently to extending filaments (Pring et al., 2002). The filaments constructed by formins are involved in a number of cellular processes such as the contractile ring required for cytokinesis, filopodia and actin stress fibres (Faix and Grosse, 2006; Goode and Eck, 2007; Kovar, 2006). Formins also nucleate actin along the endocytic pathway; RhoB has been shown to activate formin Dia1 to create actin coats around vesicles before they line up along peripheral stress fibres (Fernandez-Borja et al., 2005). Spire1 has also been implicated in endosome biogenesis; it was shown to be essential for maturation of early endosomes (Morel et al., 2009).

1.4.2 WASP Family Proteins

There are two types of nucleation promoting factors (NPFs); class I and class II. Class I NPFs function by inducing a conformational change in Arp2/3 and

presenting an actin monomer to trigger nucleation (Robinson et al., 2001). They are distinguished by their ability to bind both Arp2/3 and G-actin monomers through their VCA domain (Machesky et al., 1999; Marchand, 2001; Miki and Takenawa, 1998). Class II NPFs, such as *Saccharomyces cerevisiae* actin-binding protein-1 (Abp1), function by stabilising the interaction between Arp2/3 and F-actin filaments, but cannot bind G-actin and are subsequently less potent activators of Arp2/3 (Goode et al., 2001).

WASP family proteins are class I NPFs. These all contain the characteristic VCA domain for binding Arp2/3 and actin. The V (verprolin homology) domain, was originally named after its homology to part of the *S. cerevisiae* actin cytoskeletal protein verprolin (Symons et al, 1996). It is now more commonly known as the WASP homology 2 (WH2) domain. This region forms an amphipathic α-helix which binds directly to actin (Marchand, 2001; Miki and Takenawa, 1998). The A region is a stretch of acidic residues which nearly always contains a conserved tryptophan, and this region binds Arp2 (Machesky et al., 1999; Marchand et al., 2001; Rebowski, 2008; Rohatgi et al., 1999). The C region was originally known as the cofilin homology region, however it bears little resemblance to cofilin and is now known as the connecting or central region (Marchand et al., 2001). It increases the binding affinity of the VCA domain for both actin and Arp2/3.

The first identified class I NPF was ActA, a protein from *L. monocytogenes* (Kocks et al., 1992). This pathogen uses ActA to activate the host cell Arp2/3 complex for invasion and propulsion in the cell (Welch et al., 1998). Since then, endogenous activators of Arp2/3 have been identified in eukaryotic cells, the first being WASP (Wiskott Aldrich Syndrome Protein; Winter et al., 1999; Yarar et al., 1999). Since the discovery of WASP, several other related class I NPFs have been discovered in eukaryotes and are now known as subclasses of the WASP family of proteins. These are; Scar/WAVE, WASH and WHAMM, all discussed in detail below.

Although the WASP family proteins are defined by their conserved C terminus, the N terminal regions of each subclass are extremely variable and account for the different actin structures they direct to be built and the different roles they play in cells (fig. 1.3). WASP homologues are identified by the WASP homology 1

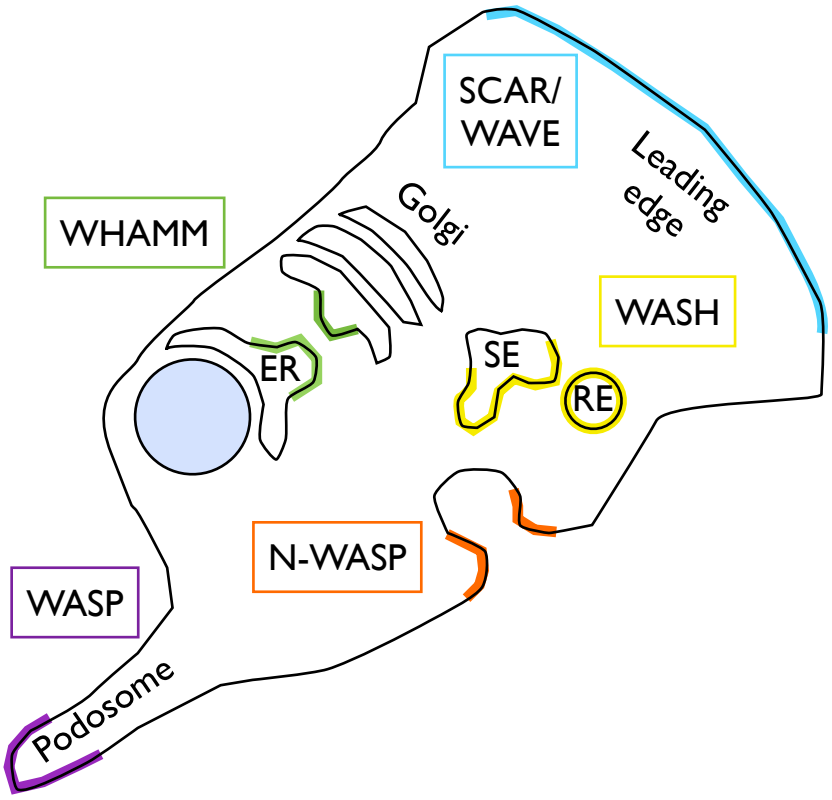

Figure 1.3 Functions of WASP family proteins

The different WASP family proteins localise to different regions of the cell. Here they activate the Arp2/3 complex to create a variety of different actin structures. At the leading edge, Scar/WAVE is responsible for creating a mesh network of actin filaments. WHAMM and WASH both create vesicular actin coats to regulate vesicle trafficking between the Golgi and ER, and through the endosomal networks, respectively. WASP and N-WASP are also responsible for the creation of actin coats around vesicles for endocytosis, and also for bundled filaments within podosomes.

(WH1) domain in the N terminal portion of the protein and a GTP-ase binding domain (GBD; Veltman and Insall, 2010). Scar/WAVE proteins similarly contain a Scar homology domain (SHD), and WASH proteins contain two WASH homology domains, WHD1 and WHD2 (Veltman and Insall, 2010). These different domains are illustrated in figure 1.4.

WASP is expressed in hematopoietic stem cells (Derry et al., 1994), although neuronal-WASP (N-WASP) is ubiquitously expressed (Miki et al., 1996). They both play a role in formation of structures such as podosomes, and in clathrin-mediated endocytosis (Campellone and Welch; 2010 Merrifield, 2004). N-WASP is also involved in endosomal rocketing (Benesch et al., 2002; Rozelle et al., 2000; Taunton et al., 2000). Pathogens *Listeria* (Tilney et al., 1992) and *Shigella* use Arp2/3 and N-WASP to propel themselves into and through cells which they invade using their own NPF, such as *Listeria* ActA (Suzuki et al., 1998, Welch et al., 1997).

WASP was discovered as the cause of Wiskott Aldrich syndrome (Derry et al., 1994). Mutations in the WAS gene encoding WASP cause defects in many cellular processes in immune cells such as proliferation, activation and motility (Dupre et al., 2002). This is an X-linked disease, characterized by thrombocytopenia, eczema and immunodeficiency (Aldrich et al., 1954), and sufferers are at higher risk of developing lymphoma or leukaemia.

The next member of the WASP family to be discovered was Scar. This protein was first identified in *Dictyostelium* as Suppressor of Cyclic AMP Receptor (Bear et al., 1998). The mammalian orthologues, of which there are three, are known as WAVE1-3 (WASP family verprolin homologous protein; Machesky and Insall, 1998; Miki et al., 1998). Scar/WAVE is found mainly at the leading edge of cells, and builds the actin network required to form lamellipodia (Hanhe et al., 2001; Stradal et al., 2001). Scar/WAVE is contained within a highly conserved pentameric complex consisting of subunits SRA1, HSPC300, NAP1 and ABI (Eden et al., 2002). IRSp53 also binds the proline-rich region of Scar/WAVE and enhances the association with active Rac1, an activator of the Scar/WAVE complex (Eden et al., 2002; Ismail et al., 2009; Miki et al., 2000).

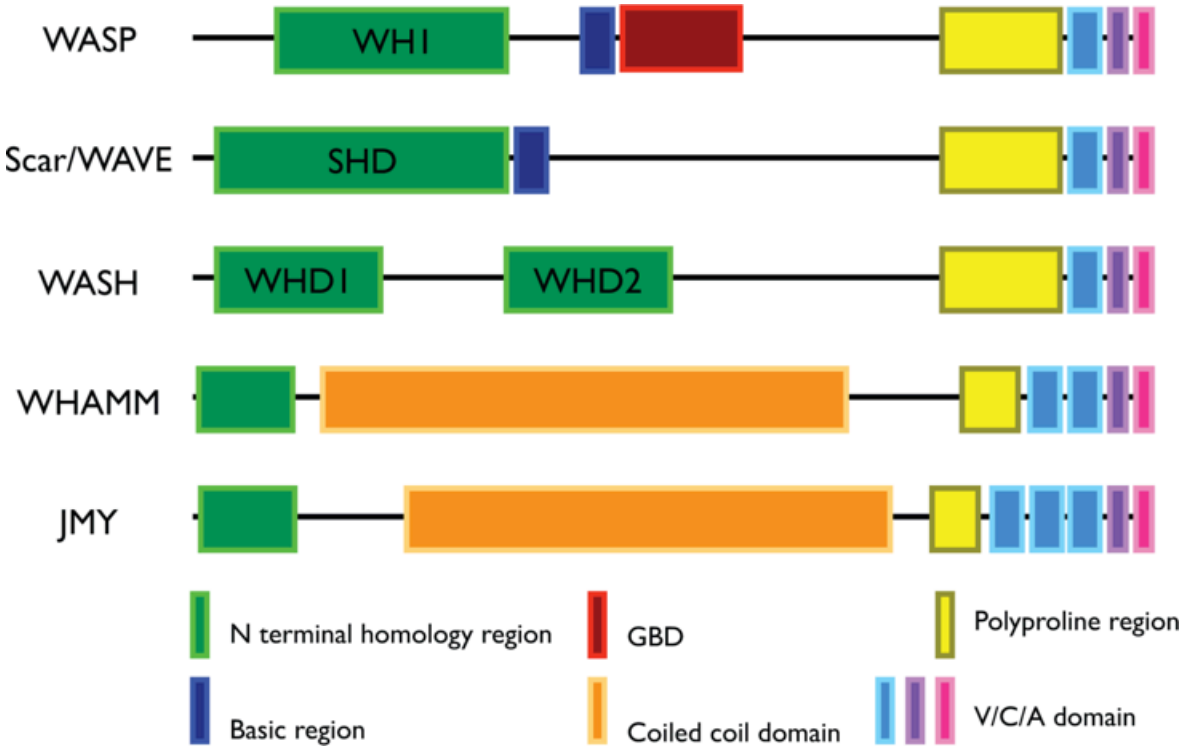

Figure 1.4 Homology and domains of nucleation promoting factors
All WASP family proteins contain a VCA domain, consisting of a verprolin homology domain (V), which binds actin monomers, a connecting or central domain (C), and an acidic domain (A) which binds the Arp2/3 complex. The distinguishing features of each group of WASP family proteins reside in the N terminal portion of the protein. For example, all WASP proteins contain a WASP Homology 1 (WH1) domain, followed by a basic region and a GTPase Binding Domain (GBD) to which Cdc42 binds and activates the protein. Scar/WAVE proteins contain the Scar Homology Domain (SHD), and a basic region. WASH proteins contain WASH Homology Domain 1 (WHD1) and WHD2, to which microtubules are known to bind.

Figure derived from Veltman and Insall, 2010; Carnell et al., 2011.

WHAMM (WASP homologue associated with actin, membranes, and microtubules) and JMY (junction-mediating and regulatory protein) are both only found in vertebrates (Veltman and Insall, 2010), however some invertebrates contain a single orthologue which is a close match for the two mammalian counterparts. This is seen in *Drosophila* which has a single protein, Whamy, however its VCA domain does not contain the essential tryptophan found in mammalian WHAMM, but has a phenylalanine in its place suggesting it may not be able to activate Arp2/3 (Campellone et al., 2008; Veltman and Insall, 2010). WHAMM localizes with the cis-Golgi and is important in anterograde trafficking from the ER, and is required to maintain Golgi shape and structure (Campellone et al., 2008). It interacts with actin and microtubules to allow membrane tubulation and elongation.

JMY was identified as a transcriptional coactivator of p53 (Shikama et al., 1999) to help control cell adhesion through cadherin expression. Unlike most other WASP proteins, it has 3 WH2 domains which gives it the ability to bind numerous G-actin monomers (Zuchero et al., 2009). This means it has intrinsic actin nucleating activity and forms unbranched filaments through this method. It can also bind and activate the Arp2/3 complex, which gives rise to the normal branched filament structure produced by Arp2/3. Originally thought to be exclusively nuclear, it appears that the localisation of JMY may vary greatly with cell motility. In mouse embryonic fibroblasts, it was seen to be mainly nuclear, whereas in highly motile cells such as neutrophils, it was seen to colocalise with actin filaments at the leading edge (Zuchero et al., 2009).

1.4.3 Discovery of WASH

Linardopoulou et al. (2007) discovered WASH using genome analysis techniques. It had not previously been identified because the gene is located in the subtelomeric regions of human DNA which are dynamic regions of DNA breaks and repair (Matise et al., 2003; Rudd et al., 2007). This makes them more difficult to sequence, and subsequently they are not as well characterized as the majority of coding regions in the human genome. Linardopoulou et al. (2007) identified WASH as a WASP family member by the presence of the VCA domain at the C terminus. Phylogenetic analysis revealed it appeared to represent a new

subclass of the WASP family, with the identification of two WASH homology domains (WHD1 and WHD2).

WASH is very highly conserved throughout the eukaryotic kingdom, with most organisms having a single copy of the WASH gene in their genome (including *D. discoideum*), and the only group seeming to lack WASH is fungi (Veltman and Insall, 2010). The human genome, however, has seven copies of the gene, although only one appears to code for a functional, full length WASH protein. This protein, WASH1, is expressed in most tissues, especially blood and brain (Linardopoulou et al., 2007). Disruption of the *Drosophila* gene, washout, showed it is essential for development, as no advance was possible from the larval stage to the prepupal stage in the absence of WASH (Linardopoulou et al., 2007). The ability to progress to the larval stage was likely due to stores of maternal mRNA. Further studies have since attempted to create mouse knockouts of WASH, which also showed loss of WASH is embryonic lethal in mammalian systems (Gomez et al., 2012).

1.4.4 Regulation of Nucleation Promoting Factors

There are different mechanisms controlling each of the NPFs, involving both activation and inhibition. Both WASP and N-WASP have autoinhibitory mechanisms whereby the GBD binds the acidic region of the VCA (Kim et al., 2000; Miki et al., 1998; Prehoda et al., 2000). This conformation prevents the VCA domain from interacting with Arp2/3 or actin. WASP interacting protein (WIP) stabilizes this binding to ensure inactivation of the proteins (Ramesh and Geha, 2009). This inhibition is released upon binding of Cdc42, which activates it by releasing the VCA domain and exposing it to bind actin and Arp2/3 (Martinez-Quiles et al., 2001; Rohatgi et al., 1999; Stradal et al., 2004; Takenawa and Suetsugu, 2007).

Whereas WASP and N-WASP are constitutively inactive due to autoinhibition, Scar/WAVE and WASH are constitutively active alone (Eden et al., 2002; Innocenti et al., 2004; Linardopoulou et al., 2007; Machesky et al., 1999). These NPFs are incorporated into complexes which prevent any inappropriate nucleating activity until activation (Derivery et al., 2009b; Ismail et al., 2009 Jia et al., 2010). Some NPFs are activated by small GTPases; Rac1 is responsible for

the activation of the Scar/WAVE complex (Eden et al., 2002; Ismail et al., 2009; Miki et al., 2000), while Cdc42 activates WASP.

1.4.5 Regulation of WASH

The regulation of WASH is through incorporation into a complex (Derivery et al., 2009; Gomez and Billadeau, 2009). There are parallels between the WASH complex and the Scar/WAVE complex (Jia et al., 2010), showing that this form of regulation is not a unique mechanism. WASH, like Scar/WAVE, is part of a pentameric complex consisting of FAM21, Strumpellin, CCDC53 and SWIP, with a more transient interaction with CapZ (Derivery et al., 2009; Gomez and Billadeau, 2009; Jia et al., 2010). Originally, the WASH complex was purified using Tandem Affinity Purification (TAP) from 3T3 fibroblasts, and the subunits were identified by mass spectrometry (Derivery et al., 2009). These results were confirmed using WASH purified from HeLa cells and bovine brain tissue, where the question was raised over the strength of the interaction between the WASH complex and CapZ (discussed later; Jia et al., 2010).

There are many similarities between the Scar/WAVE complex and the WASH complex, with both containing one NPF and four regulatory subunits. There are also parallels between the regulatory subunits themselves (Jia et al., 2010), for example SWIP and Strumpellin, like SRA1 and NAP1 of the Scar/WAVE complex, are both large proteins of predicted helical structure. SWIP appears to have some homology to SRA1, and Strumpellin to NAP1. Also, both CCDC53 and HSPC300 contain coiled-coil domains and are of a smaller size (Jia et al., 2010; Lupas et al., 1991).

Liu et al. (2009) suggest that a potential activator of WASH could be the small GTPase Rho1. Their studies in *Drosophila* show that WASH directly binds Rho1, and is released from inhibition by SpirD by the addition of Rho1, in the same way as the actin nucleators SpirC and Cappuccino (Capu). These studies have not been confirmed in mammalian cells where the case may be different, as is seen with other NPFs in *Drosophila* such as WASP and Capu, which both lack the autoinhibitory mechanisms seen in their mammalian counterparts (Liu et al., 2009; Rosales-Nieves et al., 2006). It is also generally thought that Rho1 is a regulator of linear actin nucleators, such as Capu (Rosales-Nieves et al., 2006),

rather than branched actin nucleators like WASP family proteins, however it may have the capacity to do both.

1.5 The WASH complex

1.5.1 Localisation of WASH

The first studies of GFP-WASH in mammalian cells claimed WASH was localised to actin structures at the cortex (Linardopoulou et al., 2007). Subsequent findings suggested that this was an artefact of overexpression and that WASH is not seen at the cortex, but instead localises to intracellular vesicles. In mammalian cells, WASH colocalised strongly with EEA1, a marker of early endosomes, and Rab5 which labels newly formed endosomes (Derivery et al., 2009; Gomez and Billadeau, 2009). WASH also colocalised with Rab11 and Rab4, markers of the recycling compartment where it seems to be predominantly present, although it was also observed on the lysosomal degradation pathway marked by Rab7 (Zech et al., 2011). Data also show that WASH is localised to endocytic vesicles in *Dictyostelium*, specifically neutralising lysosomes (Carnell et al., 2011).

1.5.2 WASH and Endosomal Actin

WASH was identified by Linardopoulou et al. (2007) as an activator of Arp2/3 due the presence of a VCA domain at its C terminus. This was confirmed by pyrene-actin polymerization assays in which actin monomers were shown to form filaments *in vitro* in the presence of either full length WASH, or the WASH VCA domain, with Arp2/3 (Jia et al., 2010; Linardopoulou et al., 2007; Liu et al., 2009). *In vivo*, several groups have observed colocalisation between WASH and actin on endosomal vesicles in both mammalian cells and *Dictyostelium* (Carnell et al., 2011; Derivery et al., 2009; Duleh and Welch, 2010; Gomez and Billadeau, 2009).

To establish the role of WASH in mammalian cells, several groups performed siRNA targetted knockdown of WASH which resulted in tubulation of early endosomes. This led to the idea that WASH was involved in maintaining endosomal morphology and regulating scission events (Derivery et al., 2009; Gomez and Billadeau, 2009). WASH function was also studied in *Dictyostelium*, in which a total knockout of WASH was created (Carnell et al., 2011). The actin structures previously observed on endolysosomal vesicles in *Dictyostelium* were

absent in WASH nulls. The loss of WASH and actin did not result in an obvious change in morphology of endosomes, but in a failure of lysosomes to neutralise and a total block in exocytosis. Recently, a line of WASH knockout mouse embryonic fibroblasts (WASHout MEFs) was created by Gomez et al. (2012). These cells did not display the tubulated phenotype previously seen in mammalian cells on knockdown of WASH, but instead the early endosomal and lysosomal compartments of cells were severely disrupted. The actin normally associated with these vesicles was totally absent, but was rescued on expression of GFP-WASH but not GFP-WASHΔVCA, indicating that WASH is required to produce these actin structures and maintain integrity of the endolysosomal network in mammalian cells. This data correlates with that obtained in the *Dictyostelium* WASH null cells.

1.5.3 WASH in Mammalian Cells

1.5.3.1 WASH and Receptor Recycling

WASH is important for trafficking of receptors in mammalian cells, causing defects in the transport of several cargoes when WASH is depleted. Two groups looked at Transferrin (Tf) recycling in HeLa cells which was seen to be defective in the absence of WASH (Derivery et al., 2009; Zech et al., 2011), although another two groups report that Tf recycling is unaffected by WASH (Duleh and Welch, 2010; Gomez and Billadeau, 2009), however these studies used different cell types and assays to measure the recycling. WASH knockdown also resulted in defects in EGFR and α5β1 integrin trafficking. In siWASH treated cells, EGFR is not efficiently transported to lysosomes as it should be and is held up in earlier compartments (Duleh and Welch, 2010), whereas α5β1 integrin is mis-sorted into pre-lysosomal compartments, substantially delaying its transit through the recycling compartments back to the plasma membrane (Zech et al., 2011). This integrin is known to be important in cell motility and invasion in cancer cells (Caswell et al., 2007; Muller et al., 2009; White et al., 2007), and depletion of WASH in turn decreases the motility of these cells which rely on specific receptor localisation to direct cell movement and invasion (Zech et al., 2011). In WASHout MEFs, Tf was found to be normally recycled, whereas EGFR trafficking was disrupted, as seen in siWASH cells (Gomez et al., 2012), indicating that different receptors may possibly be trafficked through WASH-

dependent and -independent pathways. These results also support the idea that WASH is important in a variety of different endocytic routes in mammalian cells.

1.5.3.2 WASH and Exocytosis

Carnell et al. (2011) had established in *Dictyostelium* WASH nulls that exocytosis was blocked, therefore they used macrophages to see whether knockdown of WASH was detrimental to mammalian cell exocytosis as well. They observed that upon feeding the yeast *Cryptococcus neoformans* to macrophages, the endocytosed yeast was contained within GFP-WASH-labelled compartments. Upon expression of the dominant negative GFP-WASHΔVCA in these cells, the yeast became trapped in the early endosomal compartments and cells had great difficulty expelling it from the cytoplasm. This shows that mammalian cells also have a requirement for WASH in progression of cargo through the endocytic cycle and exocytosis.

1.5.4 WASH in Dictyostelium

The degradation and recycling pathways seen in mammalian cells are integrated in *Dictyostelium*, with endocytosed material passing through the lysosomal stage before being recycled to the plasma membrane to be exocytosed (Maniak, 2003). Carnell et al. (2011) found that GFP-WASH recruitment to endosomal membranes was simultaneous with removal of small budding vesicles containing V-ATPase (Clarke et al., 2002), indicating WASH was recruited to neutralising lysosomes. Loss of WASH resulted in a failure of V-ATPase to be removed from lysosomes, which consequentially were unable to neutralize. This caused a block at this stage of the endocytic cycle and no further progression of vesicles was possible.

1.5.4.1 Endocytosis and Exocytosis in WASH nulls

Although exocytosis was completely blocked, endocytosis and early endosomal events were unaffected in WASH nulls (Carnell et al., 2011). Cells were still able to perform macropinocytosis as normal, and the contents of early endosomes became concentrated due to the extraction of liquid and recycling of membrane (Clarke et al., 2002; Neuhaus et al., 2002). This meant cells grown in liquid medium which contains very little indigestible material, could survive and grow at near normal rates. *Dictyostelium* cells can also be grown on bacterial plates as axenic strains still retain the ability to engulf and digest bacteria as a

food source, however this posed a problem for WASH nulls. The acidic lysosomal vesicles in WASH nulls became filled with the large amount of debris produced by digestion of bacteria (Carnell et al., 2011). This decreased the survival rate of cells substantially as the rate of division and growth was not fast enough to compensate for the build-up of intracellular debris within the endosomal system.

1.5.4.2 WASH in Lysosome Maturation

To confirm the requirement of actin for lysosomal maturation, Carnell et al. added the drug latrunculin A (latA), which blocks polymerization, to wild type *Dictyostelium* cells which had been fed the pH sensitive FITC-dextran. LatA was added to cells approximately 20 minutes after uptake of the FITC-dextran. At this point, the FITC-dextran should be beginning to enter the neutralizing stage of the endocytic cycle (Clarke et al., 2002), however addition of LatA prevented the neutralization process for the duration of treatment. Removal of the drug then allowed actin polymerization to restart, and neutralization to begin. This shows that the presence of actin is required for maturation of lysosomes into neutral post-lysosomes.

Once neutralisation is complete, WASH dissociates from the membrane of the vesicle before it is transported to the plasma membrane for exocytosis (fig. 1.5; Thomason, unpublished data). WASH was never observed at the plasma membrane in *Dictyostelium*, nor was it localised to the vesicle during the process of exocytosis. This indicates that WASH is not responsible for the actin recruited to vesicles at, and required for, exocytosis.

1.5.4.3 Mechanism of V-ATPase Removal

Carnell et al. (2011) hypothesized that the actin coat built by WASH was required to cluster the V-ATPase molecules within the lysosome membrane. This would restrict it within a subdomain of membrane where budding occurs to remove it from the membrane (fig. 1.6). The removal of V-ATPase through small budding vesicles has already been documented (Clarke et al., 2002, 2010). Several actin filament binding sites have also been identified on subunits of the V-ATPase complex (Holliday et al., 2000; Vitavska et al., 2003). The patchy distribution of WASH on membranes also supports a role in clustering and defining subdomains (Derivery et al., 2009; Duleh and Welch, 2010). Actin has

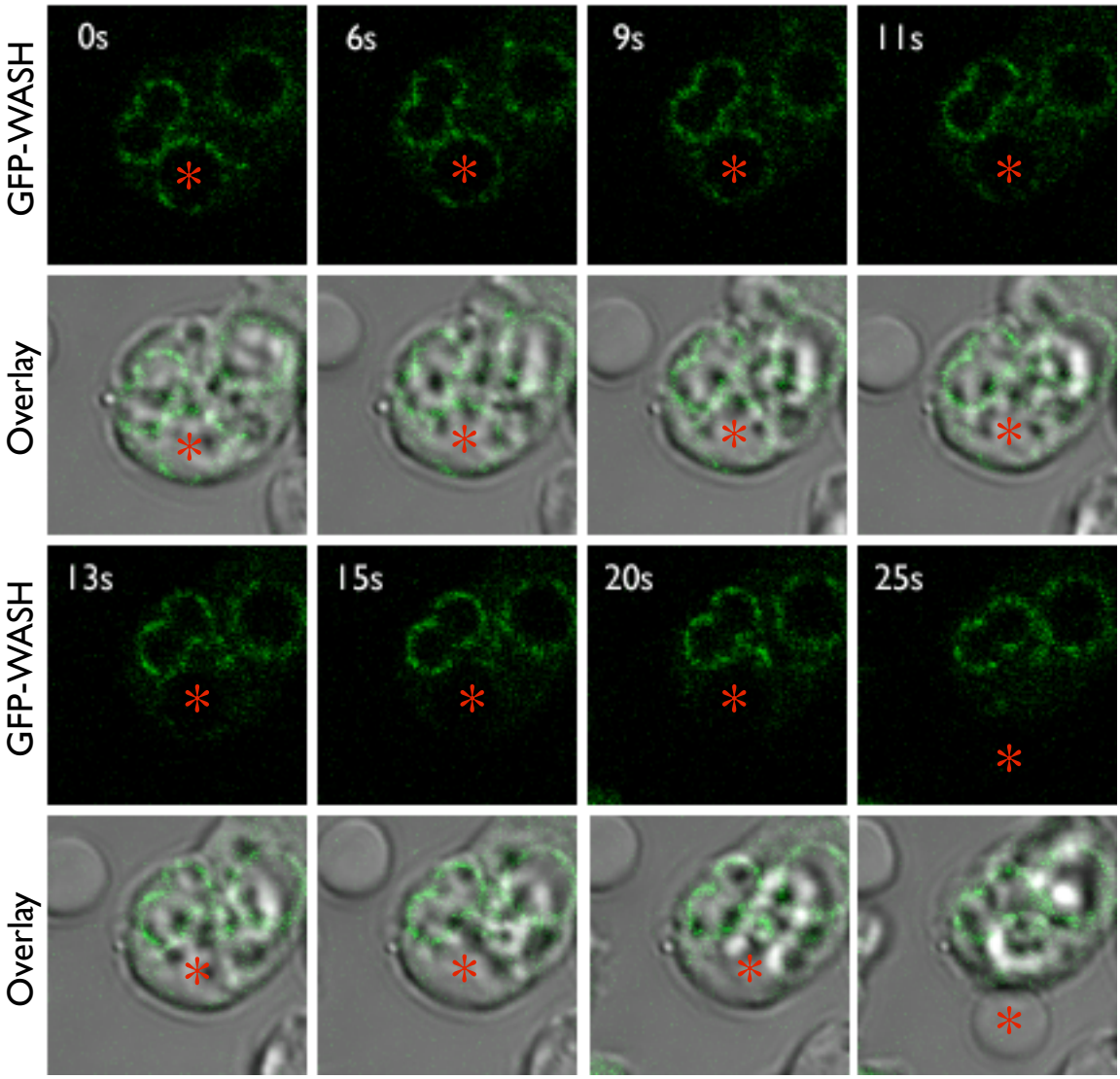

Figure 1.5 Dissociation of WASH before exocytosis

Thomason (unpublished data) fed Ax2 *Dictyostelium* cells agarose beads. These beads became decorated with GFP-WASH and were observed over time until the bead was exocytosed. The bead labelled with the red star above shows the dissociation of GFP-WASH from the bead approximately 15 seconds prior to its exocytosis from the cell.

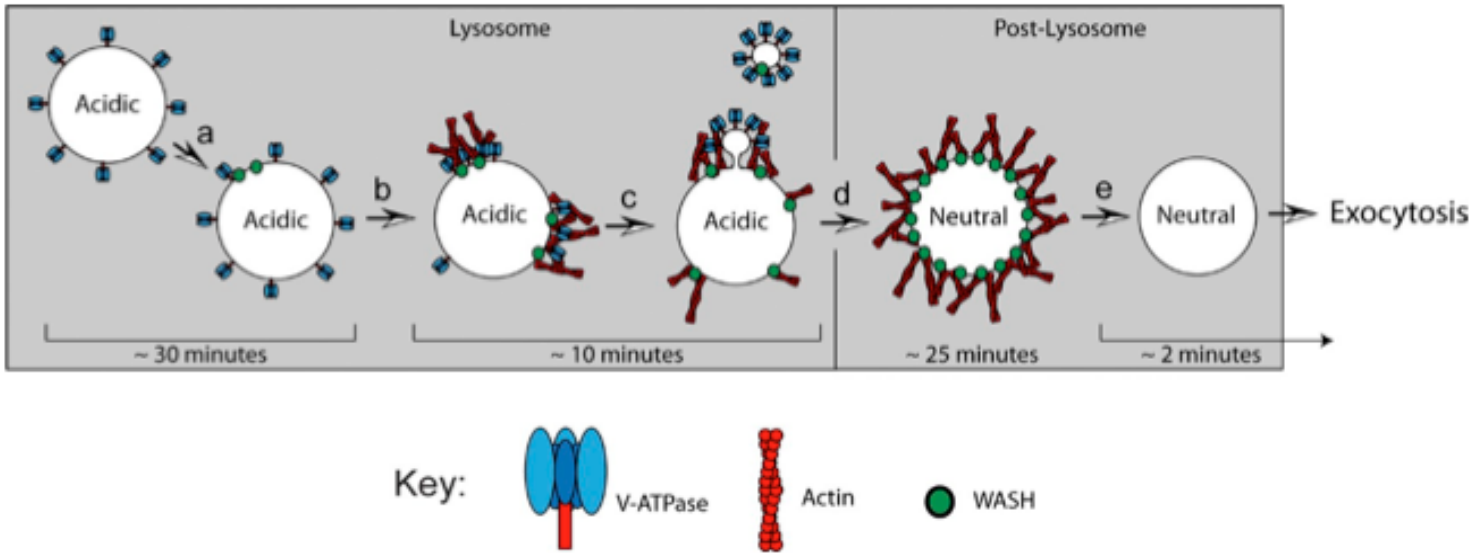

Figure 1.6 WASH mediated removal of V-ATPase from vesicle membranes
This diagram, by Carnell et al. (2011) demonstrates the idea that WASH is recruited to acidic lysosomal membranes, where it creates an actin coat which binds and clusters V-ATPase complexes. This allows sections of membrane to bud off from the vesicle, removing the V-ATPase complexes and allowing neutralization of the lumen of the vesicle, which can then progress to exocytosis.

31

been previously shown to be required for the clustering of proteins and receptors into microdomains within membranes. For example, the human immunodeficiency virus (HIV-1) uses the host cell cytoskeleton to cluster receptors, such as the T cell receptor CD4, to the site of cell-cell contact in order to bind HIV envelope proteins for invasion (Baranda et al., 2007; Dimitrov, 2004). Studies of the Tf receptor (TfR) in COS7 cells has shown that actin is required to act as a barrier against diffusion through the plasma membrane; treatment with latrunculin B resulted in a very diffuse pattern of TfR localisation, whereas treatment with the actin stabilizing drug jasplakinolide led to a much tighter clustering of the receptor (Lenne et al., 2006). These examples show that actin may be involved in actively binding and clustering membrane components, but may also play a role in acting as a barrier against diffusion. Either of these roles, or even a combination of both, may contribute to the actin coat on post-lysosomes regulating the localisation of V-ATPase within the vesicle membrane.

1.5.5 Subunits of the WASH Complex

Very little was previously known about any of the subunits before the discovery and study of the WASH complex. All five members of the complex are highly conserved throughout the eukaryotic kingdoms, and species which contain one member generally contain the entire complex (Veltman and Insall, 2010). Ccdc53 had been previously found to interact with WASH in a yeast two-hybrid screen in *Caenorhabditis elegans*, but other than that nothing is known about the protein (Li et al., 2004). Strumpellin has been identified as a cause of a specific type of spastic paraplegia. SWIP and FAM21 had not previously been identified in any known role. No known domains were identified in any of SWIP, FAM21 or Strumpellin, giving no clues as to their possible functions. The only previously studied associate of the WASH complex was capping protein, well known for its role in capping the plus ends of actin filaments (Schafer et al., 1996).

1.5.5.1 FAM21

There are several forms of FAM21 in humans; FAM21A-D of which A-C are full length, and D is a truncated form of approximately 300 amino acids of the C terminal portion. It is composed of two parts; a helical N terminal region and

the remainder of the protein which is devoid of any known domains or structure being very proline rich, except for a CapZ binding motif. The N terminal region of FAM21 is the part responsible for binding the WASH complex (Gomez and Billadeau, 2009; Harbour et al., 2010; Jia et al., 2010). The C terminus was also shown to be able to localise independently to endosomes, possibly through binding a subtype of lipids (Jia et al., 2010), suggesting it may contribute to complex localisation.

1.5.5.2 Strumpellin and Spastic Paraplegia

The gene encoding Strumpellin is expressed ubiquitously in human tissues. It has been identified in two separate screens for proteins overexpressed in prostate cancer (Duin et al., 2005; Porkka et al., 2003), however the main interest has been in its role in hereditary spastic paraplegia (HSP). HSP is a neurodegenerative disease where neurons degrade over time, causing a variety of symptoms. Pure forms of HSP involve motor neurons and affect movement of various parts of the body, whereas complicated forms also affect cognitive abilities and can produce symptoms such as retardation. Varying severity of the condition means the onset of aggressive forms can be as early as 20s, whereas other forms do not become apparent until 60s. The most common form of HSP is caused by mutations in the microtubule severing protein, Spastin, which accounts for ~40% of all dominant HSP cases (Fonknechten et al., 2000; Hazan et al., 1999). Spastin has also been shown to localise to intracellular vesicles, including early endosomes (Connell et al., 2009). It also interacts with numerous endosomal proteins including Atlastin, another protein known to cause spastic paraplegia (Evans et al., 2006; Sanderson et al., 2006). This suggests that defects in endosomal trafficking, which is strongly linked with microtubules, may contribute to the pathogenesis of the disease.

Strumpellin is involved in a pure form known as SPG8, which specifically affects the longest corticospinal neurons, resulting in loss of motor function of the lower limbs, but with no other symptoms. SPG8 is an autosomal dominant disease, and Valdmanis et al. (2007) analysed the genomes of a number of families with the disease to find that 3 specific point mutations in Strumpellin, N471D, L619F, V626F, are responsible. Over 500 controls, including members of the same families without the condition, were all negative for these mutations. All three

mutations lie in very highly conserved areas of the protein, with the L619F and V626F mutations spanning an area with 100% conservation from human to rat. The area containing the N471D mutation is also highly conserved but is missing in *Drosophila* and *Xenopus*.

All three mutations lie within α-helical secondary structures, and it is thought that the disruption of the structure by these mutations leads to malfunction of the protein (Valdmanis et al., 2007). The V626 residue is also the first amino acid of exon 15 of the protein, therefore may interfere with exon splicing, however gene analysis shows that alternative splicing is rare in the Strumpellin gene.

1.5.5.3 Capping Protein and the WASH Complex

The *Dictyostelium* proteome contains orthologues of the human CapZ α and ß subunits, which are Cap34 and Cap32 respectively (Cooper et al., 1991; Hartmann et al., 1990). Several groups have identified CapZ as being a part of the WASH complex in mammalian cells. It has been identified in immunoprecipitations of the WASH complex, as well as being shown to colocalise with WASH on endosomes (Derivery et al., 2009; Jia et al., 2010). Jia et al. (2010) state that CapZ is only present in some of the pulldowns they performed, and suggest a more transient interaction with the WASH complex, rather than it being a constitutive member. This is a possibility, as CapZ is not specific to the WASH complex and plays a part in the regulation of many other actin structures in the cell, for example Derivery et al. (2009) see it localise independently of WASH in lamellipodia. CapZ is also stable in the cell regardless of the absence of any WASH complex members, whereas other WASH complex members exhibit a co-dependency, unable to be stably expressed unless a complete complex is present in the cell (Derivery et al., 2009; Gomez and Billadeau, 2009).

The part of the complex responsible for binding CapZ, through a capping protein interaction site (CPI) is the C terminus of FAM21 (Hernandez-Valladares et al., 2010). The CPI motif is defined as LXHXTXXRPK(6X)P (Bruck et al., 2006) and is conserved in most FAM21 proteins, including *H. sapiens* FAM21, and more stringently in the *D. discoideum* FAM21 which has an exact match at position 1201 in the amino acid sequence. This interaction is supported by the fact that

purification of the C terminal portion of FAM21 alone does indeed coprecipitate CapZ (Harbour et al., 2010). Jia et al. (2010) suggest that, like with proteins such as Carmil which also contain a CPI and bind CapZ, the purpose of the interaction with FAM21 may be to sequester the capping protein away from the actin coat formed by WASH, reducing capping activity. Alternatively, FAM21 could localise capping protein to the correct site in order to enhance its activity at that position, however whether its role is cooperative or antagonistic with WASH building F-actin is yet to be tested.

1.5.6 Association of the Retromer Complex

The purpose of the retromer is to direct retrograde transport of cargo from endosomes to the Golgi. The retromer complex consists of the sorting nexins (SNX) which initiate endosomal tubulation (Cullen, 2008; Griffin et al., 2005; Rojas et al., 2007; Wassmer et al., 2007), and a cargo recognition complex, consisting of vacuolar protein sorting-associated protein 26 (VPS26), VPS29 and VPS35 in mammalian cells (Haft et al., 2000). During maturation, the lipid composition of endosomal membranes changes, and much of the phosphatidylinositol (PI) is converted to phosphoinositide 3-phosphate (PI(3)P) by the kinase PI(3)K (Panaretou et al., 1997; Vieira et al., 2001; Volinia et al., 1995). Once the membrane becomes enriched with PI(3)P, the sorting nexins are recruited and begin to elongate the vesicles in order to create a tubular structure from which the vesicles can pinch off at the ends to facilitate transport of membrane and vesicular contents (Kerr et al., 2006).

Links between WASH and the retromer were suggested due to the colocalisation seen between WASH with SNX1 and VPS29 (Gomez and Billadeau, 2009). In addition, some sorting nexins were detected in immunoprecipitations of WASH, suggesting an interaction between the retromer and WASH complexes. A direct interaction between the WASH complex and the retromer was shown by Harbour et al. (2012) to be through binding of VPS35 to the C terminal unstructured domain of FAM21. This group suggested that this interaction is essential for targeting the WASH complex to endosomes, however Gomez and Billadeau (2009) show that the complexes are not codependent on one another, as each one is able to exist and localise to endosomes regardless of the presence or absence of the other. Instead, they suggest that the loss of the WASH complex results in

the disruption to retrograde trafficking by the retromer through the deregulation of endosome morphology and lack of scission, impairing the ability of the retromer to retrieve its cargoes from tubulated endosomes.

1.5.7 Aims of This Thesis

1.5.7.1 Using Dictyostelium to Study the WASH Complex

The model organism *Dictyostelium* offered a fast route to obtaining a complete knockout cell line of WASH for Carnell et al. (2011). Mammalian systems at this time were limited by the fact that only knockdown of WASH was possible and no knockout cell line was available, therefore the phenotypes of these cells were ambiguous and it was difficult to determine the precise function of WASH. Since the publication of Carnell et al. (2011), a total knockout of WASH in mouse embryonic fibroblasts was created by Gomez et al. (2012). The data obtained using this cell line strengthened the evidence that WASH is involved in lysosomal and recycling processes, as seen in *Dictyostelium*. This indicates that the results obtained using *Dictyostelium* are widely applicable to other cell types and can lead to advances in the knowledge of actin structures and the endocytic system. We will therefore continue to use *Dictyostelium* to study the function of the regulatory subunits of the WASH complex.

1.5.7.2 WASH Complex Regulatory Subunits

Up until now, the regulatory subunits of the WASH complex have not been studied in depth. Little or no available data for each member gives few clues as to their various functions. It is yet to be determined which of the subunits are essential for the function of WASH and how each one contributes to its regulation.

The Strumpellin subunit has been identified as a cause of the disease spastic paraplegia in humans. This is due to the presence of one of three specific mutations which are shown to be responsible for causing the malfunction of the protein. It will be interesting to see whether these mutations affect the function of Strumpellin within the WASH complex in *Dictyostelium*, and if so, what effect they have on complex function.

It is currently unknown which of the subunits are responsible for the correct targetting of the complex to lysosomal membranes. This is important in ensuring that the actin coats are constructed around the correct endosomal compartments. Whether all of the subunits are required for the stability of the complex and the construction of the actin coat is also yet to be determined. It has been suggested by Gomez and Billadeau (2009) that both correct localisation and stability of the complex rely on the FAM21 subunit, however this data is again based on knockdowns using siRNA, therefore we will determine whether the same in true in a total knockout cell line using *Dictyostelium*, currently unavailable for mammals.

1.5.7.3 FAM21 and Capping Protein

FAM21 is the only subunit to have been studied to any degree in mammalian cells. It has been shown by Jia et al. (2010) that FAM21 interacts with the WASH complex through its N terminus, and that the C terminal region interacts with capping protein, through a CPI domain, and possibly lipids. No other capping protein binding sites have been identified within the complex but it has not been tested whether or not this truly is the only domain through which capping protein can interact with the complex. The functional significance of the interaction between the complex and capping protein is as yet unknown. There have been suggestions, such as the theory of Jia et al. (2010) that the role of FAM21 is to sequester capping protein away from the actin coat created by WASH and Arp2/3, but the precise function of this interaction has not been investigated.

CHAPTER 2

MATERIALS AND METHODS

2 Materials and Methods

2.1 Molecular Biology

2.1.1 Restriction Digests and Ligations

All restriction digests were performed using NEB restriction enzymes and buffers unless otherwise stated, as per protocol. Ligations were performed using T4 DNA Ligase and T4 DNA Ligase Buffer (Fermentas) as per protocol. Ligations were incubated for a minimum 1 hour at room temperature before transformation or storage at -20^{o}C.

2.1.2 Bacterial Strains, Preparation and Transformation

Ligations and plasmids were propagated in chemically competent DH5α E. coli cells, generated using the CaCl2 method (Sambrook et al., 1989). 5µl of ligation, or 0.5µl of plasmid, were incubated on ice with 50µl cells for 30 minutes. The cells were then heat shocked at 42^{o}C for 40s, before adding 500µl LB. Cells were incubated at 37^{o}C for 1 hour to allow them to start expressing antibiotic resistance before plating 200µl of mixture onto a LB agar plate containing the appropriate antibiotic for selection (100µg/ml ampicillin or 50µg/ml kanamycin).

2.1.3 Minipreps

Single colonies from transformed ligations/plasmids were used to inoculate 2ml LB and grown at 37^{o}C, 200rpm overnight. Bacterial cultures were spun at 3000g for 5 minutes and supernatant was discarded. Plasmid DNA was then purified from the pellets using a Qiagen 8000 Bio-Robot robot.

2.1.4 Cloning of WASH Complex Genes

cDNA was prepared from *Dictyostelium* Ax2 cells using the SuperScript® III CellsDirect cDNA Synthesis Kit (Invitrogen) as per protocol. Genes were amplified from cDNA using primers designed against the specific cDNA sequences listed on dictyBase, with additional BamHI/BglII and SpeI/AvrII sites on the 5' and 3' ends respectively. Human WASH1 and FAM21C cDNA clones were obtained from ImaGenes. HsFAM21 was also amplified with added restriction sites using gene specific primers. These PCR products were then blunt-ligated into pDM368 and fully sequenced. The genes were then digested out of the

vector using the appropriate pair of restriction enzymes and subcloned into the desired expression vector.

2.2 DNA Constructs

2.2.1 Knockout Constructs

Strumpellin cDNA in pDM368 was digested with MslI to remove the mid-section of the gene, and a blunt ligation was performed to insert the blasticidin resistance (BsR) cassette, digested out of pLPBLP using SmaI (Faix et al., 2004). The FAM21 knockout construct was made in the same way, with digestion using MfeI.

The ccdc53 construct was acquired from Douwe Veltman (unpublished); 5' and 3' arms of the gene were amplified from cDNA and ligated into pDM368 either side of the BsR cassette as described.

2.2.2 Fluorescent Tag Expression Constructs

All fluorescent tag expression vectors used were obtained from Veltman et al. (2009) for single and coexpression. For single gene expression, genes were cloned into pDM448 to create an N-terminal GFP tag construct for expression in Dictyostelium, selected by hygromycin. pDM318 was used for N-terminal RFP tagged constructs, selected with neomycin. Genes were cloned into pDM602 shuttle vector for coexpressing with an N terminal RFP tag, and excised with NgoMIV. The destination vector expressing a GFP-tagged gene was linearized with NgoMIV, and the RFP shuttle ligated in. All clones were checked by digestion patterns to ensure presence and correct orientation of inserted genes.

GFP-CRAC (pDM631) used as a positive control for the lipid blot assay, pDM317, an expression vector for GFP alone for use as a control, and RFP-actin (pDM463) were all obtained from Veltman et al. (2009). vatB-GFP was obtained from Carnell et al. (2011). Human GFP-WASH was created by cloning human WASH1 into pEGFP-C1 (Invitrogen).

2.2.3 Mutant Constructs

FAM21ΔCPI; PCRs were performed using primers oLP067/166 and oLP167/140 to amplify FAM21 regions upstream and downstream of the CPI site, which is

located at base pairs 3601-3651 of the FAM21 cDNA sequence. The primers oLP166 and oLP167 contain a region of complementary sequence which allowed the two products to anneal together, but also eliminated the CPI from the sequence. The full length gene was then amplified using primers oLP067 and oLP140. The product was then ligated into pDM368 and fully sequenced.

FAM21ΔCT and FAM21ΔNT; PCRs were performed using primers oLP067/SoapNT and FAM21CT/oLP140 to amplify the head region (nucleotides 1-765) and tail region (735-4440) of FAM21 cDNA. These products were then ligated into pDM368 and fully sequenced, named FAM21ΔCT and FAM21ΔNT respectively.

hyFAM21; The *D. discoideum* N terminal portion of FAM21 was amplified using primers oLP067/oLP069. The C terminal portion of *H. sapiens* FAM21C was amplified using primers oLP132/oLP133. The primers oLP132 and oLP069 contain a region of complementary sequence which allows the two products to anneal together, and the full length hybrid gene to be amplified using primers oLP067/133. The product was then ligated into pDM368 and fully sequenced, and the resulting construct coded for a protein consisting of residues 1-255 of *D. discoideum* FAM21, and 265-1341 of *H. sapiens* FAM21.

SPG8 Strumpellin mutants; the Strumpellin gene 5' region was amplified using F primer oLP001 with R primer oLP011, oLP013 or oLP015 for creation of the L607F, V614F or N459D mutant respectively. Each R primer contains the altered nucleotide triplet to introduce the relevant mutation, followed by a short complementary sequence corresponding to that on the 3' regions, amplified by F primers oLP012, oLP014 and oLP016 respectively, with R primer oLP002. The corresponding 5' and 3' templates were then combined and the entire gene with the amplified triplet was in each case amplified with oLP001/oLP002. The full length gene was then blunt ligated into pDM368 and fully sequenced.

2.3 Cell Biology

2.3.1 Transfection of Dictyostelium Cells

Dictyostelium transfections were performed by adding 10-50ng plasmid to a 2mm gap Electroporation Cuvettes Plus™ cuvette (BTX Harvard Apparatus) and adding between 3 x 10^6 and 1 x 10^7 cells suspended in 400ml E-buffer. Cells

were electroporated at 500V using an ECM 399 Electroporation System (BTX Harvard Apparatus), left on ice to recover for 10 minutes and then plated out onto a 10cm dish containing 10ml HL5 medium. Selective antibiotics were added 24 hours later if required (50µg/ml hygromycin or 10µg/ml neomycin).

2.3.2 WASH Complex Null Cell Line Generation

WASH Ax2 knockouts were acquired from Carnell et al. (2011). FAM21 Ax4 and SWIP Ax4 knockouts were acquired from Torija et al. (2006). Ccdc53 Ax3 and Strumpellin Ax2 knockouts, and WASH/FAM21 double knockouts, were made by gene disruption through recombination. A knockout construct containing the BsR cassette and at least 1kb of flanking sequence either side, was amplified by 8 x 50ul PCR reactions. Primers oLP179/064 were used to amplify the Strumpellin construct, oLP101/oLP069 for FAM21 and oDM552/oDM553 for ccdc53. The PCR products were pooled for each gene and purified using a Zymo Research DNA Clean and Concentrator^{TM}-25 Kit as per protocol, and eluting in 30ul H_2O. The DNA was then transfected into cells as described. After transfection, cells were resuspended in 60ml HL5 medium and plated out in aliquots of 100ul into 96-well plates. After 24 hours, another 100ul of HL5 containing 20ug/ml (2 x concentration) blasticidin was added to select for transformants. Recombinant clones were screened for by western blot probed with anti-Strumpellin generated against peptide CSHFQRPDSNPYPSD (BioGenes GMBH). The ccdc53 Ax3 knockout cell line was screened by Peter Thomason (unpublished).

To make the double WASH/FAM21 knockout, WASH- cells were transfected with pDEX-NLSCRE to express Cre-recombinase in the cells. This allowed the excision of the BsR cassette, which is flanked by loxP sites. Transformants were plated out and selected for pDEX-NLSCRE expression using neomycin. Clones in which the BsR cassette had been successfully excised became blasticidin sensitive. These clones were then removed from neomycin selection and grown for 3 days, then tested for loss of the extrachromosomal pDEX-NLSCRE plasmid by sensitivity to neomycin. PCR was used to confirm the removal of the BsR cassette, and these clones were then transfected with the FAM21 knockout construct and selected as described.

2.3.3 Microscopy

Unless otherwise stated, cells were incubated in LoFlo medium containing 5% dextran for 3 hours prior to imaging. Cells were visualized using a Nikon A1R confocal microscope using a 60x 1.4 NA objective and images were captured using NIS-Elements AR3.1 software (Nikon). Cells were imaged on MatTek 35mm glass bottom culture dishes. Images were captured using 488nm excitation, and 500-550 emission for FITC/GFP, and 561.4nm excitation, 570–620-nm emission for TRITC/RFP.

2.3.4 GFP-Trap Purification

40ul of bead suspension of GFP-Trap beads (Chromotek) were transferred to an eppendorf and washed twice with TNE buffer then resuspended in 40ul TNE. Cells were grown in shaking culture and $1x10^8$ cells were harvested and washed 3 times in ice cold KK_2 buffer before resuspending in 1ml TNE plus 1:100 HALT™ Protease Inhibitor Single-Use Cocktail (ThermoScientific). Cells were left on ice for 20 minutes to lyse, then lysates were spun at 4°C for 20 minutes at maximum speed to pellet debris. Supernatant was decanted into a clean tube and 40ul of beads were added to each sample. Samples were rotated overnight at 4°C to allow GFP-tagged protein to bind to the beads. Beads were then pelleted by spinning at 3000rpm for 3 minutes and supernatant was removed. Beads were washed in TNE 3 times before resuspending in 1x NuPAGE LDS Sample Buffer (Invitrogen) and boiling for 3 minutes. Samples were cooled and then run on a gel as described.

2.3.5 SDS-PAGE

Dictyostelium cells were lysed by heating in 1x NuPAGE LDS Sample Buffer (Invitrogen) at 70°C for 10 minutes, then cooled on ice. Samples were run in an Invitrogen tank using pre-cast Invitrogen SDS-PAGE Bis-Tris 10% gels for 50 minutes at 200V. Gels were then washed in water for 10 minutes before western blotting or analysis by mass spectrometry.

2.3.6 Western Blotting

Proteins were transferred from an SDS-PAGE gel onto an Amersham Hybond-P PVDF membrane (G E Healthcare) in a BioRad transfer tank at 240A for 2 hours. Membranes were blocked for 1 hour at room temperature in 5% dried skimmed

milk in TBS. Membranes were then washed in TBS and incubated with primary antibody diluted in 2ml TBS in a 50ml Falcon tube by rotating at 4°C overnight. The membranes were washed 5 times in TBST before addition of secondary antibody at a dilution of 1:10,000 in TBST and incubation with rotation at room temperature for 40 minutes. To develop, membranes were washed 3 times in TBST for 10 minutes each, before addition of Immobilon™ Western Chemiluminescent HRP Substrate (Millipore), wrapping in Saranwrap (Dow Chemical) and visualization using a transilluminator.

2.4 Dictyostelium Cellular Assays

2.4.1 Exocytosis and Endocytosis Assays

For the exocytosis assay, cells were grown in shaking culture overnight in HL5 medium containing 2mg/ml FITC-dextran to take up fluorescent material. Cultures were then spun at 2000rpm for 2 minutes to pellet cells, washed twice in cold KK_2 to remove the FITC-dextran, before resuspending cells in 20ml HL5 medium and continuing to shake. Samples of 0.5ml of medium were taken, starting immediately, at timepoints from 0-300 minutes. Each sample was spun to pellet the cells, and washed twice in KK2 to remove any external fluorescent material. Pellets were stored on ice until all timepoint samples were taken. Pellets were resuspended in 200µl of lysis buffer to release the FITC-dextran endocytosed by the cells before transferring the lysate to a quartz cuvette. The fluorescence of the lysate was measured using a PTI fluorimeter with 470nm excitation and 515nm emission, to determine the amount of FITC-dextran remaining in the cells at each timepoint.

For the endocytosis assay, cells were grown in shaking culture overnight in 20ml HL5 medium. Cell numbers were adjusted to $1x10^6$ cells/ml before adding 2mg/ml FITC-dextran to the flask. Starting immediately, to measure the rate of endocytosis of the fluorescent dextran, samples were taken and measured as per the exocytosis assay. Fluorescence levels of samples was normalized to protein content, measured using Precision Red (Cytoskeleton).

2.4.2 Neutralization Assay

Cells were plated onto a glass bottomed dish and incubated in HL5 medium containing 5% unlabelled dextran, 0.4mg/ml FITC-dextran and 4mg/ml TRITC-

dextran for 3 hours to allow the dextran to transit through the endocytic compartments. For each experiment, Ax2 cells were examined using a Nikon A1R confocal. The power for each of the 488 and 561 lasers was adjusted to maximum intensity possible without saturation to visualize both fluorophores in the vesicles. These normalized settings were then maintained to examine the other cell lines in order to detect FITC fluorescence in neutralized compartments.

2.4.3 Cell Fixation

Cells were seeded on coverslips and incubated in HL5 plus 10% dextran for 2 hours before picrate/paraformaldehyde fixative was added for 15 minutes to fix cells. Coverslips were then washed in PBS and transferred into 70% ethanol for 2 minutes. Coverslips were washed sequentially in PBS, then PBS containing 0.1M glycine. To stain for actin, cells were incubated in 33nM Texas red-phalloidin (Invitrogen) in PBS for 1 hour before washing in PBS, then water, and mounting on slides using ProLong Gold antifade reagent (Invitrogen).

2.4.4 Enlarged Vesicle Imaging

Cells were plated onto a glass bottomed dish in HL5 medium containing 10% dextran for a minimum of 3 hours to cause vesicle enlargement (in Ax2 cells, a slight enlargement made it easier to visualize vesicles, and in FAM21- cells, a gross enlargement was induced by addition of dextran). To label endocytic compartments, 1mg/ml of TRITC-labelled dextran was added to the medium for 3 hours prior to imaging unless otherwise stated. For labelling whilst simultaneously imaging GFP constructs, TRITC-dextran was added at the lower concentration of 0.1mg/ml to prevent saturation which masked the GFP signal, to ensure GFP-labelled proteins were visible.

2.4.5 Distribution of GFP-WASH

The ratio of vesicular GFP-WASH on the enlarged post-lysosome membrane in a FAM21 null compared to total vesicular GFP-WASH in the cell was quantified using ImageJA v1.45. The pixel intensity of a cytoplasmic area of a cell expressing GFP-WASH was measured, and this value was used as the threshold; only signal above this value was counted to rule out autofluorescence or cytoplasmic protein. For FAM21 nulls, the threshold was set, then a whole cell

was selected and the total signal measured to give total vesicular GFP signal for that cell. Then the membrane of the enlarged post-lysosome within the cell was selected individually, using a circular band selection tool of band width 0.5µm, and the GFP signal measured for the enlarged vesicle only. For Ax2 cells, the same was done except up to 3 large vesicles in the cell were measured and pooled, and compared to total vesicular GFP-WASH.

2.4.6 Doubling Times

Cells were added to flasks containing 10ml HL5 medium with or without 10% w/v dextran (60-90kDa) at a density of $1x10^5$ cells/ml and grown in shaking culture overnight. Aliquots were taken at 24 and 42 hour timepoints and cell density measured using a Casy cell counter (Roche). Doubling times of cultures were calculated by using the formula;

$$\text{Doubling time} = \frac{T}{\log_2(C(T)/C(T_0))}$$

Where T = timepoint (hours of incubation), C(T) = cell number/ml at time T, $C(T_0)$ = cell number/ml at start

2.4.7 Lipid Blot Assay

The lipid blot assay was performed using the PIP Array™ (Echelon) kit. Lipid membranes supplied were blocked for 1 hour at room temperature with 1% skimmed milk in PBS. *D. discoideum* cells transfected with pDM317 (GFP control), GFP-CRAC, GFP-FAM21 or GFP-FAM21ΔCT were grown overnight in shaking culture in HL5 medium. Cells were washed and resuspended in TBS containing HALT™ Protease Inhibitor Single-Use Cocktail (ThermoScientific). Cells were filter lysed using a syringe to pass the cells through a 5.0µm TMTP Isopore™ Membrane Filter (Millipore) and cell lysate protein concentration was measured by adding 10µl of sample to 1ml of Precision Red protein assay reagent (Cytoskeleton) and measuring the concentration on a photometer. The experiment was done twice, once using 1µg/ml and once using 2µg/ml of protein suspended in TBS with 1% skimmed milk to incubate the membrane at 4°C overnight. The membrane was washed with TBST 3 times before incubation with mouse HRP-conjugated anti-GFP antibody (Abcam, AB5450) at 1:1,000 in TBS at

room temperature for 1 hour. The membrane was then washed again 3 times with TBST and secondary anti-mouse antibody was added at 1:10,000 at room temperature for 1 hour. The membrane was washed again 3 times in TBST before adding Immobilon™ ECL substrate for 5 minutes and visualizing on a transilluminator.

2.4.8 Fluorescence Recovery After Photobleaching (FRAP) in Dictyostelium cells

Dictyostelium Ax2 and FAM21 null cells, both transfected with GFP-WASH were imaged with an inverted confocal microscope (Fluoview FV1000, Olympus) equipped with an uPlanSApo 60×/1.35 oil objective. The images were acquired using software FV10-ASW1.7. FRAP analysis of cells was performed using images of 520 x 520 pixels, and 5% power of 488 nm laser. Effective photo-bleaching of GFP was achieved with 405 nm laser, 10 µs/pixel dwell time for one frame in the region of interest. Images were captured at 1 frame/second over 10 seconds to observe recovery.

2.5 Mammalian Cell Assays

2.5.1 Transfection and Knockdown

A2780 ovarian carcinoma cells were cultured in RPMI medium. They were grown to confluency on a 15cm dish, before medium was aspirated and cells were washed in PBS. 2ml of 0.25% trypsin was added to cells and incubated at 37°C for 10 minutes to release adherence of cells from the surface of the plate. 10ml RPMI containing serum was then added to inactivate the trypsin. Cells were transferred to a 15ml falcon tube and centrifuged at 1000g for 5 minutes. Medium was aspirated and cells were washed in PBS and centrifuged again. Cells were resuspended in 100ml Amaxa T solution and transferred to a cuvette containing 20mM siRNA. Cells were then resuspended in 6ml RPMI medium and 2ml of this was added to a 15cm dish containing 20ml RPMI.

The siRNA against human WASH1 used was pooled siW1 (Hs_WASH1_1) and siW4 (Hs_FLJ00038_1) obtained from Qiagen. The siRNA against human FAM21C used was pooled siF1-4 (Hs_FAM21C_9, Hs_FAM21C_10, Hs_FAM21C_11, Hs_FAM21C_12 respectively) obtained from Qiagen.

To test knockdown efficiency, cells were lysed by addition of 200µl lysis buffer to a 10cm dish and use of a cell scraper. 30µl of SDS-PAGE sample buffer was added to 90µl of lysate before heating the sample at 70°C for 10 minutes. The samples were cooled on ice before being run on a gel and analysed by western blotting as described (fig. 2.1).

2.5.2 Integrin Recycling Assay

An ELISA Maxisorb 96 well plate (Invitrogen) was prepared by coating overnight with 5µg/ml anti-human α5 integrin antibody (Pharmigen, 555651) in 0.05M Na_2CO_3, pH 9.6. The plate was then blocked with 5% BSA in PBST before washing with PBST. 12 confluent 6cm dishes of cells for each of cells treated with siFAM21, siWASH or untreated were used, 11 of each were labelled with 0.13mg/ml sulfo-NHS-SS-Biotin at 4°C for 30 minutes, and one dish of cells for each was left unlabelled as control until the lysis step. Dishes were washed with PBS and incubated at 37°C for 30 minutes to allow internalization of labelled integrins. Dishes were then washed again and incubated at 4°C with 15mg/ml MesNa in pH 8.6 buffer for 20 minutes to remove surface labelled proteins which were not internalized. Dishes were either kept on ice for T=0 sample, or transferred to 37°C incubator for 15 or 30 minutes to allow recycling. After recycling period, dishes were again washed and treated with MesNa to remove recycled, labelled proteins from cell surface. Lysis buffer was then added to all dishes, and lysates were added to the ELISA plate and incubated overnight at 4°C. The ELISA plate was then washed with PBST before adding streptavidin-conjugated horseradish peroxidase (BD Biosciences) at 1:1000 in PBST. The plate was incubated for 1 hour before washing with PBST and developing using 0.56mg/ml o-phenylenediamine dihydrochloride detection reagent in detection reagent buffer. The reaction was stopped after 10-20 minutes by addition of 8M H_2SO_4 and imaged using a plate reader to measure absorbance at 490nm.

2.5.3 FRAP in A2780 Cells

FRAP was performed with Tobias Zech as described for *Dictyostelium*, with A2780 cells transfected with EGFP-WASH treated with siFAM21 or untreated, in an atmosphere of 5% CO_2 at 37°C. FRAP analysis of cells was performed using

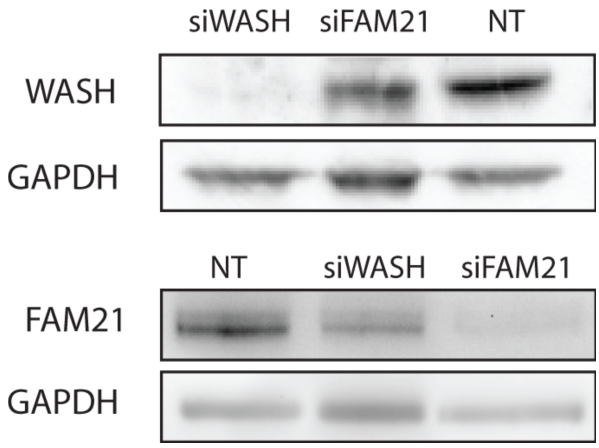

Figure 2.1 Knockdown efficiency for siFAM21 and siWASH
A2780 cells were transformed with siRNA against WASH or FAM21C as described. Anti-WASH and anti-FAM21C antibodies were then used to measure the efficacy of the knockdown by comparing the level of protein with that of non-transformed cells.

images of 640 x 640 pixels and 3% power of the 488 nm laser. Effective photo-bleaching of EGFP was achieved with 405 nm laser, 10 µs/pixel dwell time for one frame in the region of interest. Images were captured over 66 seconds at 1 frame/3 seconds.

2.6 Antibodies

For testing efficacy of knockdowns in A2780 cells, anti-WASH complex subunit FAM21C (Millipore, ABT79) and anti-WASH1 (Atlas Antibodies, HPA002689) were used. For detection of *Dictyostelium* WASH, peptide anti-WASH antibody obtained from Carnell et al. (2011) was used. For detection of *Dictyostelium* CAP32 subunit of capping protein, Anti-CapZ against human CapZß (Abgent, AP2888a) was used.

2.7 List of Primers

oLP001 F
CTGTGAGAATTCATGGTAAAAGAATTTTTAGGGGAAGGTAGTCAAGC
oLP002 R
CTGCTGGGATCCTTAATTATTATAATAATCAAAAATATAAGGTGGTACATAACC
oLP011 R
CCAATACCTTTCTAACATAACCAACGAATTCACCAGAGTAATACTCTGATACCG
oLP012 F
CGTTGGTTATGTTAGAAAGGTATTGG
oLP013 R CATTTGTTTTGGTACGATTTCCAAAAACTTTCTAACATAACCAACCAATTCACC
oLP014 F
TTTTTGGAAATCGTACCAAAACAAATG
oLP015 R
GATTTCACCAAACCATTTTTGAAGATCTTCATTCTTTTTCACACGAGTTAACG
oLP016 F
GATCTTCAAAAATGGTTTGGTGAAATC
oLP064 R
CATCATGGATCCTTACCAATATATTTGACAACTCTTTTAGACG
oLP067 F
CATCATGGATCCAAAATGCCTGAAGAACAACCACAACAACAACAACAACCAGTTCGTGAA
CAACCATCGAACCC
oLP069 R

ACTAGTTTCCTCATCTTCTTCATCTGAAGAATCAG

oLP101 F

CCAACTCGTTCTTTCACTGCCACTG

oLP132 F

CTGATTCTGATTCTTCAGATGAAGAAGATGAGGAATCTGAGAAGGAGGA

GGAAGATATTGAG

oLP133 R

AGCTGAGGATCCCTGGCCTCCAAAGGCATTCAGG

oLP140 R

CCCGGGCCTAGGATCAAATAAATTTTCCACATTTTTAGCTTTTGGTTTTGATTTCGAAGG

oLP166 R

GACTCTGAAGAAAGTGAACCAGTTAAAGAAACTCGTAAATCTGGTACTTCTGCTCC

oLP167 F

GGAGCAGAAGTACCAGATTTACGAGTTTCTTTAACTGGTTCACTTTCTTCAGAGTC

oLP179 F

CGATCATTGCTGAATTACTTCGTTTAAGTGC

FAM21NT R

GGGGACCACTTTGTACAAGAAAGCTGGGTATCTAGATTATTCCTCATCTTCTTCATCTGA

AGAATCAGA

FAM21CT F

GGATCCAAAATGGATTCTGATTCTTCAGATGAAGAAGATGAGG

2.8 Buffer Recipes

DNA Gel Loading Buffer x6 (40ml);

2.5% glycerol

2.5mg/ml bromophenol blue

10mM Tris pH 8.0

E-Buffer

16.5mM KH_2PO_4

3.8mM K_2HPO_4

50mM sucrose

ELISA Detection Reagent Buffer

25.4mM Na_2HPO_4

12.3mM citric acid
0.003% H_2O_2
pH 5.4

Endocytosis/Exocytosis Lysis Buffer
50mM Na_2HPO_4
0.2% TritonX-100
pH 9.3

Fixative (Dictyostelium cells)
2% (w/v) paraformaldehyde
15% v/v saturated picric acid
10mM PIPES
pH to 6.5

HL5 axenic medium
1.43% peptone (Oxoid, L34)
0.72% yeast extract (Oxoid, L21)
3.6mM Na_2HPO_4, 3mM KH_2PO_4
30% glucose
0.5mg/ml vitamin B12
1mg/ml folic acid, pH 9.0
pH to 6.4

KK_2
16.5mM KH_2PO_4
3.8mM K_2HPO_4

LB
1% Bacto-tryptone (Difco)
0.5% Bacto-yeast extract (Difco)
17mM NaCl
pH to 7.0
(LB agar – 1.5% Bacto-agar (Difco))

LoFlo Medium (1 litre)

11g glucose

5mM KH_2PO_4

5g Casein peptone

500mM NH_4Cl

200mM $MgCl_2$

10mM $CaCl_2$

5mM $FeCl_3$

4.84mg Na_2-EDTA.$2H_2O$

2.3mg $ZnSO_4$

1.11mg H_3BO_4

0.51mg $MnCl_2.4H_2O$

0.17mg $CoCl_2.6H_2O$

0.15mg $CuSO_4.5H_2O$

0.1mg $(NH_4)6Mo7O_2.4H_2O$

pH to 6.5

Mammalian Cell Lysis Buffer

150mM NaCl

10mM Tris

1mM EGTA

1mM EDTA

pH to 7.5

1% v/v NP-40

PBS

137mM NaCl

2.68mM KCl

7.98mM Na_2HPO_4

1.47mM KH_2PO_4

pH to 7.2

TAE

40mM Tris-acetate

1mM EDTA

TE

10mM Tris-HCl pH 7.5

1mM EDTA

TNE

50mM Tris

150mM NaCl

1mM EDTA

1% TritonX-100

Western blotting buffer

48mM Tris-Cl pH 6.8

96mM glycine

20% v/v methanol

CHAPTER 3
IDENTIFICATION AND ANALYSIS OF THE WASH COMPLEX SUBUNITS

3 Identification and Analysis of the WASH Complex Subunits

3.1 The *D. discoideum* WASH complex

Dictyostelium discoideum has been shown by Carnell et al. (2011) to be a good model in which to study WASH complex function. It has also been used extensively to study the actin cytoskeleton, and NPFs such as Scar/WAVE (Bear et al., 1998; Ibarra et al., 2005; Machesky and Insall, 1998; Pollit and Insall, 2008). We began by examining the fully sequenced genome of *D. discoideum* to confirm the presence of genes encoding the WASH regulatory subunits. The human protein sequences for the five known members of the WASH complex, WASH, FAM21, Strumpellin, SWIP and ccdc53 (Derivery et al., 2009; Gomez and Billadeau, 2009), were entered into the basic local alignment search tool (BLAST) on the *Dictyostelium* database (dictyBase.org, 2010) to search for homologous predicted protein sequences. All known WASH complex members were present in *D. discoideum* and the details of the relevant genes and proteins are listed in table 3.1. As well as the five core members, capping protein is also thought to interact with the WASH complex and play a role in its regulation (Derivery et al., 2009; Gomez and Billadeau, 2009; Jia et al., 2010), and orthologues of the human CapZ α and ß subunits were also identified in *D. discoideum*; Cap34 and Cap32 respectively.

To ensure the subunits were all expressed and did indeed form a complex *in vivo*, as was seen in mammalian cells (Derivery et al., 2009; Gomez and Billadeau, 2009) we performed several coimmunoprecipitations. We first created a GFP-tagged WASH construct, which was expressed in Ax2 cells then purified from the cells using GFP-Trap beads (Chromotek) as described in Materials and Methods. GFP alone was also expressed in Ax2 and used as a negative control. GFP-WASH and any bound proteins were eluted from the beads by boiling in SDS-PAGE sample buffer, and the eluent was then run on a Bis-Tris SDS-PAGE gel. Sections of gel thought to contain unique bands were then cut out and analysed by mass spectrometry. A large number of proteins were identified, many of which were also present in the GFP control, however some were exclusively present in the GFP-WASH sample. These

Protein Name	dictyBase Gene ID	Chromosome	Position	Exons	Protein Length	Molecular Weight	*Homo sapiens* NCBI Gene ID	*Homo sapiens* Gene Name
WASH	DDB_G0292878	6	2194668 to 2196220	2	472	50.92	100287171	WASH1
							375260	WASH2P
							374666	WASH3P
							374677	WASH4P
							375690	WASH5P
							653440	WASH6P
							653635	WASH7P
FAM21	DDB_G0276221	2	6307785 to 6312539	4	1479	160.37	387680	FAM21A
							55747	FAM21B
							253725	FAM21C
							653450	FAM21D
Strumpellin	DDB_G0288569	5	1693802 to 1697605	4	1164	134.9	9897	KIAA0196
SWIP	DDB_G0283355	4	559359 to 563272	4	1135	131.57	23325	KIAA1033
ccdc53	DDB_G0267948	1	1170991 to 1172151	2	354	38.41	51019	CCDC53
Cap34	DDB_G0272104	2	1562720 to 1564080	4	281	31.16	829	CAPZA1
							830	CAPZA2
							93661	CAPZA3
Cap32	DDB_G0267374	1	1647348 to 1648410	3	272	30.69	832	CAPZB

Table 3.1 Identification of *Dictyostelium discoideum* WASH complex members

Homo sapiens protein sequences of the known WASH complex members were analysed using dictyBase.org BLAST (blastp, E = 0.1) to search the *D. discoideum* proteome for orthologues. All five members of the WASH complex were identified, each encoded by a single gene. Basic gene and protein information is given here about each of the five subunits and their H. sapiens counterparts, as well as the genes in *D. discoideum* which encode the two subunits of the capping protein heterodimer.

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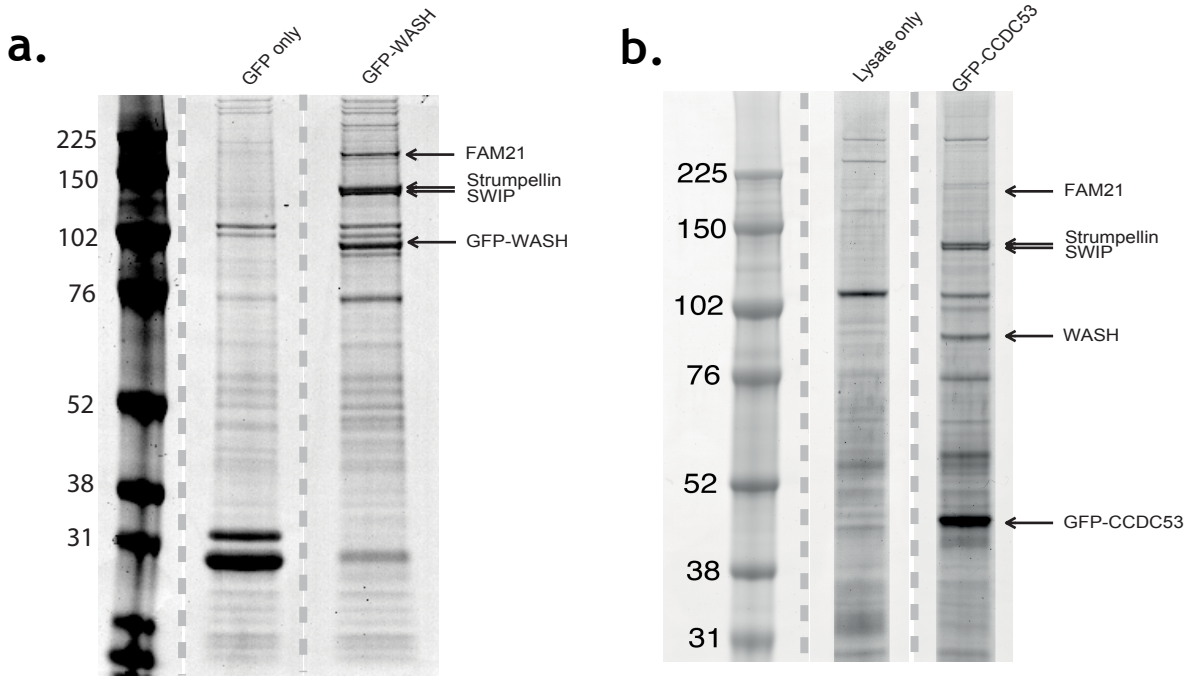


c.

Protein Name	Accession Number	Molecular Weight (kDa)	Number of Peptides GFP-FAM21	Number of Peptides GFP only
FAM21	in|LAP002|LAP002_R06		164	51
Strumpellin	DDB0234050|DDB_G0288569	135	71	2
SWIP	DDB0234041|DDB_G0283355	132	60	0
ccdc53	DDB0349216|DDB_G0267948	38	3	0
WASH	DDB0305648|DDB_G0292878	51	18	0
Cap34	DDB0191243|DDB_G0272104	31	13	1
Cap32	DDB0191202|DDB_G0267374	31	20	2

Figure 3.1 Coimmunoprecipitation of the WASH complex

GFP-WASH, GFP-CCDC53 and GFP-FAM21 were purified from Ax2 cells using GFP-Trap beads. The purified proteins were analysed by electrophoresis on a 4-12% Bis-Tris gel, and mass spectrometry was used to identify the bands. (a) The complex members FAM21, Strumpellin and SWIP were all identified using GFP-WASH. (b) The subunits FAM21, Strumpellin, SWIP and WASH were all identified using GFP-ccdc53. (c) All WASH complex subunits, as well as the two subunits of the capping protein heterodimer, Cap34 and Cap32, were identified by mass spectrometry using GFP-FAM21.

included the complex members FAM21, Strumpellin and SWIP (fig. 3.1a), and also identified was the capping protein subunit, Cap34. Although some of the other proteins identified were potentially interesting, these are not discussed further as we decided to focus solely on the known complex members.

This initial coimmunoprecipitation did not appear to contain ccdc53 which was previously stated as being a core complex member in mammalian cells (Derivery et al., 2009; Gomez and Billadeau, 2009), and was identified in the *D. discoideum* genome. To confirm whether it was part of the complex, we created a GFP-ccdc53 construct which was expressed in Ax2 cells and purified as described for GFP-WASH. Analysis by mass spectrometry revealed all complex members copurified with GFP-ccdc53; WASH, FAM21, Strumpellin and SWIP, however neither of the capping protein subunits were identified (fig. 3.1b). It has already been suggested that capping protein is not a constitutive member of the complex, and was only found in a proportion of the immunoprecipitations performed by Gomez and Billadeau (2009). Also, evolutionarily, the five core subunits are always found together in an organism, however capping protein is found in many other organisms and has many other known roles (Veltman and Insall, 2010). Finally, a third subunit, FAM21, was tagged with GFP, expressed in Ax2 cells and purified using GFP-Trap. Analysis of this sample by mass spectrometry confirmed the presence of all complex members, including ccdc53, and both subunits of capping protein (fig. 3.1c). Unfortunately, the gel was damaged and couldn't be imaged, however the mass spectrometry data identifying the complex members is shown instead.

FAM21 is known to contain a capping protein interaction site (CPI), the only one identified in the WASH complex (Hernandez-Valladares et al., 2010), and we have now shown that FAM21 copurifies with both subunits of capping protein. It may be that the interactions between capping protein with WASH and/or ccdc53 were not strong enough to remain intact through the purification process, however the direct interaction with FAM21 was plainly sufficient to allow purification of capping protein. (Note; this immunoprecipitation was done later in conjunction with other constructs, described in Chapter 5). Combining the results of these three immunoprecipitations, it is clear that WASH is present in *Dictyostelium* and indeed forms a complex within the cell with the same four

subunits identified in mammalian cells. It also suggests that there is a transient interaction between the complex and capping protein, potentially through FAM21.

3.2 WASH subunits form a constitutive complex

In both mammalian cells and Dictyostelium, WASH has been shown to localise to a subset of endosomal vesicles, where it is required for the formation of intermediate endosomal F-actin coats (Carnell et al., 2011; Derivery et al., 2009, Duleh and Welch, 2010; Gomez and Billadeau, 2009; Zech et al., 2011). We decided to investigate the localisation of the remaining subunits of the WASH complex in *Dictyostelium* to see whether the regulatory subunits were localised to the same endosomal vesicles, and constitutively colocalised with WASH.

Each subunit was N-terminally tagged with GFP and transfected into Ax2 cells. These cells were then fixed with paraformaldehyde and the actin stained with Texas Red-phalloidin. The GFP-tagged subunits all colocalised to vesicular structures in the cells with actin (fig. 3.2a) but not to any other part of the cell. To verify that the subunits were indeed constitutively colocalised with WASH, a single subunit, FAM21, was tested. A construct for coexpressing GFP-WASH and RFP-FAM21 was created and transfected in to Ax2 cells. The two proteins were observed by live cell imaging using a confocal microscope and appeared to be colocalised at all times (fig. 3.2b).

These results show that the five members of the WASH complex appear to be constitutively together as a complex, and do not have separate localisation to any other part of the cell individually. Colocalisation between FAM21 and WASH was also previously observed in HeLa cells, supporting this data (Gomez and Billadeau, 2009).

3.3 WASH complex nulls are blocked in exocytosis

WASH and the vesicular actin coat it creates are clearly important for correct trafficking of material through the endocytic cycle (Derivery et al., 2009; Gomez and Billadeau, 2009; Harbour et al., 2012; Zech et al., 2011) and accordingly, *Dictyostelium* WASH mutants have a defect in exocytosis (Carnell et al., 2011).

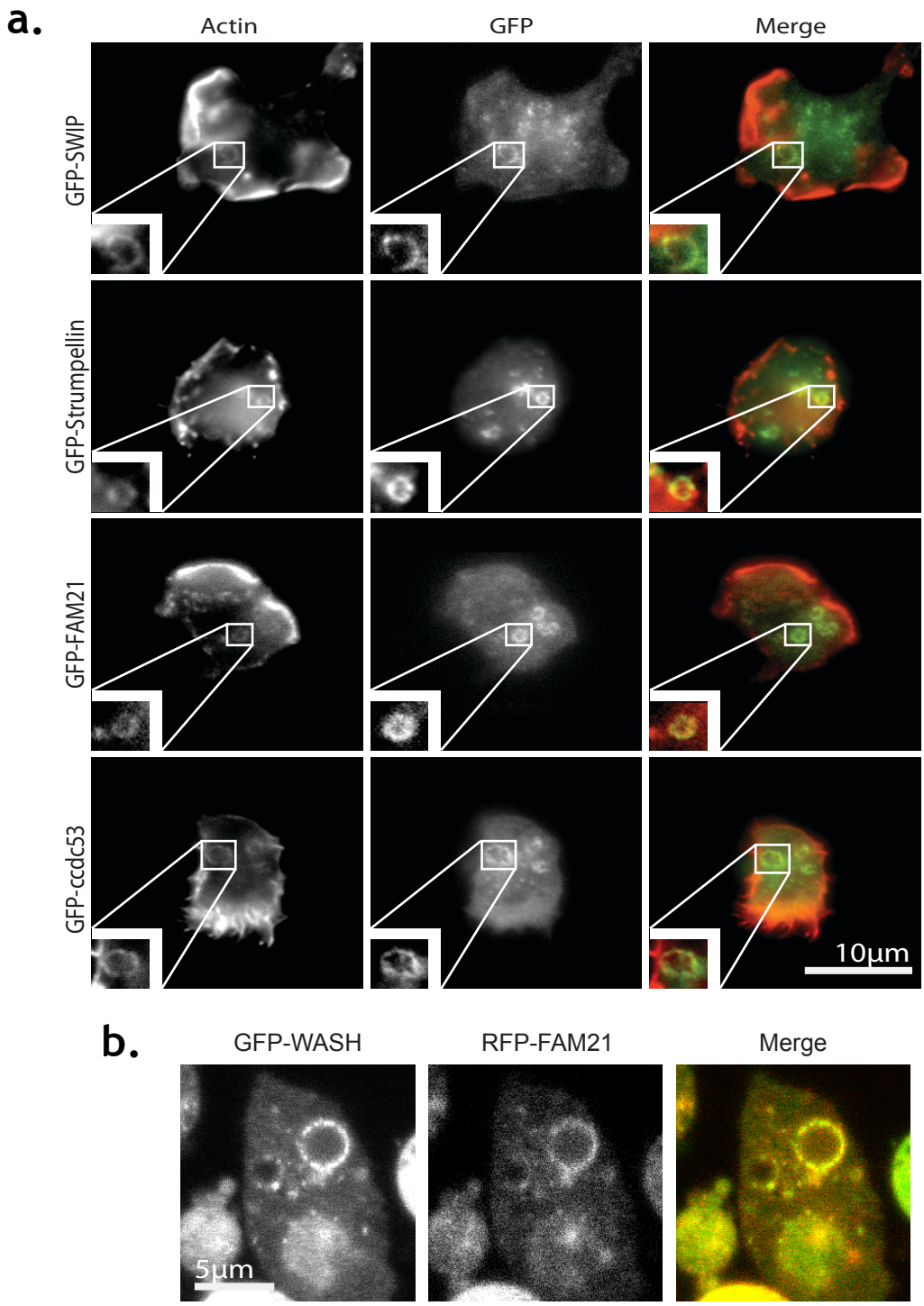


Figure 3.2 Colocalisation of WASH subunits

(a) GFP-SWIP, GFP-Strumpellin, GFP-FAM21 and GFP-ccdc53 were all expressed in Ax2 cells. Cells were fixed with paraformaldehyde and stained for actin with Texas Red-phalloidin. (b) GFP-WASH and RFP-FAM21 were coexpressed in Ax2 cells. Live cells were imaged on a confocal microscope.

The importance of each of the regulatory subunits in the function of the WASH complex has not been studied as yet, therefore we created a total knockout cell line by gene disruption of each of the subunits of the WASH complex. We first performed an exocytosis assay with each knockout cell line to determine whether exocytosis was blocked in the absence of each subunit.

Strumpellin and ccdc53 knockout cell lines were created by inserting a blasticidin resistance cassette into the corresponding gene. This cassette introduced a STOP codon at the site of insertion, and the disruption cassette was then transfected into cells whereby the endogenous gene would be disrupted by homologous recombination. Blasticidin was then used to select for cells successfully transfected, and PCR and/or western blotting was used to ensure the wild type gene had been replaced. FAM21 null and SWIP null cell lines were obtained from Torija et al. (2006). To perform the exocytosis assay, cells were grown in shaking culture overnight in medium containing FITC-dextran. The next day, cells were washed and resuspended in medium without dextran and shaken for another 5 hours. A sample of cells was taken at t=0 to calculate starting intracellular fluorescence, then samples were taken at intervals for 5 hours. The fluorescence of the subsequent samples after t=0 was recorded as a percentage of the initial value to indicate the amount of FITC-dextran which had been exocytosed from the cells.

We found that in all five knockout cell lines, WASH, FAM21, Strumpellin, SWIP and ccdc53 nulls, after 5 hours the internal fluorescence of the cells remained above 80% of the original level (fig. 3.3a) as previously seen in WASH null cells and were therefore blocked in exocytosis (Carnell et al., 2011). As none of the knockout cell lines were able to perform exocytosis, we can confirm that all five of the subunits are required for a fully functional WASH complex and that loss of any one subunit results in a block in exocytosis.

To confirm that there were no secondary effects and that the block in exocytosis was a direct result of the selected gene disruption in each case, we expressed the corresponding GFP-tagged protein in each cell line (for example, GFP-SWIP in SWIP nulls) to rescue the knockout phenotype. We repeated the exocytosis assay for each cell line and found that in all cases, the block in exocytosis was at

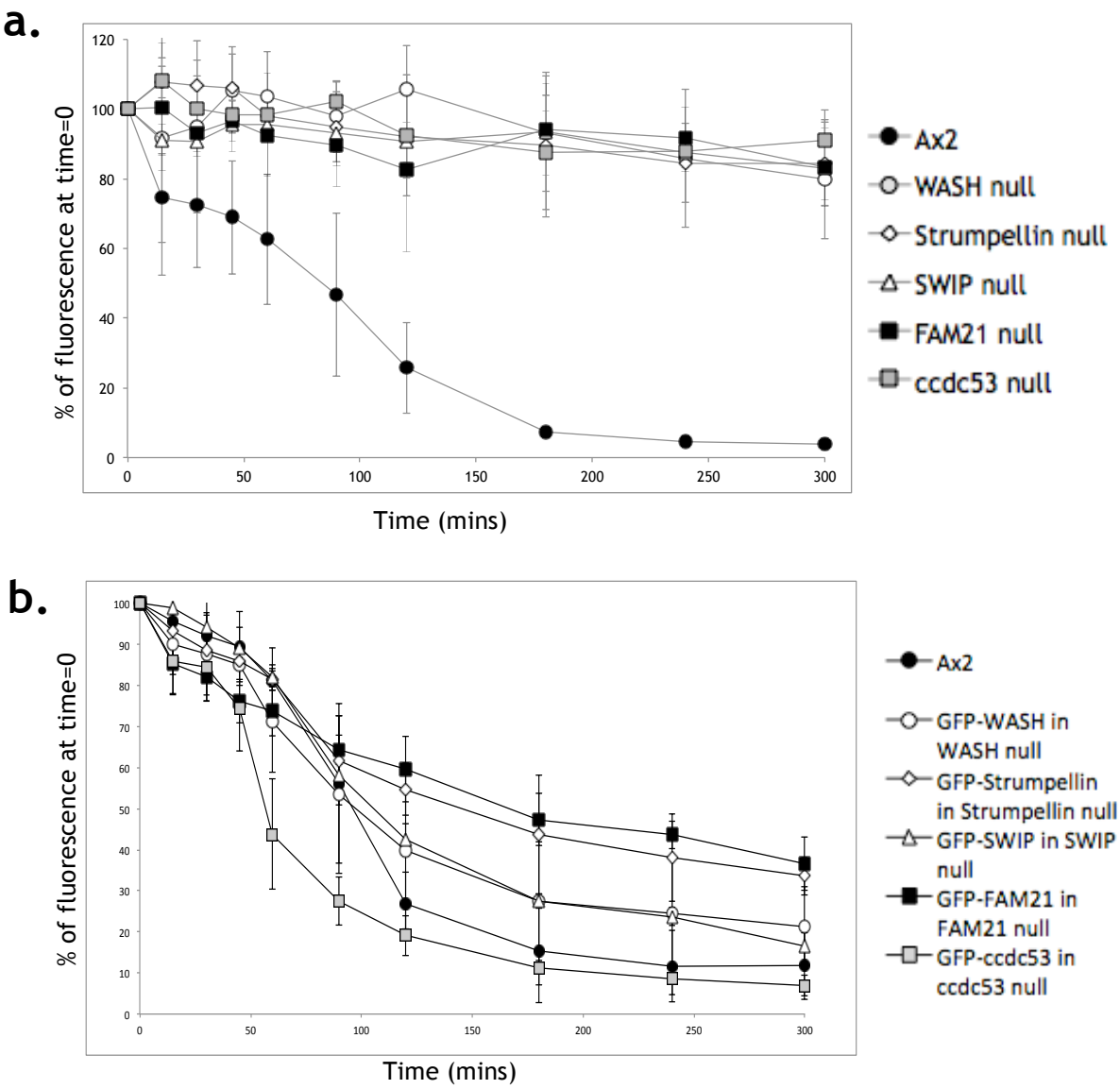


Figure 3.3 Exocytosis assay

(a) An exocytosis assay was performed for each WASH complex subunit knockout. Cells were incubated overnight in shaking culture in medium containing FITC-dextran. Cells were washed and resuspended in medium without dextran, and cell samples were taken at timepoints over 5 hours. The cell samples were lysed and the amount of FITC-dextran remaining in the cells at each timepoint was calculated by measuring the lysate fluorescence using a fluorimeter (n=3; error=SD). (b) Each null cell line was transfected with the corresponding GFP-tagged protein in order to rescue the null phenotype, and the exocytosis assay was performed as described.

least partially released, and the knockout phenotype rescued (fig. 3.3b).

3.4 Endocytosis is normal in WASH complex nulls

In WASH null cells, the process of endocytosis is not affected, and cells are still able to efficiently take up fluid by macropinocytosis at a normal rate (Carnell et al., 2011). The same has not yet been established for the remaining subunits. In order to confirm that the exocytosis defect we see in each knockout cell line is not due to a problem with initial endocytosis, we performed an endocytosis assay.

The endocytosis assay was very similar to the exocytosis assay except the FITC-dextran was added at t=0 rather than removed. Cells were grown in shaking culture overnight, and the next day FITC-dextran was added to the flask. At this time, t=0, a sample of cells was taken as a start point, then samples were taken at intervals up to 5 hours. The intracellular fluorescence was recorded and the increase in fluorescence relative to t=0 is shown in figure 3.4. All subunit knockout cell lines were able to endocytose the FITC-dextran at a normal rate for the first two hours, after which internal fluorescence continued to increase until the end of the experiment.

In Ax2 cells, equilibrium is reached after approximately 2 hours, where the amount of endocytosed FITC-dextran equals that being exocytosed from the cell (Klein and Satre, 1986; Neuhaus et al., 2002). WASH null cells do not reach this equilibrium; they are unable to perform exocytosis therefore as they continue to endocytose the FITC-dextran, the fluorescence continues to increase indefinitely (Carnell et al., 2011). Our results show that the subunit mutants appear to have no problem with the process of endocytosis, but as expected the inability to exocytose the FITC-dextran prevents the cells reaching equilibrium and internal fluorescence increases indefinitely.

3.5 Growth rates of WASH complex nulls

The liquid medium in which laboratory strains of *Dictyostelium* are normally grown contains nutrients for growth and survival but very little indigestible material. Indigestible products such as dextran can be added to the medium, and this has been shown to build up and cause a growth defect in WASH null

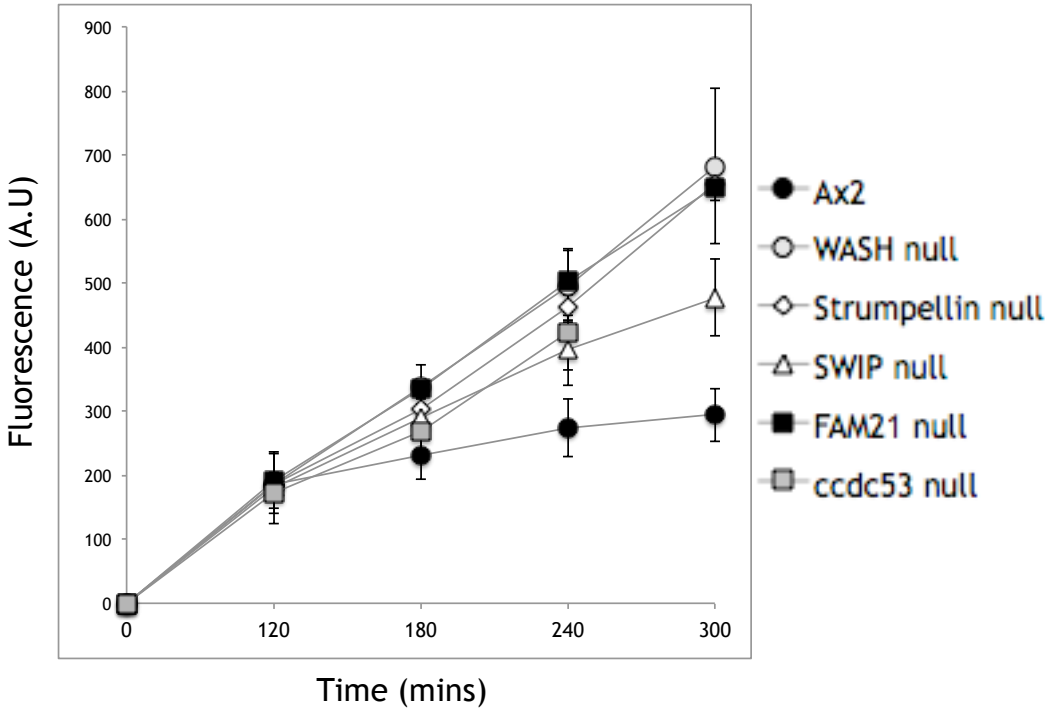


Figure 3.4 Endocytosis assay

An endocytosis assay was performed for each WASH complex subunit knockout. Cells were incubated overnight in shaking culture in medium. Cells were washed and resuspended in medium containing FITC-dextran, and cell samples were taken at timepoints over 5 hours, starting immediately. The cell samples were lysed and the amount of FITC-dextran taken up by the cells at each timepoint was calculated by measuring the lysate fluorescence using a fluorimeter (n=3; error=SD).

cells due to the fact that they can't perform exocytosis (Carnell et al., 2011). This is also true when WASH nulls are grown on bacterial plates, due to the large amount of intracellular debris produced by bacterial digestion. We hypothesized that the loss of any of the other WASH subunits would also incur a growth defect, as these cell lines were also unable to perform exocytosis.

In order to determine whether this was true, each knockout cell line was grown in medium with or without 10% unlabelled dextran (w/v) for two days. The cells were seeded at a known density, and at two timepoints over 48 hours, the cell densities of the cultures were measured again to calculate the doubling time (fig. 3.5). We found that the doubling times of WASH, SWIP and FAM21 nulls were not significantly different to that of Ax2 in medium without dextran, about 10 hours. Both Strumpellin and ccdc53 nulls surprisingly appeared to have a growth defect in medium without dextran, their doubling time increasing to approximately 15-20 hours. With the addition of dextran in the medium, all nulls exhibited a growth defect, however this was more severe in some than others. WASH, FAM21 and Strumpellin nulls all appeared to have an approximately 1.5 fold increase in doubling time in medium containing dextran, with WASH and FAM21 null times increasing to ~15 hours, and Strumpellin to ~25 hours. However ccdc53 and SWIP nulls both increased by 3-4 fold, to up to 40 hours.

Ax2 cells are able to exocytose indigestible material (Klein and Satre, 1986) therefore the addition of dextran to the medium does not affect their growth rate at all. The slow growth rate of Strumpellin and ccdc53 nulls in medium without dextran suggests that they play roles outside of the WASH complex in growth and/or division, as the cells appear to have a growth defect which is possibly independent of the endocytic cycle. They may be responsible in some way for the extraction of nutrients from the vesicles on which they are located, or cause the remaining partial complex to hinder these processes some way in their absence. The differences in growth rates in medium containing dextran, specifically for SWIP and ccdc53, are more likely attributed to their individual roles within the function of the WASH complex, which are as yet undefined.

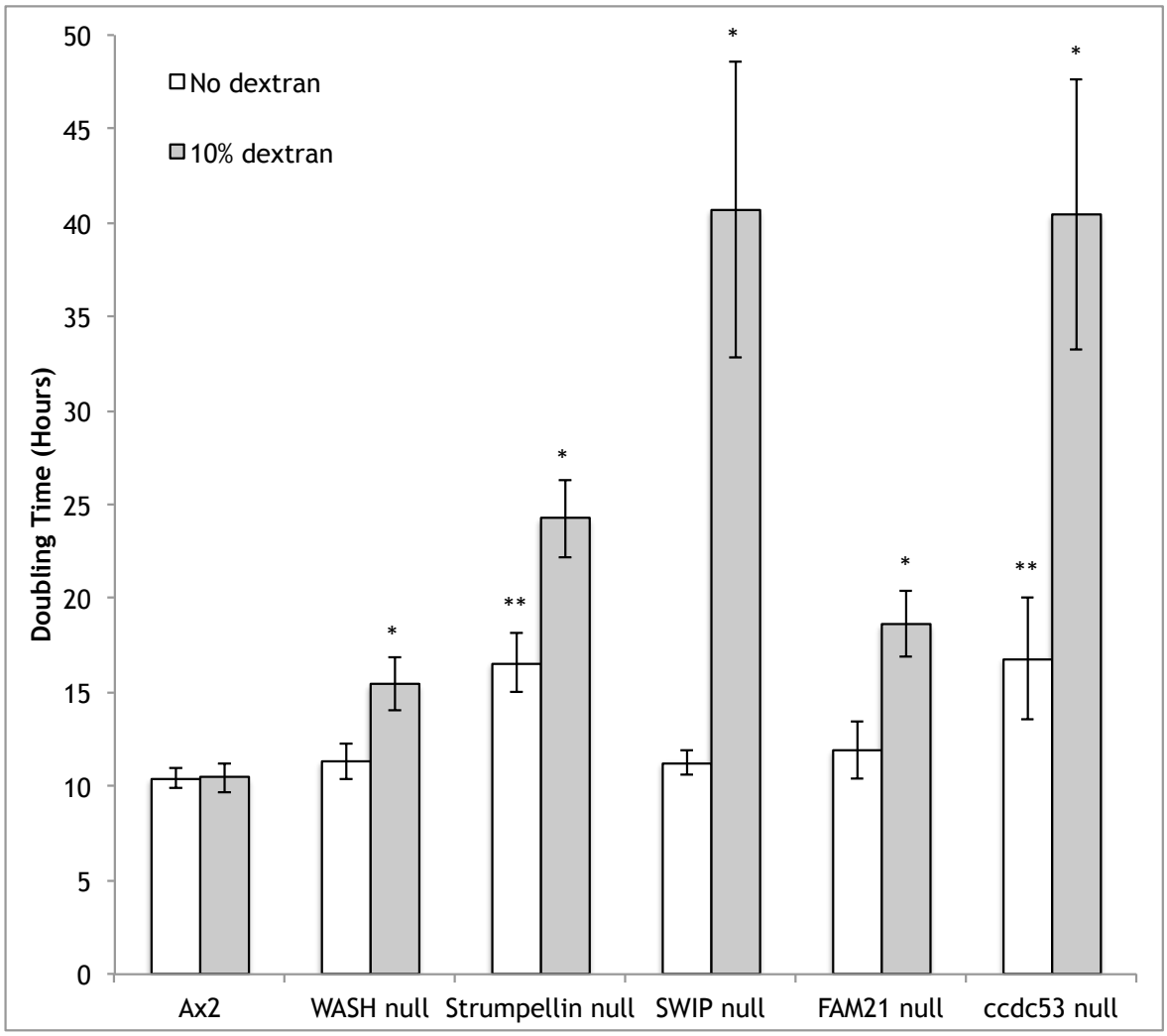


Figure 3.5 Doubling times of null cell lines

Null cell lines were grown in shaking culture in medium with or without 10% unlabelled dextran for two days. 10ml cultures were started at a cell density of $1x10^4$/ml. Cell counts were done at two timepoints, 24 and 42 hours (*significantly different to Ax2 in presence of 10% dextran, **significantly different to Ax2 without dextran, p=0.01, error=SD).

3.6 Localisation of subunits

In order to start to determine the individual roles of the subunits of the WASH complex, we decided to look into which of them were required for the correct localisation of the complex to endosomal vesicles. It has already been suggested in mammalian cells that the FAM21 subunit is the essential component for localisation of WASH (Gomez and Billadeau, 2009) therefore we wanted to see whether this was true for the *Dictyostelium* complex.

We expressed each of the GFP-tagged subunits in each of the null cell lines and determined whether or not the vesicular localisation of the protein was maintained in each case. We found that in all cases, vesicular localisation was maintained, except in the absence of SWIP (fig. 3.6 and table 3.2). Loss of any other subunit did not appear to affect the localisation of any member of the WASH complex, however in SWIP nulls, neither GFP-WASH nor GFP-Strumpellin were localised to vesicles, and instead were cytoplasmic. GFP-WASH was also expressed at a very low level. Both GFP-FAM21 and GFP-ccdc53 were unaffected by the loss of SWIP and maintained their localisation.

These results indicate that SWIP is the only subunit required for correct localisation of the WASH complex. It may act as a platform for binding of WASH and Strumpellin to endosomal membranes. Both FAM21 and ccdc53 can independently localise and therefore are likely to have their own localisation signals, not relying on any other subunits for recruitment. One interesting finding is that, unlike data obtained in mammalian cells, FAM21 is not required for WASH localisation in *Dictyostelium*.

3.7 Complex stability

The extrachromosomal vectors used to express the GFP-tagged subunits in *Dictyostelium* are usually expressed far more highly than endogenous genes. Therefore, as GFP-WASH appeared to be expressed at a very low level in SWIP nulls, this was indicative that endogenous WASH may also be reduced in these cells. Studies in mammalian cells also suggest that loss of FAM21 also causes a reduction in endogenous WASH protein (Gomez and Billadeau, 2009).

	GFP-WASH	GFP-FAM21	GFP-STRU	GFP-SWIP	GFP-CCDC53
AX2	Vesicular	Vesicular	Vesicular	Vesicular	Vesicular
WASH$^-$	Vesicular	Vesicular	Vesicular	Vesicular	Vesicular
SWIP$^-$	No localisation	Vesicular	No localisation	Vesicular	Vesicular
Strumpellin$^-$	Vesicular	Vesicular	Vesicular	Vesicular	Vesicular
FAM21$^-$	Vesicular	Vesicular	Vesicular	Vesicular	Vesicular
ccdc53$^-$	Vesicular	Vesicular	Vesicular	Vesicular	Vesicular

Table 3.2 Localisation of GFP-tagged constructs in mutants

As shown in figure 3.6, each GFP-tagged subunit was expressed in all subunit null cell lines. Localisation remained vesicular for all subunits in all nulls except SWIP null cells. GFP-WASH and GFP-Strumpellin were delocalised to the cytoplasm in SWIP- cells, however GFP-FAM21 and GFP-ccdc53 were unchanged. Loss of no other individual subunit affected complex localisation.

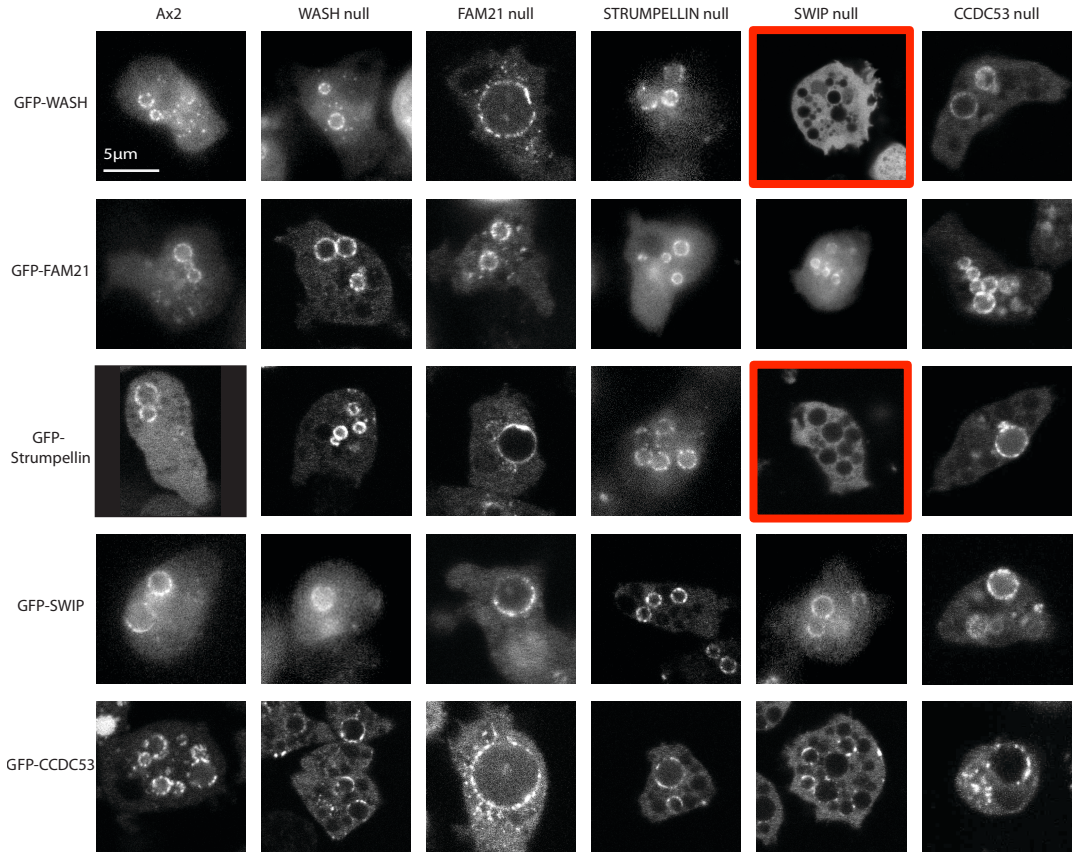


Fig. 3.6 Expression of subunits in all mutants

The results in Table 3.2 are illustrated here. Each GFP-tagged subunit was expressed in all null cell lines and imaged on a confocal microscope. A representative cell was chosen to indicate whether or not the GFP construct was able to maintain vesicular localisation in the cell line in which it was expressed. All subunits were able to maintain vesicular localisation except for those highlighted in red boxes; although GFP-WASH and GFP-Strumpellin were able to be expressed (albeit at low levels) in SWIP nulls, neither was able to localise to vesicles. In contrast, the absence of SWIP did not affect the localisation of either GFP-FAM21 or GFP-ccdc53.

As we had an anti-WASH antibody available, we decided to investigate the consequences of loss of each of the subunits on levels of endogenous WASH protein. Cells from each null cell line were lysed, and lysates were analysed by western blot to determine the relative amounts of endogenous WASH in each one. To ensure equal loading for all samples, total protein levels were measured on a photometer after addition of Precision Red protein assay reagent, and were normalized before loading. We found that the level of WASH in SWIP, Strumpellin or ccdc53 nulls was reduced to less than 13% that of the protein level in Ax2, however FAM21 nulls still retained 66% of WASH (fig. 3.7).

This confirms that not only SWIP, but also Strumpellin and ccdc53 are required to form a stable WASH complex, and that loss of any one subunit appears to lead to the degradation of WASH itself, with the exception of FAM21. This would explain the WASH null-like phenotype seen in these cell lines and why they are all blocked in exocytosis. This also supports the parallel seen between the WASH and Scar/WAVE complexes (Jia et al., 2010), whereby both complexes cannot survive incomplete.

3.8 Loss of endosomal actin coats

In mammalian and *Dictyostelium* cells, loss of WASH has been shown to cause a total absence of actin from the endocytic network (Carnell et al., 2011; Gomez et al., 2012). As WASH levels are drastically reduced in most of the subunit nulls, we wanted to confirm whether the intermediate vesicular actin coats were still present in these cells.

The null cell lines of each subunit were fixed with paraformaldehyde, and stained for actin using Texas Red-phalloidin, as was described previously. GFP-FAM21 was expressed in the cells in order to label the correct population of vesicles. We chose this marker as we have already seen that it constitutively colocalises with WASH, and is stable and able to localise to vesicles in all the mutants. To label the correct vesicles in FAM21 null cells, they were transfected with GFP-WASH. Over 50 cells were observed for each cell line, and none of WASH, Strumpellin, ccdc53 or SWIP null cells contained any visible F-actin on GFP-FAM21 labelled endocytic vesicles (fig. 3.8a). Fixation was unsuccessful for many of the FAM21 null cells for reasons discussed later,

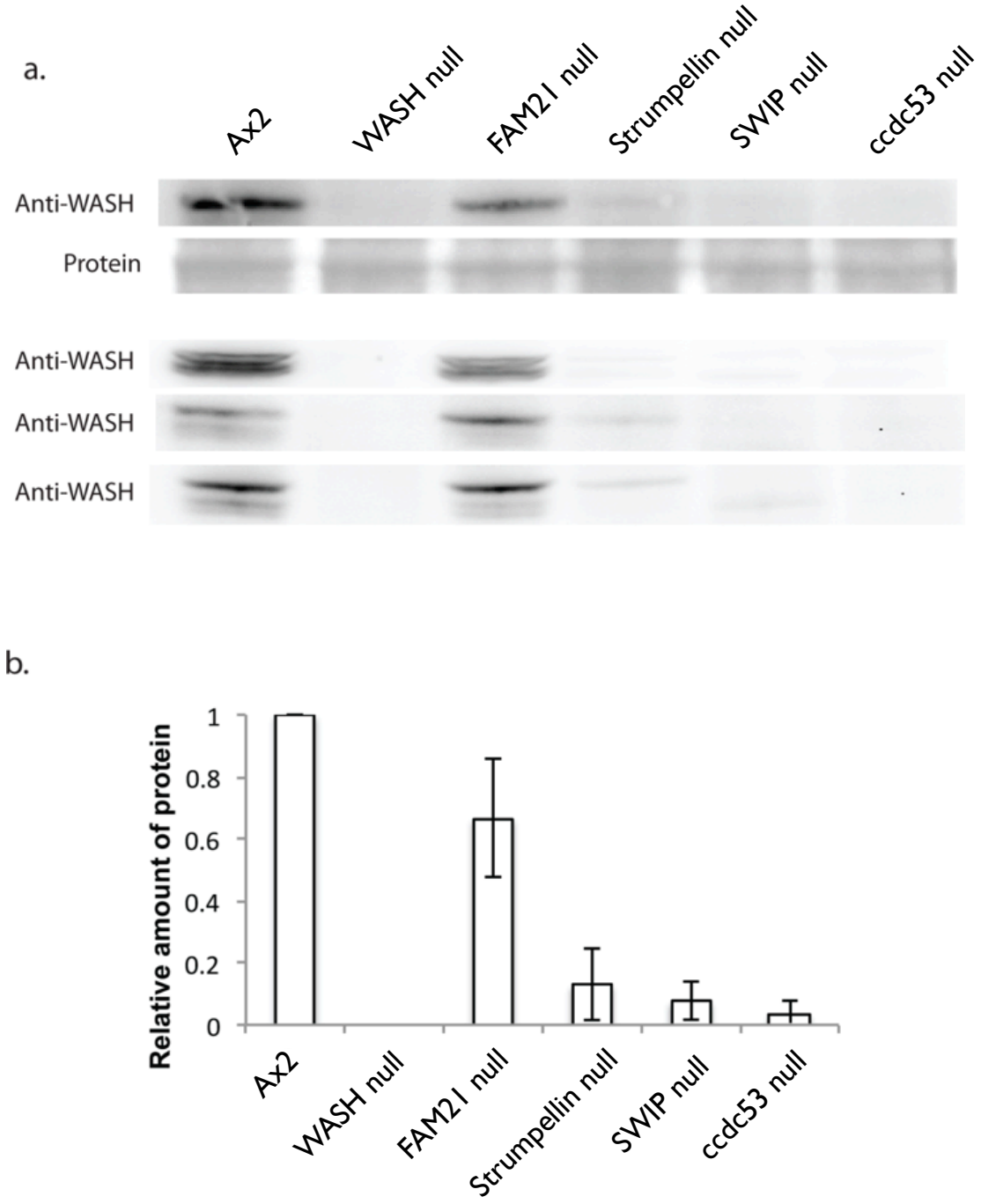


Figure 3.7 Endogenous levels of WASH protein in subunit nulls

(a) Cell lysates were taken from all subunit nulls. Total protein level of each lysate was measured by using Precision Red protein assay reagent and a photometer. Equal amounts of total protein were loaded into wells of a 4-12% Bis-Tris SDS-PAGE gel, before performing a western blot and detecting with anti-WASH. (b) The bands were analysed using ImageJ and the ratio of the amount of WASH between mutant and Ax2 was quantified (n=4; error=SD).

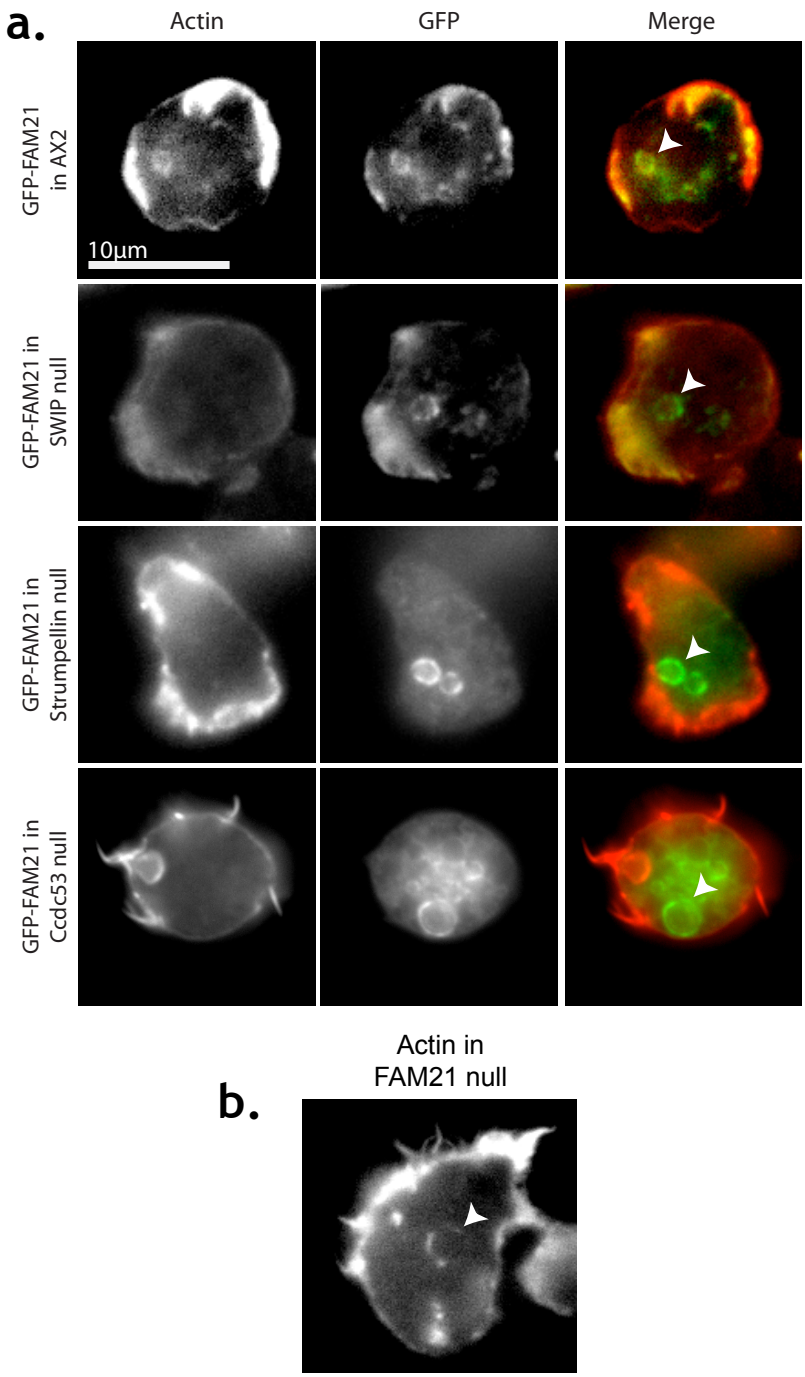


Figure 3.8 Detection of endocytic actin structures in nulls

(a) Subunit null cell lines were transfected with GFP-FAM21 to visualise WASH complex-decorated vesicles. Cells were then fixed with paraformaldehyde and stained with Texas Red-phalloidin to co-visualize actin. (b) GFP-WASH was expressed in FAM21 null cells to label endosomal vesicles. The GFP was disrupted by the fixation, however actin structures were visible on endosomal vesicles within the cells.

however a few cells were observed and vesicular actin structures were clearly visible (fig. 3.8b). Unfortunately, in no FAM21 null cells were the GFP-WASH and the actin coat both successfully fixed, therefore alternatives such as live cell imaging were used to confirm the presence of actin in these cells. This is discussed in detail in Chapter 4.

The absence of WASH in each of the subunit nulls, excluding FAM21, has resulted in the absence of intermediate endosomal actin coats in these cells. This further supports the idea that all the subunits except FAM21 are required for formation and stability of a WASH complex, and that partial complexes are unable to exist or function.

3.9 Lysosome maturation in nulls

Carnell et al. (2011) showed that the intermediate actin coats in Dictyostelium are required for the maturation of acidic lysosomes to neutral post-lysosomes. They show that the absence of WASH results in a total failure of lysosomes to neutralize, and that the endocytic pathway is blocked at this point in WASH nulls, with no further progression to exocytosis. This results in the accumulation of acidic lysosomes in the cytoplasm. As we know that the other subunit nulls also lack endosomal actin structures, we performed a neutralization assay to see whether neutral vesicles were absent from the cells, as with WASH nulls.

Dextran-conjugated fluorophores can be used to visualize the different stages of the endocytic pathway. Dextran specifically labels the endocytic vesicles of the cell, as opposed to the contractile vacuole system (Jenne et al., 1998). Red TRITC-dextran labels all endocytic vesicles, independently of pH, however green FITC is pH dependent, therefore FITC-dextran only fluoresces in vesicles with a neutral lumen (Aubry et al., 1993). This results in acidic vesicles appearing red, while neutral vesicles, which fluoresce red and green, appear yellow. We fed the cells a ratio of TRITC-dextran and FITC-dextran for approximately three hours to allow it to transit through the endocytic pathway of the cells. We then observed the cells to see whether there were any neutral vesicles present. The laser intensities of the microscope were normalized using control Ax2 cells which contain populations of both acidic lysosomes and neutral post-lysosomes. None of WASH, Strumpellin, SWIP or ccdc53 nulls contained neutral vesicles, all

containing only red, acidic lysosomes (fig. 3.9). In contrast, the FAM21 nulls did contain yellow, neutral post-lysosomes.

Again, the lack of WASH in the nulls gives them a WASH null-like phenotype, whereas the FAM21 nulls have a different phenotype. This is consistent with our data so far, that FAM21 null cells contain a partial but stable WASH complex, and endosomal actin structures unlike any of the other nulls. Because of this difference, we decided to study specifically the FAM21 subunit in more depth. These cells are still blocked in exocytosis however it appears it may not be at the same acidic lysosomal stage as the other nulls.

3.10 Strumpellin mutations in *Dictyostelium*

The absence of Strumpellin in *Dictyostelium* cells causes a WASH null-like phenotype, where cells are unable to perform exocytosis. Human Strumpellin has been identified as the protein which causes a form of spastic paraplegia known as SPG8 (Hedera et al., 1999; Reid et al., 1999). There are three specific mutations, N471D, L619F and V626F, each of which can individually result in SPG8 (Valdmanis et al., 2007). We were interested to see whether the *Dictyostelium* Strumpellin protein contained these mutated sites and if the mutations had an effect on the function of the WASH complex.

We aligned the *D. discoideum* and *H. sapiens* Strumpellin proteins to reveal that they are extremely conserved, with over 50% homology between them, and are 100% homologous in the regions containing the mutations (fig. 3.10a). We therefore engineered the corresponding *Dictyostelium* mutations, N459D, L607F and V614F, into expression constructs for *Dictyostelium* to see whether these mutations had a direct detrimental effect on the function of the WASH complex. We transfected Strumpellin null cells with each of the three mutant constructs to see how effectively each one could rescue the null phenotype. To test this, we performed the exocytosis assay on each of the cell lines. We found that all three mutants were able to rescue the exocytosis defect of the cells as efficiently as wild type GFP-Strumpellin (fig. 3.11b). It appears that these mutations do not hinder the function of Strumpellin when incorporated into the WASH complex, and that cells containing these mutants still have a functional endocytic cycle.

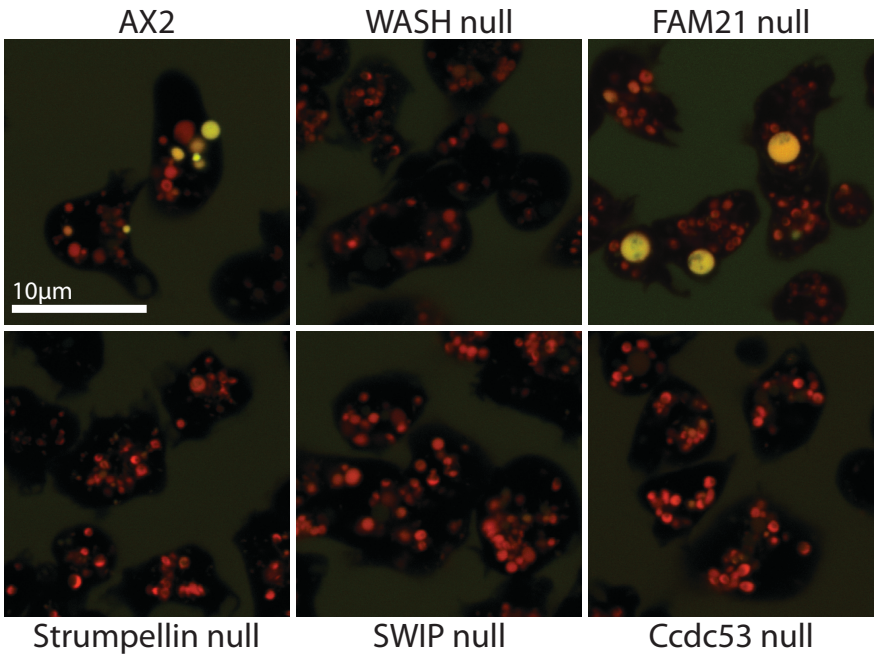
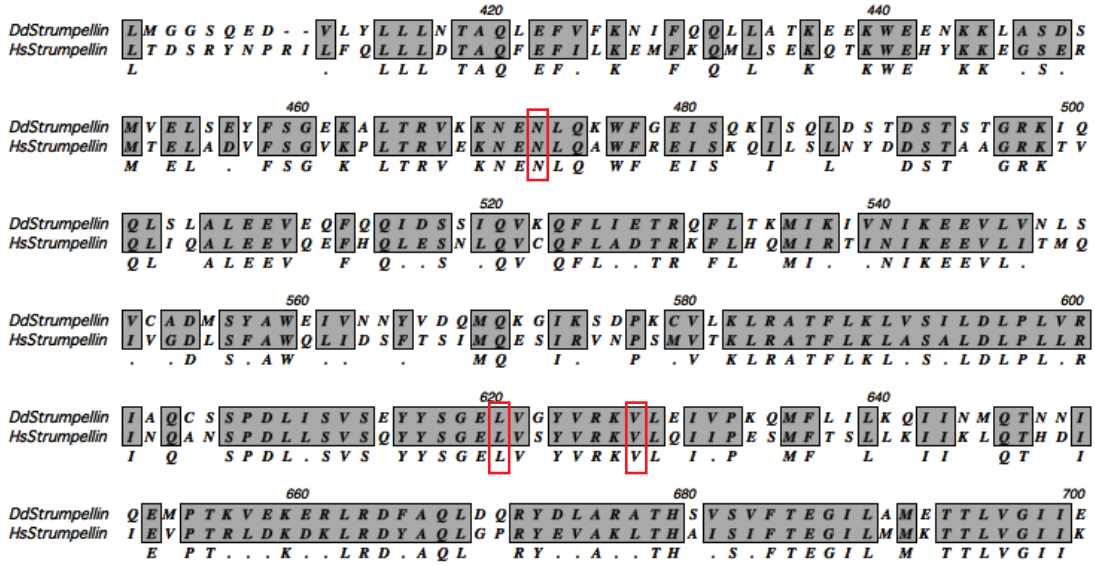


Figure 3.9 Neutralization assay with WASH subunit nulls

Cells were fed 0.4mg/ml FITC-dextran and 4mg/ml TRITC-dextran for 3 hours before being imaged on a confocal microscope. Ax2 control cells were used to adjust the laser intensities to visualize the FITC-dextran at a point just below saturation, and the TRITC-dextran accordingly to ensure the contrast between the populations of vesicles containing fluorescent FITC (neutral) and quenched FITC (acidic).

a.



b.

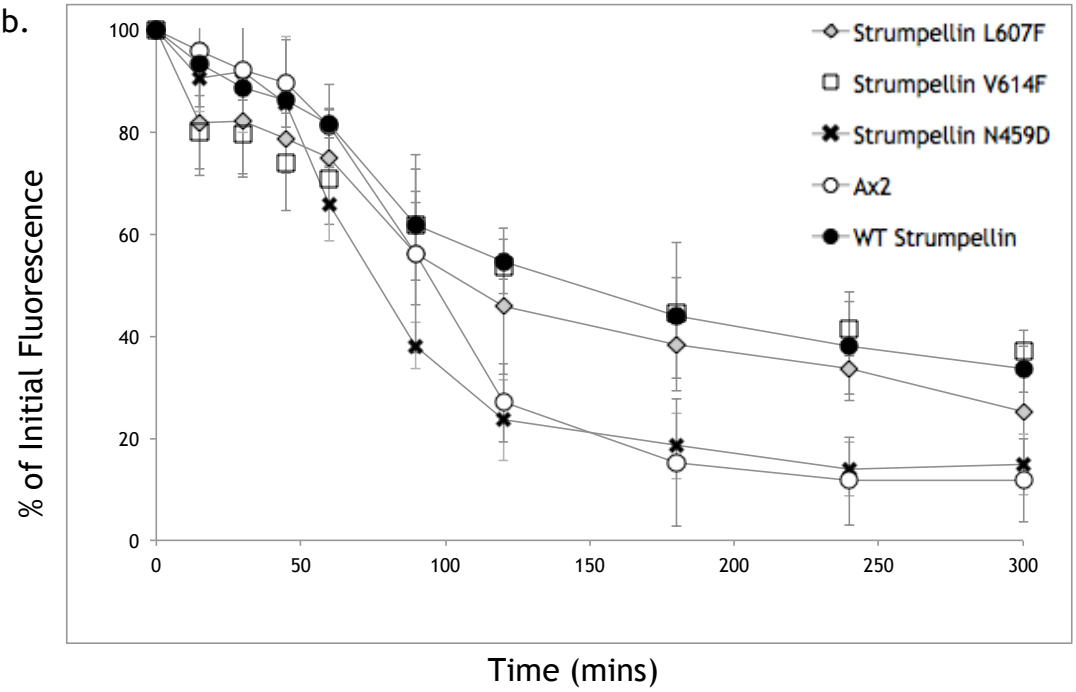


Figure 3.10 Strumpellin SPG8 mutants

(a) Human and *Dictyostelium* Strumpellin sequences were aligned. The sites of the human mutations and their corresponding position in the Dictyostelium sequence are highlighted in red. (b) The three *Dictyostelium* Strumpellin mutant constructs were each transfected into Strumpellin null cells and the exocytosis assay was performed (n=3; error=SD).

CHAPTER SUMMARY

In this first chapter, we have shown that the WASH complex requires all four of the regulatory subunits in order for WASH to function as an NPF and direct the building of intermediate endosomal actin coats. These actin coats are required for the maturation of lysosomes to post-lysosomes by the removal of V-ATPase. A failure to remove this prevents exocytosis, therefore loss of any one member of the WASH complex blocks exocytosis.

We also have shown that the subunit SWIP is essential for the recruitment of the WASH complex to endosomal membranes, however FAM21 and ccdc53 both also contain their own localisation signals and are able to localise independently of any other subunits. Also, the mutations in Strumpellin that cause spastic paraplegia in humans do not appear to affect the function of the WASH complex in *Dictyostelium*.

The most interesting finding is that one of the subunits, FAM21, has a different phenotype to any of the other subunits. Loss of WASH, Strumpellin, SWIP and ccdc53 causes a block at the same acidic lysosomal stage of the endocytic cycle in cells. This is because these four subunits are all required to form a stable complex in cells. FAM21 is dispensable for complex formation, and stable WASH protein is expressed in the cells. Because loss of this particular subunit gives rise to such a different phenotype, we have made it the main focus of our study.

CHAPTER 4
THE ROLE OF FAM21 IN THE WASH COMPLEX

4 The Role of FAM21 in the WASH complex

4.1 FAM21 nulls contain enlarged endosomal vesicles

The loss of any one of the WASH complex subunits gives rise to a WASH null-like phenotype, with the exception of FAM21. One of the key differences we have shown between FAM21 and WASH null cells is that FAM21 cells contain neutral endosomal vesicles (fig. 3.10). While performing the neutralization assay, we observed that the neutral vesicles contained in the FAM21 nulls appeared noticeably larger than those seen in Ax2 cells. This swelling of endosomal vesicles seemed to be a result of feeding the cells dextran, which is indigestible and would therefore accumulate in endosomes in cells which are unable to perform exocytosis. In order to confirm this, we fed the cells labelled dextran and compared the size of the vesicles they contained.

Ax2, WASH null and FAM21 null cells were all incubated in 5% unlabelled dextran in medium overnight, except for one dish of FAM21 nulls kept in medium without dextran as a control. The next day, cells were all incubated with 1mg/ml TRITC-dextran in the medium for 3 hours before being imaged to label the endosomal compartments. Figure 4.1a illustrates the vesicles observed in each cell type. The WASH null cells contained numerous TRITC-filled vesicles which were all slightly bigger than the vesicles seen in Ax2 cells. The FAM21 nulls incubated in unlabelled dextran overnight all contained a single, giant TRITC-filled vesicle in each cell. The control FAM21 nulls which had not previously been incubated in unlabelled dextran did not appear to contain any vesicles bigger than those seen in Ax2 cells. To quantify the size difference, the diameter of the biggest vesicle in each cell was measured. These measurements were used to calculate an average of the cross-sectional area for the largest vesicles present in cells of each cell line (fig. 4.1b). Ax2 cells rarely contained vesicles greater than 3μm^2. WASH null vesicles were on average double the size of those in Ax2, but never greater than 8μm^2, whereas FAM21 null cells commonly contained vesicles exceeding 30μm^2, 10 times that of Ax2 cells.

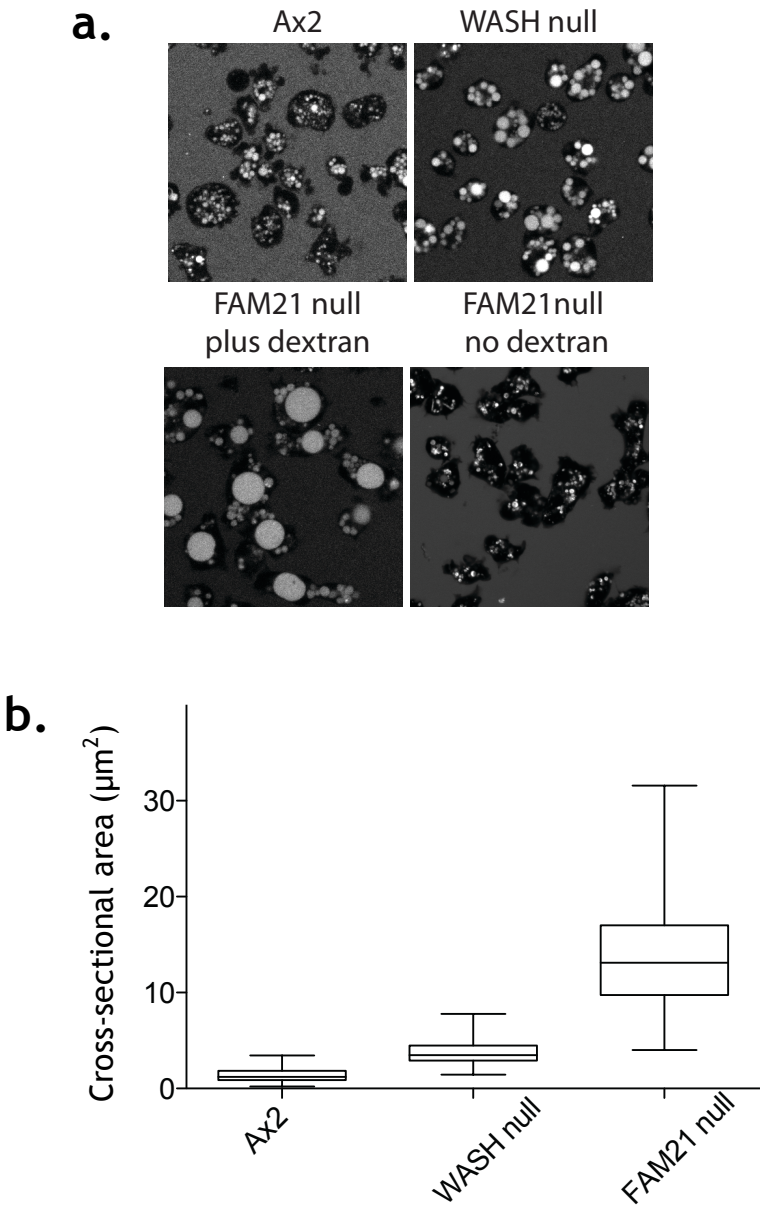


Figure 4.1 Imaging and quantification of vesicle size in FAM21 null cells Ax2, WASH and FAM21 null cells were incubated overnight in medium plus 5% unlabelled dextran. One control dish of FAM21 null cells was incubated in medium without dextran. (a) The following day, all cells were incubated with TRITC-dextran for 3 hours before imaging on a confocal microscope. Experiment was performed on 3 separate days, each time 10 images for each cell line were taken. (b) Cross-sectional area of the largest vesicle in each cell was calculated by measuring the diameter using the images captured. Average vesicle area was 1.4μm^2 for Ax2 cells, 3.7μm^2 for WASH null cells, and 14μm^2 for FAM21 null cells (n=100).

Ax2 cells are able to exocytose indigestible material such as dextran, therefore it never builds up within the endosomal system to a degree which causes much of an increase in endosomal vesicle size. WASH nulls are unable to exocytose the dextran, therefore their endosomal vesicles are prone to swell as the cells take up more and more dextran. The fusion of acidic lysosomes is highly regulated, and after initial fusion events, the lysosomes such as those seen in WASH nulls remain relatively separate from one another (Clarke et al., 2002). This is why numerous, slightly larger vesicles are present in these cells. In the control FAM21 null cells, the small amount of TRITC-dextran fed to label the cells is not sufficient to cause vesicle enlargement, however the FAM21 null cells fed a large amount of unlabelled dextran overnight each contained a single vesicle of an immense size. The fact that the size of this vesicle is dependent on the presence of indigestible material in the medium, and that there is only one per cell in nearly all cases, suggests that all the dextran is being trafficked into this one compartment, or that all dextran-containing vesicles are fusing together.

4.2 Presence of intermediate endosomal actin

We had previously detected endosomal actin structures in fixed FAM21 null cells (fig. 3.9) however these cells, unlike the other subunit null cell lines, appeared to be difficult to fix effectively. It is apparent now that this may have been due to the presence of the enlarged vesicles within these cells. In order to better observe actin structures in FAM21 nulls, we tried a different tact by using live cell imaging. In Ax2 cells, endosomal actin structures are visible with live cell imaging, but image quality is poor due to the small size of the vesicles and the thinness of the actin coat. Therefore for most cells, fixation and staining is a much better way to observe these structures. As FAM21 nulls have larger vesicles, we hypothesized that the actin might be more clearly visible in live cells.

We transfected FAM21 nulls, and Ax2 cells for comparison, with RFP-actin. The cells were then incubated in unlabelled dextran overnight before imaging. Faint actin coats were observed in the Ax2 cells (fig. 4.2a), however the actin observed on the vesicles in FAM21 nulls was extremely clear (fig. 4.2b). On the enlarged vesicles in FAM21 nulls, huge comets of actin were seen streaming off

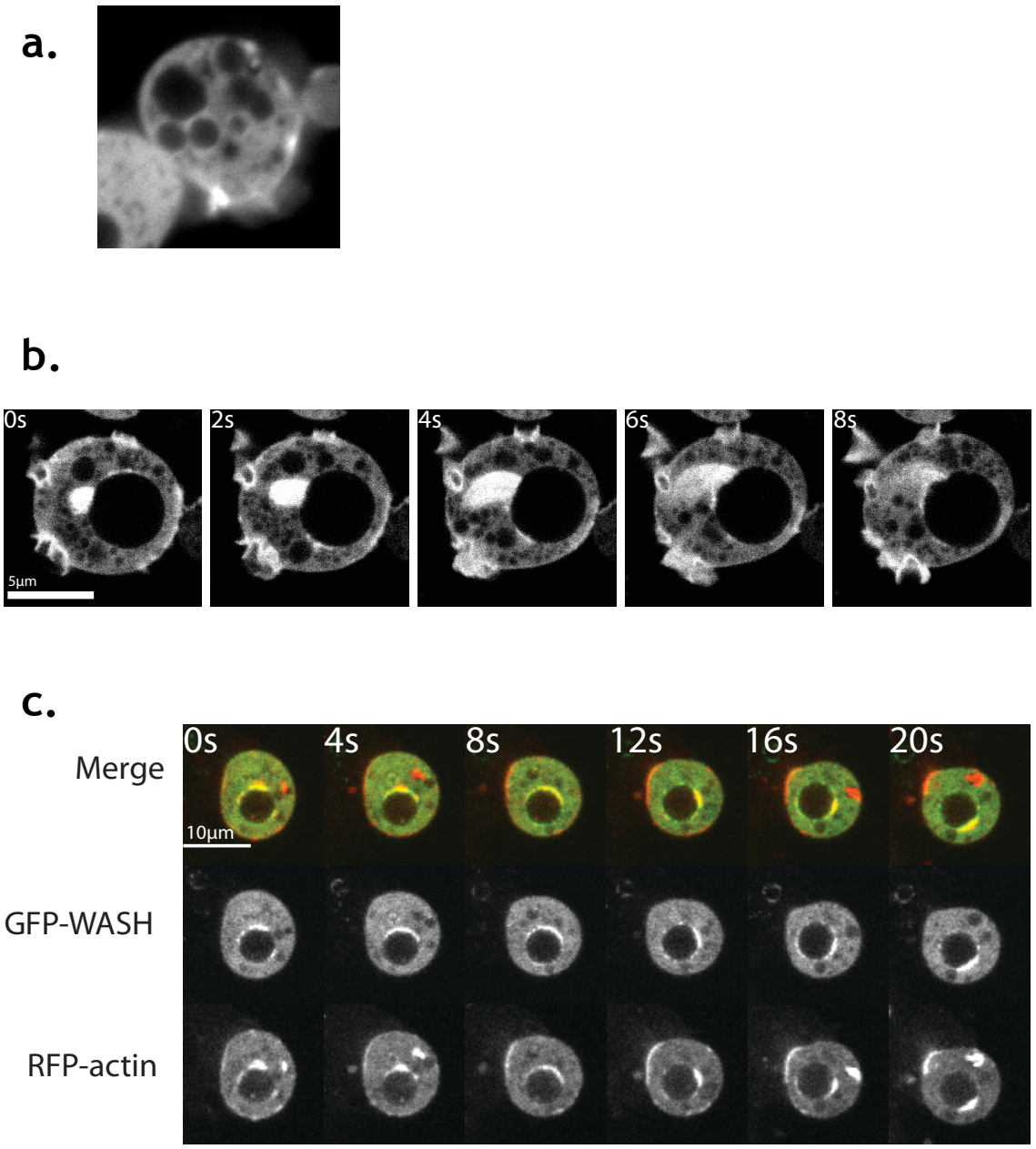


Figure 4.2 Actin in FAM21 null cells

RFP-actin was expressed in Ax2 and FAM21 nulls, and cells were imaged on a confocal microscope. (a) Images were captured of Ax2 cells, and an actin coat is indicated by the red arrow. (b) A sequence of images was captured for FAM21 nulls, with frames taken every 2 seconds to record the dynamic movement of the actin comet on the enlarged vesicle. (c) GFP-WASH was coexpressed with RFP-actin in FAM21 nulls and images were captured every 4 seconds on a confocal microscope.

the vesicles. These streams seemed to travel around the surface of the vesicle, although it was unclear whether this was due to the rotation of the vesicle, or the actin actively moving across the membrane. This actin comet did not appear to be responsible for movement as the enlarged vesicles remain relatively stationary in cells, unlike smaller vesicles which move rapidly within the cytoplasm.

To confirm that WASH was responsible for this excessive actin production, we coexpressed GFP-WASH with RFP-actin in FAM21 nulls. These cells were again incubated overnight in unlabelled dextran before imaging. Again the actin comets were observed in the enlarged vesicle membranes and we saw that these colocalised to patches of GFP-WASH on the membrane (fig. 4.2c). This confirms that WASH appears to be polymerizing excessive actin to form these comet structures on the surface of the enlarged vesicles in FAM21 nulls.

4.3 Lysosome neutralization and maturation

As we see neutralization occurring in FAM21 nulls, we hypothesized that potentially the vesicles were progressing further along the endocytic pathway in a FAM21 null than in a WASH null. This would mean that V-ATPase was being removed from lysosomes and they were able to mature to post-lysosomes. Alternatively, there could be deregulation of fusion between compartments in FAM21 nulls. For example, Jenne et al. (1998) show that loss of vacuolin B results in deregulation of fusion between endocytic compartments, resulting in the aberrant fusion between early and late endosomes. To confirm that the neutral vesicles in FAM21 nulls were true post-lysosomes, we decided to look at the dynamics of the V-ATPase complex in the cells.

Ax2, WASH null and FAM21 null cells were transfected with vatB-GFP, the subunit used to observe the V-ATPase complex by Carnell et al. (2011). The cells were fed a short pulse (10 minutes) of TRITC-dextran for which we then observed the transit through the endocytic cycle (fig. 4.3). In Ax2 cells, after approximately 1 hour, the TRITC-dextran had been trafficked into vesicles which were no longer labelled with vatB-GFP. WASH null cells were left for an extended time of 3 hours, but even after this time, all the TRITC-dextran remained contained within vesicles decorated with vatB-GFP. FAM21 nulls

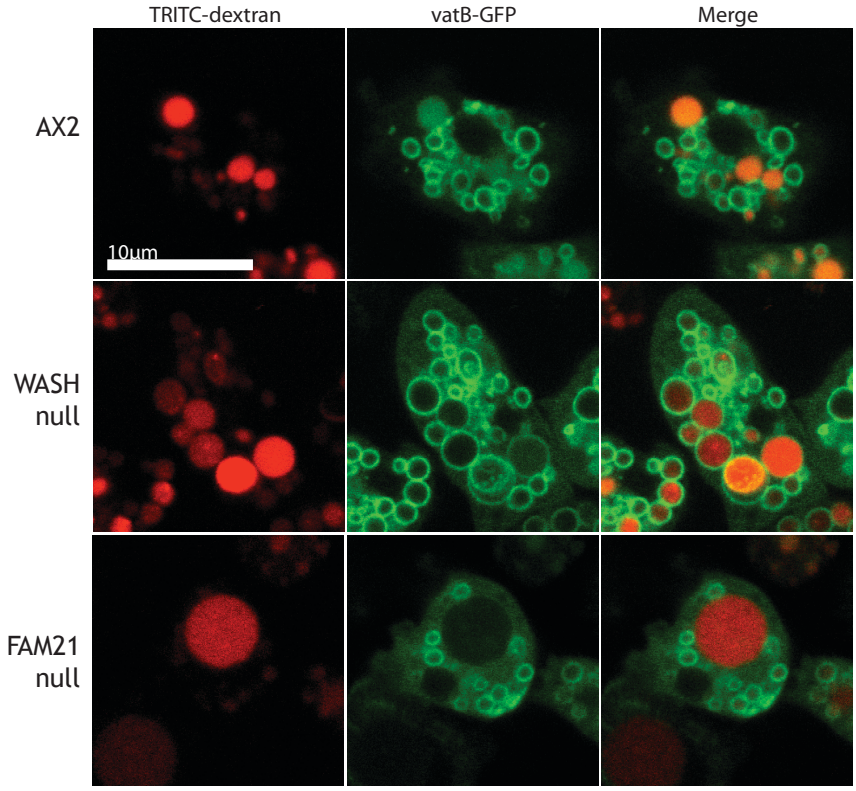


Figure 4.3 V-ATPase localisation in cells

Ax2, WASH null and FAM21 null cells expressing vatB-GFP were fed a pulse of TRITC-dextran for 10 minutes, which was then washed off and chased through the cells with LoFlo medium containing unlabelled dextran. Cells were observed over time for up to 3 hours. Images were captured on a confocal microscope after 1 hour for Ax2, and after 3 hours for WASH and FAM21 nulls.

seemed to transit the TRITC-dextran more slowly through the cells, however TRITC-dextran was transited through acidic compartments into the enlarged compartment within 3 hours, which was not labelled with vatB-GFP.

In an Ax2 cell, dextran takes approximately 40-60 minutes to transit through to neutral compartments (Clarke et al., 2002) which is what we observed. As expected, in the WASH nulls, the TRITC-dextran remained within V-ATPase-decorated vesicles from which progression is blocked. In the FAM21 nulls, the TRITC-dextran was able to progress from the acidic compartment into the neutral compartment. We can confirm from this that V-ATPase is indeed being removed actively from lysosomes in FAM21 null cells, to allow maturation into post-lysosomes. This, in combination with the presence of endosomal actin in these cells, confirms that the WASH complex is functional in removing V-ATPase without the presence of the FAM21 subunit.

4.4 FAM21 functions downstream of WASH

We have now shown that WASH is able to function, at least in part, in the absence of FAM21, however the function of FAM21 is still vital to the completion of the endocytic cycle, as FAM21 nulls are still unable to perform exocytosis. Vesicles are also clearly able to progress further along the endocytic cycle in FAM21 nulls than in WASH nulls, therefore these data suggest that FAM21 has a function in the endocytic cycle which is downstream of WASH itself. In order to confirm this, we created a double WASH null/FAM21 null cell line.

The WASH null cell line was made sensitive to blasticidin by expression of Cre recombinase in cells, removing the blasticidin resistance cassette flanked by loxP sites. This allowed us to insert another knockout construct for FAM21 into these cells, and select cells using blasticidin resistance again. The double knockout phenotype was then confirmed using PCR. To determine the phenotype of the double null cells, we performed a neutralization assay, as previously described. We found that the double nulls contained no neutral vesicles, showing a WASH null-like phenotype (fig. 4.4a). We then expressed either GFP-WASH or GFP-FAM21 in the cells to see whether this would rescue the phenotype, at least in part. Upon expression of GFP-WASH, the cells converted to a FAM21 null-like phenotype, displaying enlarged neutral vesicles (fig. 4.4b).

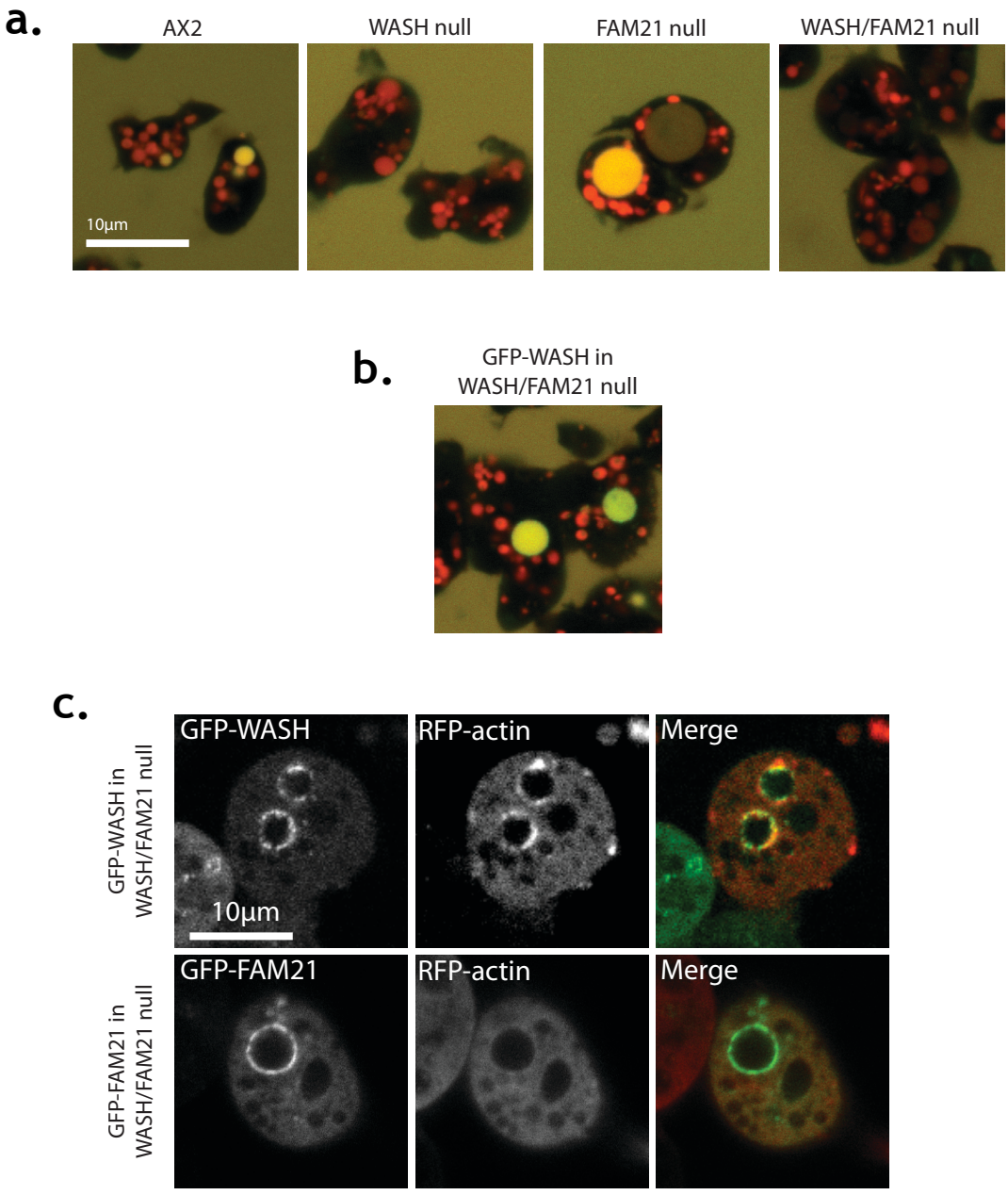


Figure 4.4 Double WASH/FAM21 null cells

(a) The neutralization assay was performed as previously described, revealing that the double WASH/FAM21 null cells did not contain any neutral vesicles. (b) Expression of GFP-WASH in the double nulls partially rescued the phenotype of the cells, and enlarged neutral vesicles were observed. Note; GFP-WASH is not visible with the acquisition settings used for the neutralization assay. (c) Double null cells coexpressing GFP-WASH or GFP-FAM21 with RFP-actin were imaged using a confocal microscope.

We also looked at the presence of actin in the cells. Coexpression of GFP-WASH with RFP-actin revealed the presence of endosomal actin coats (fig. 4.4c). In contrast, the expression of GFP-FAM21 in the cells did not alter the phenotype at all. Cells remained WASH null-like, and when coexpressed with RFP-actin there were no visible endosomal actin structures within the cells.

Expression of FAM21 is unable to rescue the phenotype of the double null cells because its function is downstream of the point at which the cells are blocked by loss of WASH. However, expression of WASH can partially rescue the cells because its action is upstream of the blockage caused by loss of FAM21. This shows how these two subunits, although constitutively part of the same complex, function at different points within the endocytic cycle, with the function of WASH preceding that of FAM21.

4.5 WASH and FAM21 in mammalian cells

It has been previously shown that knockdown of WASH in A2780 ovarian carcinoma cells impairs the ability of the cells to recycle internalized α5β1 integrin back to the cell surface via the recycling pathway (Zech et al., 2011). The integrin is retained within the cells, and may be mis-sorted into the degradative pathway. The recycling pathway of mammalian cells is the most similar endocytic route to that of *Dictyostelium* (Maniak, 2003), therefore we used this assay to determine whether FAM21 and WASH also have separate roles in mammalian cells, as we have shown in *Dictyostelium*.

NT, siWASH and siFAM21 A2780 cells were incubated with biotin to label cell surface proteins. The cells were then incubated at 37^{o}C to allow the internalization of the labelled proteins. After removing the remaining biotin-labelled proteins from the surface, the cells were incubated again for a specified time period to allow internalized proteins to be recycled back to the plasma membrane. The cell surface was stripped of biotin once more before cells were lysed. The lysate was then used in a capture ELISA assay to immobilise the biotin-labelled proteins that had remained inside the cell. An anti-integrin antibody was then used to detect the amount of α5β1 on the ELISA plate. This quantified the amount of biotin-labelled α5β1 integrin which had remained in the cell rather than being recycled back to the plasma membrane.

Figure 4.5 shows that recycling in siWASH cells was reduced compared to NT cells, as seen before by Zech et al. (2011). However siFAM21 cells were able to recycle the integrin as efficiently as NT cells, and the integrin was not retained inside the cells.

These results can be paralleled to the V-ATPase recycling in *Dictyostelium* cells; WASH is required for removal and recycling of V-ATPase complexes (Carnell et al., 2011), however we have shown that FAM21 is not required for this process, and V-ATPase recycling occurs normally in its absence. In a similar way, mammalian cells require WASH for recycling of α5β1 integrin, however we have now shown that they do not require FAM21. This confirms that in both *Dictyostelium* and mammalian cells, WASH and FAM21 appear to have separate functions within the endocytic pathway.

4.6 Delay in lysosome neutralization

We noticed whilst observing the transit of TRITC-dextran through FAM21 null cells that the dextran took longer to reach the neutral compartment than in Ax2 cells. We decided to quantify this by observing the neutralization process in the cells.

Ax2 and FAM21 nulls, incubated overnight in unlabelled dextran or not, were fed FITC- and TRITC-dextran in order to mark endosomal compartments and indicate pH, as previously described. We performed the assay with FAM21 null cells both pre-fed and not fed unlabelled dextran to rule out the possibility that the presence of the enlarged vesicle in the cells was the cause of the delay in transit. In Ax2 cells, the first neutral vesicles began to appear after approximately 40-60 minutes (fig. 4.6). In FAM21 nulls, whether the cells had been pre-fed unlabelled dextran or not, no neutralization was observed for approximately 3 hours.

FAM21 nulls are able to mature lysosomes into neutral post-lysosomes as they contain functional WASH which removes V-ATPase. However, the process of neutralization appears to take 3 times as long as in Ax2 cells. This is not dependent on whether the post-lysosomal vesicles are swollen and enlarged in

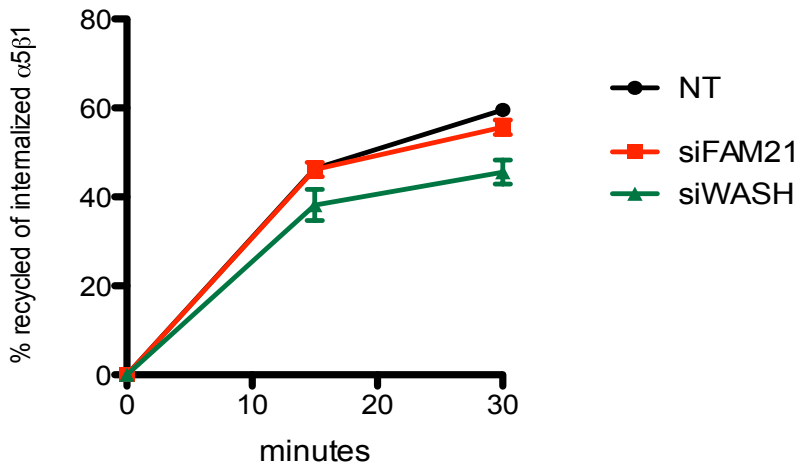


Figure 4.5 Integrin recycling assay

Surface proteins of NT, siWASH and siFAM21 A2780 cells were labelled with biotin. Cells were incubated to internalize labelled proteins, and biotin was removed from the cell surface. Cells were incubated again to allow recycling of labelled proteins to the surface. Cells were lysed, and labelled proteins which remained intracellular were purified by capture-ELISA. α5β1 integrin was then detected using an anti-integrin. A control whereby no recycling was permitted was used to calculate the total intracellular α5β1 integrin, and above is shown the relative percentage of α5β1 integrin recycled by each cell line (n=3, error=SD).

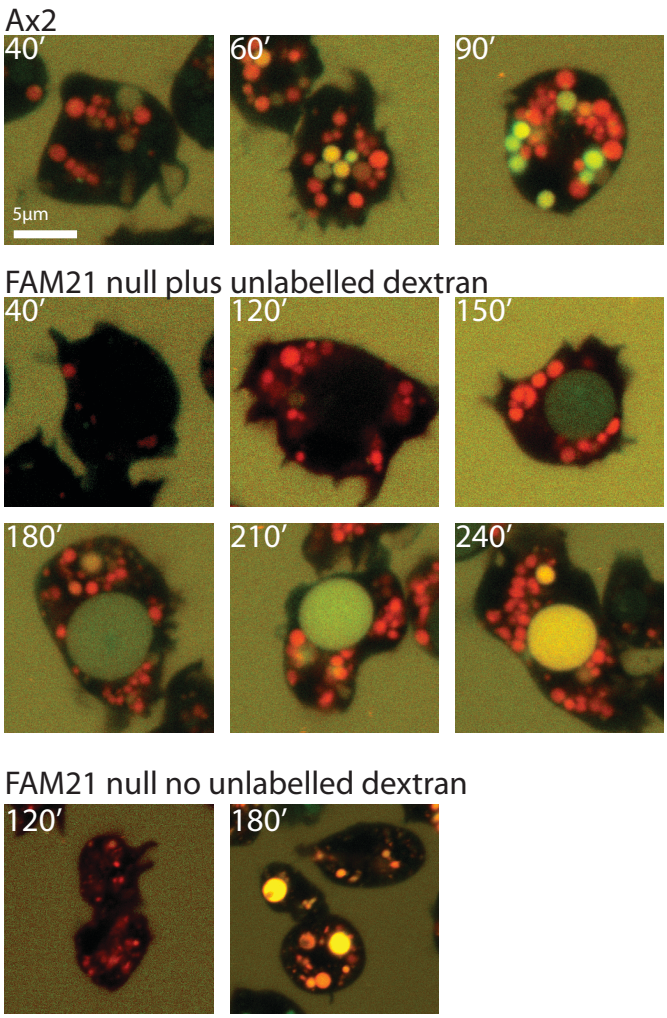


Figure 4.6 FAM21 null delay in neutralization

Ax2 and FAM21 nulls, fed unlabelled dextran overnight or not, were used to perform the neutralization assay as previously described. At t=0, the FITC- and TRITC-dextran combination was added, and cells were observed and imaged over time on a confocal microscope until neutral vesicles became visible. Ax2 cells displayed neutralized vesicles after 60 minutes. FAM21 nulls did not display neutralized vesicles until ~180 minutes, whether they had been previously incubated in unlabelled dextran or not.

the cells, as FAM21 nulls not previously fed unlabelled dextran also took the same length of time to neutralize. As previously stated, the relatively small quantity of dextran fed to cells during the course of this particular assay does not cause dramatic swelling of vesicle size, although some enlargement is visible by the end of the 3 hours in the cells not pre-fed dextran.

4.7 Post-lysosomal exocytic block

We have shown that vesicles in FAM21 null cells can progress at least to the post-lysosomal stage of the endocytic cycle. This is further than the progression seen in the null cell lines of other WASH complex subunits, which are blocked at the earlier, acidic lysosome stage. Despite this, FAM21 nulls are still unable to perform exocytosis (fig. 3.4) therefore this process is still blocked in this cell line. We wanted to determine at exactly what stage the FAM21 null cells were blocked in the endocytic cycle, and whether there is any further progression towards exocytosis from the post-lysosomal stage. To do this, we performed a TRITC-dextran chase assay to determine the end point in the cycle to which the dextran was trafficked.

FAM21 null, WASH null and Ax2 cells were all fed a pulse of TRITC-dextran for 30 minutes. This was then washed off the cells and they were incubated overnight in medium containing unlabelled dextran. The next day, cells were observed to see which endosomal compartments retained the TRITC-dextran (fig. 4.7). Ax2 cells no longer contained any TRITC-dextran, whereas WASH null cells contained numerous vesicles of TRITC-dextran. The FAM21 null cells had accumulated all the TRITC-dextran into the single, enlarged vesicle within each cell.

The Ax2 cells are able to exocytose the TRITC-dextran in a matter of hours, which is why there were no traces of the labelled dextran left in the cells after overnight incubation. WASH nulls contain the TRITC-dextran in numerous acidic lysosomal vesicles which cannot be exocytosed (Carnell et al., 2011). FAM21 nulls appear to have trafficked all the TRITC-dextran into the enlarged post-lysosomal compartment in each cell, and here the labelled dextran has persisted for >12 hours, indicating that it will progress no further within the cell. It also supports our earlier data, that FAM21 nulls do not perform exocytosis. This

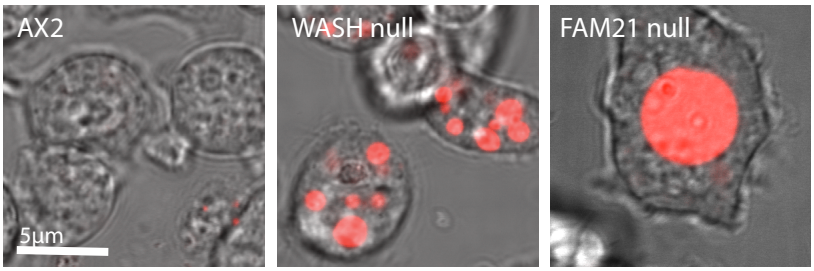


Figure 4.7 Trafficking of TRITC-dextran in FAM21 nulls

Cells were fed a pulse of TRITC-dextran for 30 minutes, then washed and incubated overnight in LoFlo containing 5% unlabelled dextran. The next day, cells were imaged on a confocal microscope. Above are images of representative cells for each cell line. Assay was repeated on two additional days.

confirms that FAM21 null are blocked in exocytosis at the post-lysosomal stage of the endocytic cycle.

4.8 Fusion of post-lysosomes

We have established that the enlarged compartment of a FAM21 null is a neutral post-lysosome. This giant vesicle is the end point of the blocked cycle in these cells, and accumulates indigestible material. Unlike the tightly controlled fusion between early endosomal compartments, post-lysosomes normally remain in the cell for a very short period of time, minutes at most, before being exocytosed (Lee and Knecht, 2002). As the post-lysosomes which form in FAM21 null cells are permanently stuck within the cell, it is possible that they fuse together to form the enlarged vesicles.

To see whether this was the case, we incubated FAM21 null cells in unlabelled dextran overnight before feeding them FITC-dextran. We watched the transit of the FITC-dextran through the cells and observed that small neutral vesicles form in the cells, but these seem to fuse with the enlarged compartment after a while, gradually trafficking the FITC-dextran into this compartment and enlarging it. The fusion events were difficult to predict, and the smaller neutral vesicles difficult to follow for more than a few seconds. Despite this, we managed to capture the fusion between two slightly larger neutral vesicles, which themselves had most likely formed through numerous fusion events, into a single, giant vesicle (fig. 4.8). This confirms that the formation of the enlarged vesicle is through fusion of numerous, smaller neutralizing vesicles.

4.9 WASH sequestration on post-lysosomes

FAM21 null cells have a delay in neutralization, taking approximately 3 times as long to traffic material into neutral compartments compared with an Ax2 cell (fig. 4.6). To try and determine the cause of this delay, we decided to investigate the localisation of GFP-WASH in these cells.

We expressed GFP-WASH in FAM21 nulls and incubated the cells in unlabelled dextran to allow differentiation between the enlarged post-lysosome and other earlier endosomal vesicles in FAM21 nulls. We observed that the majority of the GFP-WASH expressed in a FAM21 null appeared to be localised to the membrane

FITC-dextran in FAM21 null

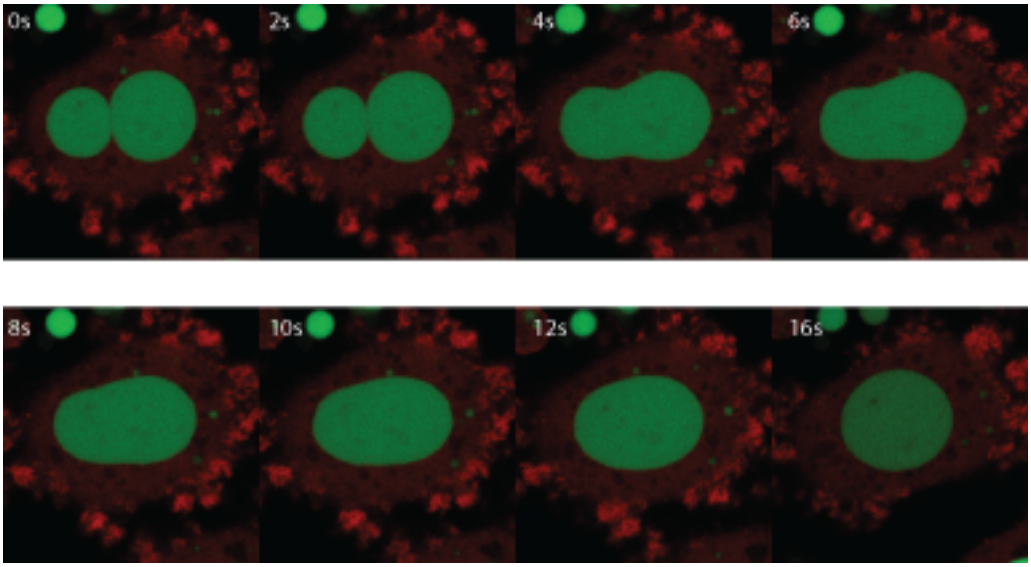


Figure 4.8 Fusion of post-lysosomes in FAM21 nulls

FAM21 null cells were fed FITC-dextran for 10 minutes, then incubated in unlabelled dextran for 2-3 hours. Cells were imaged using a confocal microscope during this time, and fusion between FITC-containing post-lysosomal vesicles was observed in the cells. One of these events was captured as a movie and is shown above. This cell contained two large, neutral vesicles which fused together to form one. NB. The cell is also expressing RFP-actin.

of the enlarged post-lysosome (fig. 4.9a). Quantification of this revealed that an average of 70% (71.4% ±12.0) of the vesicular GFP-WASH in each cell was located on this single membrane, with up to 90% in some cells (fig. 4.9b;). For comparison, we repeated this experiment in Ax2 cells. The distribution of GFP-WASH in Ax2 cells revealed that no more than 20% (13.38% ±6.88) of the vesicular GFP-WASH was ever located on a single vesicle within the cell.

This biased distribution of WASH in FAM21 nulls indicates the possibility that it is being sequestered on the enlarged post-lysosomal membrane. This would explain why there is a delay in neutralization in these cells, as the WASH may normally be recycled from post-lysosomal membranes back to new acidic lysosomes. Sequestration of WASH on post-lysosomes would prevent this recycling and cause a delay in newly formed lysosome neutralization.

In order to confirm whether this sequestration was of the entire complex, not just WASH itself, we also expressed the other GFP-tagged subunits in FAM21 nulls and observed the same biased localisation to the enlarged post-lysosomes (fig. 4.9c).

4.10 WASH complex dynamics

Due to the size difference between a post-lysosome in a FAM21 null and an Ax2 cell, it is difficult to conclude whether WASH truly is ‘stuck’ on the membrane in a FAM21 null. The relative amounts of membrane and cell space dedicated to post-lysosomes in each cell type are already so different that we cannot confirm that the ratio of WASH distribution on post-lysosomes versus other vesicles is also different. To better quantify the ability of WASH to recycle from post-lysosomes in FAM21 nulls, we performed a FRAP (fluorescence recovery after bleaching) experiment.

Ax2 and FAM21 nulls, both expressing GFP-WASH, were incubated in unlabelled dextran for 3 hours. Selected GFP-WASH-decorated vesicles in Ax2 cells were then bleached and the recovery of fluorescence measured. An entire vesicle was selected and bleached, and then followed for a 10 second period to measure the amount of recovery. Unfortunately, vesicles in Ax2 cells move extremely rapidly therefore longer recovery times were unachievable, despite attempts to

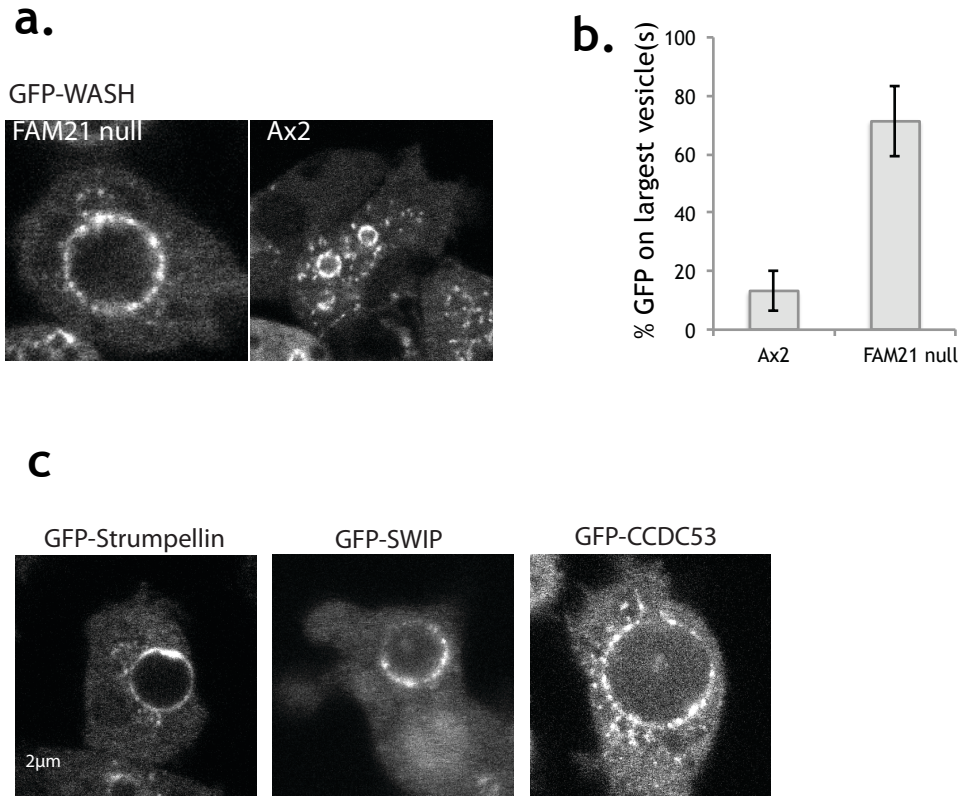


Figure 4.9 GFP-WASH localisation in FAM21 nulls

Ax2 and FAM21 null cells expressing GFP-WASH were fed unlabelled dextran overnight before being imaged on a confocal microscope. (a) Example images of GFP-WASH distribution in FAM21 null and Ax2 cells. (b) GFP-WASH distribution was quantified using 10 images taken over 3 days for each cell type. In FAM21 nulls, the ratio of GFP-WASH localised to the enlarged vesicle membrane versus total vesicular GFP-WASH was determined using ImageJ software (n=33). In Ax2 cells, the ratio of the total GFP-WASH on the 1-3 largest vesicles in each cell versus total vesicular GFP-WASH was determined (n=11; error=SD). (c) FAM21 null cells expressing either GFP-Strumpellin, GFP-SWIP or GFP-ccdc53 were imaged using a confocal microscope to show vesicular distribution.

overlay cells with agarose. For the FAM21 nulls, the enlarged post-lysosome was selected and bleached, and the recovery of fluorescence measured over the same time period.

During the 10 second recovery period, we did see recovery of fluorescence in Ax2 cells (fig. 4.10), indicating that there is indeed dynamic exchange of the complex on vesicle membranes. In the FAM21 nulls, the recovery of fluorescence on the enlarged post-lysosome membrane was relatively low, indicating that there is little exchange of WASH complex occurring on these membranes. This confirms that WASH is being sequestered on the post-lysosomes in FAM21 nulls, and a role for FAM21 in promoting removal and recycling of the WASH complex.

4.11 WASH recycling in mammalian cells

We have determined that FAM21 and WASH play distinct roles within the endocytic cycle in both Dictyostelium and mammalian cells. We wanted to see whether the role of FAM21 in complex recycling was also conserved across cell types. In order to do this, we repeated the FRAP experiment in A2780 cells treated with siFAM21, measuring recovery of WASH on vesicle membranes.

In non-targetted (NT) cells, we selected EGFP-WASH-decorated vesicles, and bleached the whole vesicle. The recovery of EGFP-WASH on the vesicle membrane was measured over a period of over 60 seconds (possible in mammalian cells due to the relative stability of cytoplasmic vesicles). FRAP was performed in the same way in siFAM21 treated cells however the relative amount of recovery in FAM21 knockdown cells was still substantially less (<10%) than that seen in NT cells (~25%; fig. 4.11; thanks to Tobias Zech for performing the mammalian FRAP experiment).

This shows that the role for FAM21 in recycling of the complex that we observed in *Dictyostelium* cells is likely to be conserved in mammalian cells. It has not been quantified in mammalian cells whether siFAM21 treated cells have any difference in endosomal vesicle size compared to wild type, although there were no obvious enlarged compartments observed during this experiment. Despite

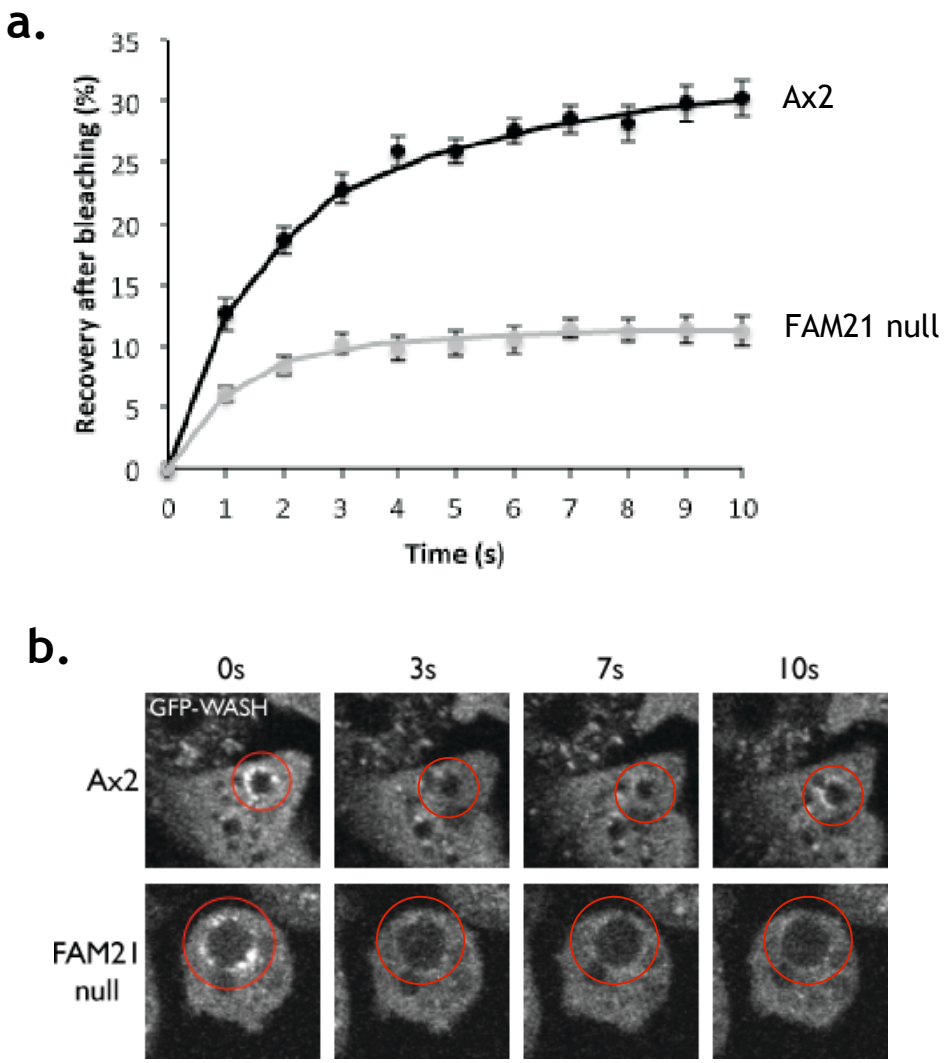


Figure 4.10 FRAP of GFP-WASH in Dictyostelium

(a) FRAP was performed with Ax2 and FAM21 null cells expressing GFP-WASH. Cells were fed unlabelled dextran overnight. For Ax2 cells, a GFP-WASH coated vesicle was selected and bleached for 1 frame before imaging recovery over 10 seconds at 1 frame/sec. For FAM21 nulls, the enlarged, GFP-WASH coated vesicle was bleached and imaged in the same way. The experiment was repeated twice more and images were used to quantify recovery for each cell type (n=28; error=SEM). (b) Example images of an Ax2 and a FAM21 null cell recovering after bleaching, bleached vesicle is highlighted by a red circle.

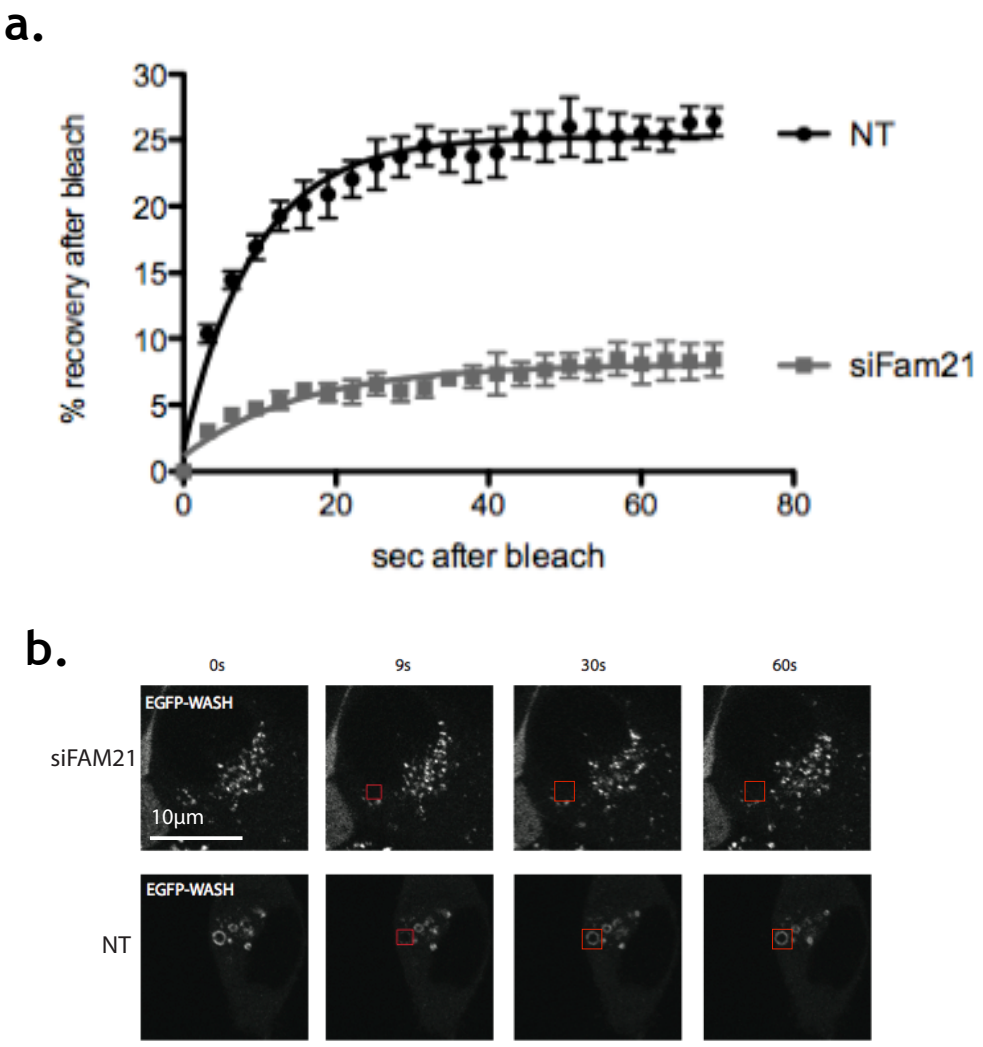


Figure 4.11 FRAP of GFP-WASH in A2780 cells

(a) FRAP was performed as described for *Dictyostelium* cells, with NT and siFAM21 A2780 cells expressing EGFP-WASH. Cells were bleached for 1 frame before imaging recovery for 66 seconds at 1 frame/3 secs (n=30; error=SEM). (b) Example images of NT and siFAM21 cells recovering after bleaching, bleached vesicle is highlighted by a red box.

this, it appears that WASH may still be sequestered on some compartments of the endosomal system in mammalian cells.

4.12 WASH complex removal and actin

FAM21 nulls are blocked at the post-lysosome stage and progression to exocytosis is prevented. We have shown that that the WASH complex is sequestered on post-lysosomal vesicles at this point, and excessive actin is polymerized as a result. These actin comets seen on the FAM21 null post-lysosomes may act as a barrier to prevent removal of the WASH complex directly, or to prevent budding or fission of the post-lysosomes for progression. In order to test whether this was true, we treated cells with latA to remove the potential actin barrier and see whether any progress towards exocytosis occurred.

FAM21 nulls expressing GFP-WASH were fed a pulse of TRITC-dextran, and were then incubated in medium with unlabelled dextran to swell the post-lysosomal compartment. LatA was then added to cells and this abolished the endosomal actin structures associated with the post-lysosomes (fig. 4.12a). We observed the cells for 2 hours, until secondary effects of the drug became apparent and cells begin to round up and detach from the plate. For the entire duration of the treatment, the post-lysosomes remained within the cells, filled with TRITC-dextran, and did not appear to change in any way. GFP-WASH also continued to decorate the membrane (fig. 4.12b). We then repeated the experiment, treating the cells for 30 minutes with LatA before washing off the drug and resuspending in medium in an attempt to see whether brief removal of the actin could shunt the pathway along, however again there was no change to the cells (data not shown).

It appears that removal of the excessive actin coat/comets from the enlarged post-lysosomes in FAM21 nulls does not cause any visible dissociation of WASH from the vesicle membrane. There was also no budding of smaller vesicles of removal of the TRITC-dextran from the lumen of the vesicle in any way. This suggests that actin is not acting as a barrier preventing WASH dissociation from the membrane, nor is it preventing budding events from ‘breaking up’ the enlarged vesicle.

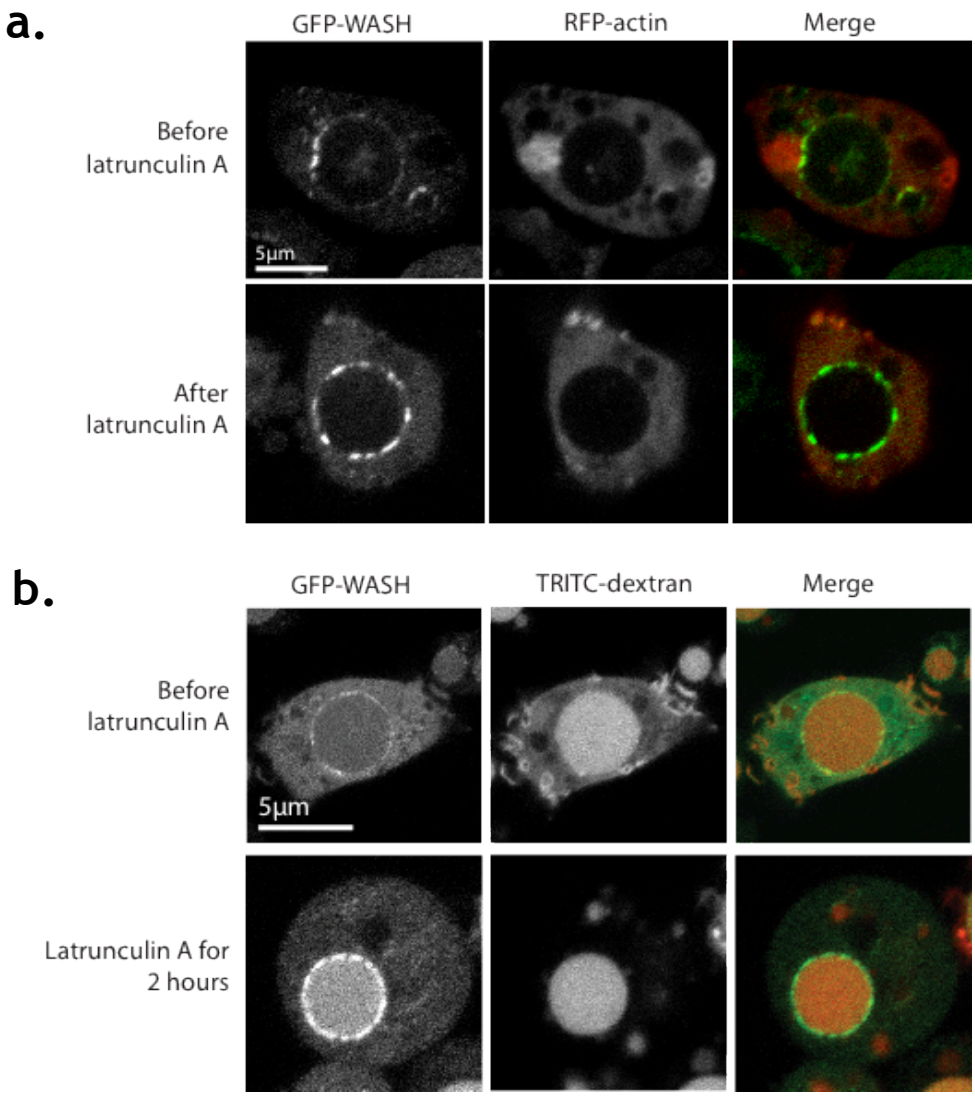


Figure 4.12 Latrunculin A treatment of FAM21 nulls

(a) FAM21 null cells coexpressing GFP-WASH and RFP-actin were treated with 10µg/ml latA in LoFlo containing unlabelled dextran. Addition of the drug prevented formation of endosomal actin coats. (b) FAM21 nulls coexpressing GFP-WASH and RFP-actin were fed TRITC-dextran for 1 hour. Cells were washed and incubated in LoFlo medium containing unlabelled dextran for 3 hours. LatA was added to cells, which were then imaged on a confocal every 20 mintues for 2 hours.

CHAPTER SUMMARY

We have now determined that loss of FAM21 still causes a block in in the endocytic cycle, but at a different stage from loss of WASH. Vesicles are able to progress further along the cycle in FAM21 nulls, indicating that the block in these cells is further downstream. From this, we have determined that FAM21 has a separate function from WASH, which is also downstream from that of WASH itself.

The partial WASH complex that remains in FAM21 nulls is able to efficiently remove V-ATPase from lysosomal membranes, but the failure in these cells is in removal of the WASH complex from post-lysosomal membranes which must occur for progression to exocytosis. FAM21 nulls contain very enlarged post-lysosomes which form through fusion of neutral vesicles, and it is on the membrane of these vesicles which the WASH complex is sequestered in these cells.

We hypothesize that the function of FAM21 is to remove the WASH complex from the membrane at this point in the cycle in order to allow progression to exocytosis. We will now therefore focus on this function of FAM21 and look into the mechanisms of how it might achieve this.

CHAPTER 5
FAM21 STRUCTURE AND INTERACTION WITH CAPPING PROTEIN

5 FAM21 Structure and Interaction with Capping Protein

5.1 FAM21 is composed of two distinct regions

The FAM21 mutant is unique among the WASH complex subunit mutants in having a different phenotype. It clearly plays a vital but separate role from WASH itself, and to further elucidate its function, we looked in more detail at the structure and domains of the protein. Analysis of the human and *Dictyostelium* FAM21 protein sequences using SMART (Simple Modular Architecture Research Tool; Schultz et al., 1998) revealed that the proteins contained no known domains. We then aligned FAM21 protein sequences from different eukaryotic species using ClustalW (v1.83) multiple sequence alignment in order to try and detect any particular conserved regions. We found that the N terminal region of FAM21 appeared to be far more conserved between species than the remainder of the protein sequence. This is clearly illustrated in Appendix I, where an example alignment of 8 species is shown, and the histogram under the sequences represents the level of homology of the residues. Analysis of the *D. discoideum* FAM21 by Scratch Protein Prediction (SSpro v 4.5; Cheng et al., 2005), a program to predict the secondary structure of proteins, indicated that the N terminal ~300 amino acids, or 'head' region of FAM21 contains a number of predicted α-helices, however the remaining 'tail' of the protein is very unstructured (fig. 5.1). This is likely due to the large number of proline residues present in this region of the sequence (Gomez and Billadeau, 2009). These data correlate with findings in mammalian cells that the conserved N terminal head of FAM21 is responsible for binding the WASH complex (Gomez and Billadeau, 2009; Harbour et al., 2010).

5.2 FAM21 tail structure

The tail sequences of the various FAM21 proteins at first appeared to have a relatively low conservation. A more in depth look revealed that the tail regions appeared to contain a number of short repeats. Using a Pustell protein matrix analysis, where regions of a protein are aligned against itself to detect repeats, we found this was indeed the case. Repeats were present throughout the tail of

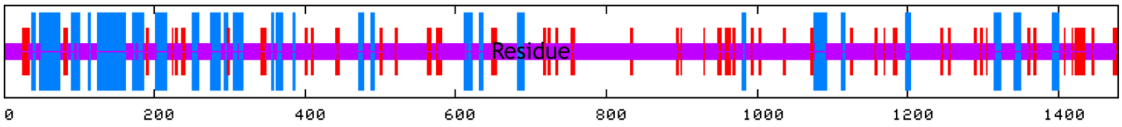


Figure 5.1 Predicted secondary structure of FAM21

Dictyostelium discoideum FAM21 was analysed by SSpro v4.5 (Cheng et al., 2005) and the predicted secondary structure is shown here. The blue bands represent preicted α-helices, and the red bands represent predicted ß-sheets.

the human FAM21 protein (fig. 5.2a). Analysis of other species revealed that, although the actual sequence of the repeats was not always conserved, there were distinct repeats present in FAM21 proteins from all species analysed. A particularly good example is the FAM21 protein of *Trichomonas vaginalis,* a parasitic protozoan, in which a large number of stringent repeats was identified throughout the tail region of the protein (fig. 5.2b). The examples in figure 5.2 also both show that the repeats are confined to the tail region of the protein, and little if any are present in the first ~300 amino acids composing the head region. The repeats in the sequences of *H. sapiens*, *D. discoideum* and *T. vaginalis* FAM21 proteins were identified and aligned by hand. Figure 5.2c shows that the number and stringency of the repeats was highly variable, with *D. discoideum* having around 22 repeats, following a relatively weak consensus. In contrast, the *T. vaginalis* protein has 42 repeats with high fidelity to the consensus. These repeats are likely to bind a ligand of low complexity such as a lipid.

5.3 FAM21 head and tail are both essential

We wanted to determine how important each of the two regions of the FAM21 protein were, and whether one region alone was sufficient for some or any of FAM21 function. To do this, we created two *Dictyostelium* FAM21 fragments, one of the head region only and one of the tail region only, which we expressed in FAM21 nulls to see whether they were able to rescue the phenotypes of the null cells.

The head region, FAM21ΔCT, consisted of the first 255 amino acids of the protein. The tail region, FAM21ΔNT constituted the remaining amino acids 245-1480 of unstructured sequence. First of all, these constructs were both tagged with GFP and transfected into FAM21 null cells. The exocytosis assay was performed as previously described in order to see whether either portion of the protein alone was able to rescue the block in exocytosis. We found that on expression of either FAM21ΔCT or FAM21ΔNT, the internal fluorescence of cells did not decrease below 80% over 5 hours, indicating that these cells were unable to perform exocytosis (fig. 5.3). This shows that the full length FAM21 protein is

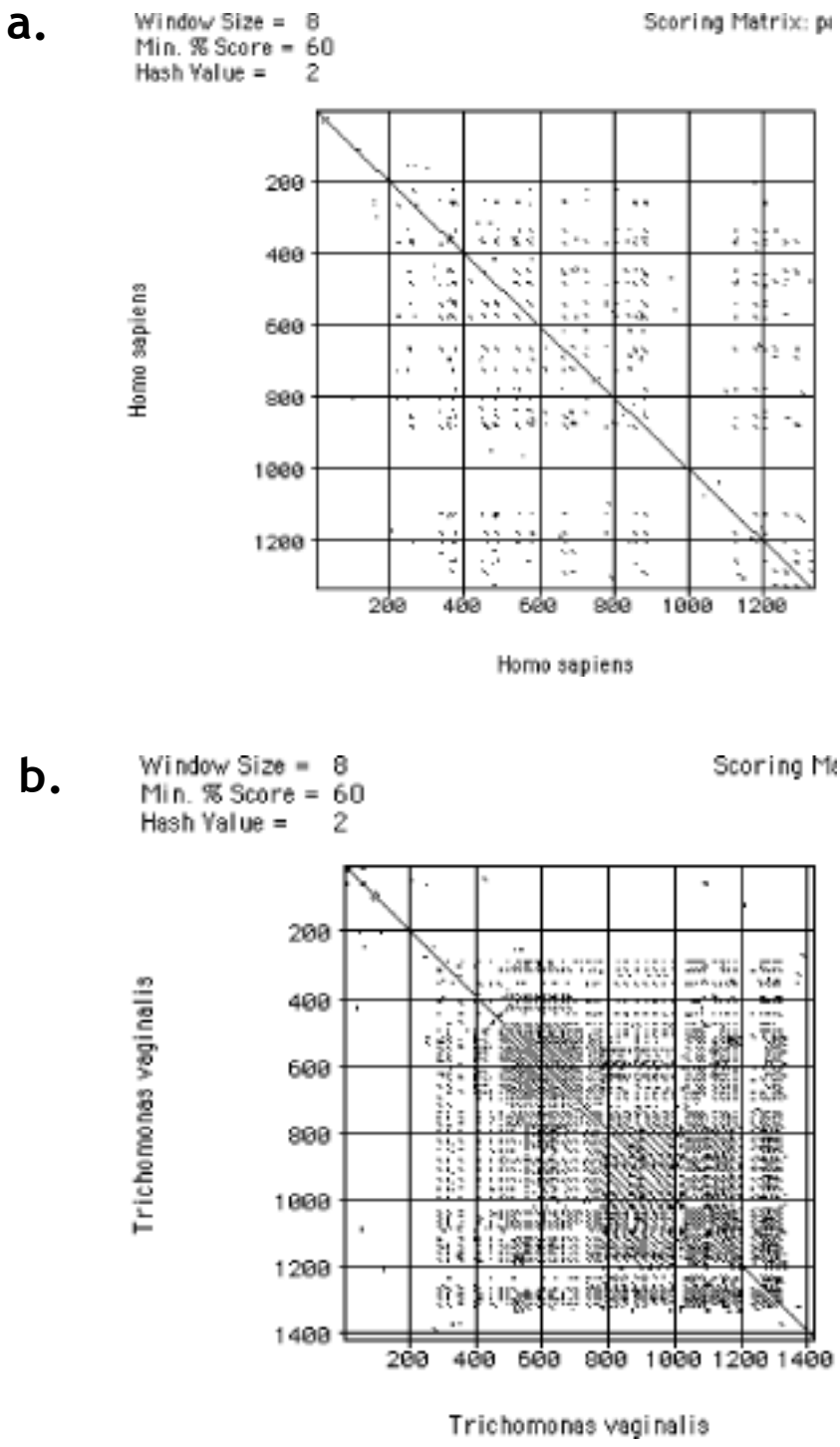


Figure 5.2 FAM21 tail structural analysis

A Pustell protein matrix analysis (scoring matrix: pam250) of FAM21 protein sequences was used to detect repeats. The black marks on the graph represent repeated sequence. The x and y axis values correspond to the two positions within the protein of the repitition. (a) Human FAM21 protein analysis. (b) Trichomonas vaginalis FAM21 protein analysis.

C.

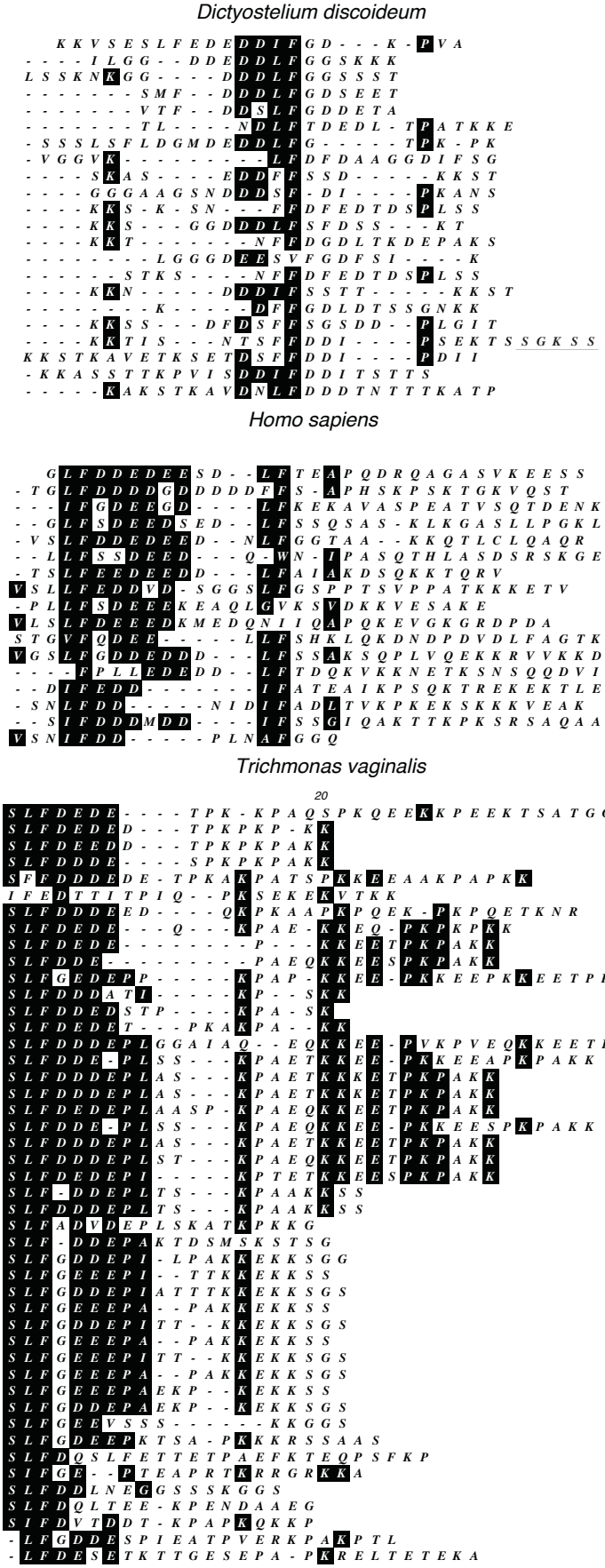


Figure 5.2 FAM21 tail structural analysis *cont*.

(c) The repeats in D. *discoideum*, *H. sapiens*, and *T. vaginalis* FAM21 proteins were identified and aligned by hand.

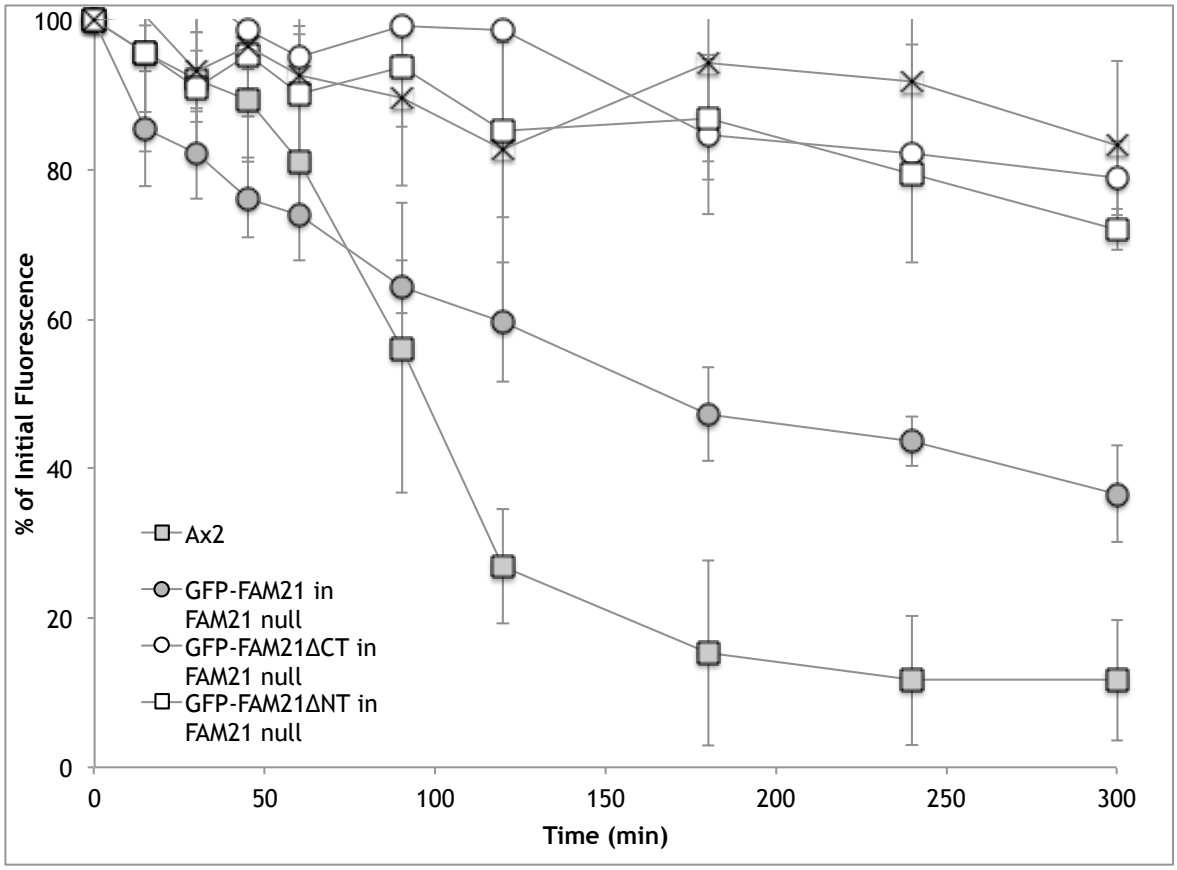


Figure 5.3 Exocytosis assay with FAM21 fragments

GFP-FAM21ΔCT and GFP-FAM21ΔNT were expressed in FAM21 nulls. The cells were used to perform the exocytosis assay as previously described. Full length GFP-FAM21 was used as a control.

necessary for exocytosis, and that the head or tail region alone are not functional.

5.4 Head and tail localisation

The FAM21 subunit does not require any of the remaining subunits of the WASH complex to localise correctly in cells, therefore the protein must contain localisation signals of its own. To determine which of the regions of FAM21 was responsible for localising the protein, we expressed the two fragments in the WASH complex mutants and observed their localisation.

GFP-FAM21ΔCT and GFP-FAM21ΔNT were first each expressed in FAM21 null cells and observed on a confocal microscope. We found that both regions were able to localise correctly to endocytic vesicles (fig. 5.4a). We then expressed each fragment in each of the WASH complex null cell lines to see whether any other subunits were required for localisation of either fragment. Figure 5.4b and table 5.1 illustrate that the GFP-FAM21ΔNT construct was able to maintain its localisation in all of the knockout cell lines. In contrast, GFP-FAM21ΔCT was too unstable to be expressed in any knockout cell lines, except for FAM21 null, in which it was localised to vesicles.

These results show that the tail of FAM21 appears to require no other subunit for either stability or localisation in cells. It must therefore contain its own localisation signal. The head of FAM21 appears to be dependent on the existence of a stable WASH complex for expression, and as loss of any complex member apart from FAM21 causes instability, this is the only cell line in which it was successfully expressed. We can see, however, that the head must have an additional localisation signal, not requiring the tail region for vesicular localisation in FAM21 nulls.

5.5 The head region binds the WASH complex

As previously stated, FAM21 has been shown to bind the WASH complex through the head region in mammalian cells (Gomez and Billadeau, 2009; Harbour et al., 2010). We wanted to establish whether this is also true for *Dictyostelium*.

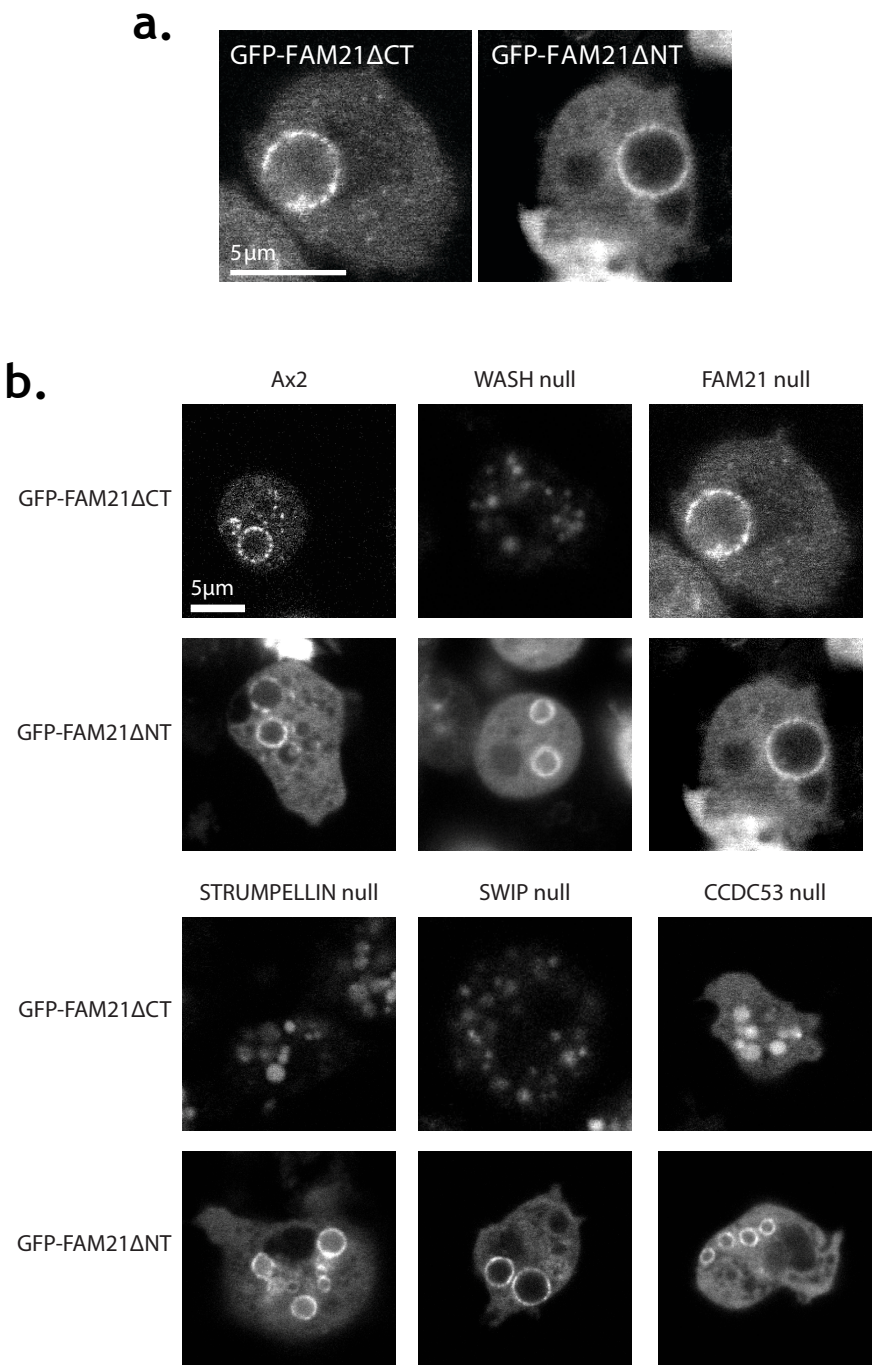


Figure 5.4 Localisation of head and tail fragments

GFP-FAM21ΔCT and GFP-FAM21ΔNT were expressed in (a) FAM21 nulls and (b) the remaining WASH complex subunit knockout cell lines. The cells were fed unlabelled dextran in LoFlo medium and imaged on a confocal microscope. The localisation of each fragment in the different cell lines is summarized in table 5.1.

	GFP-FAM21ΔCT	GFP-FAM21ΔNT
Ax2	Vesicular	Vesicular
WASH null	Not expressed	Vesicular
FAM21 null	Vesicular	Vesicular
SWIP null	Not expressed	Vesicular
Strumpellin null	Not expressed	Vesicular
Ccdc53 null	Not expressed	Vesicular

Table 5.1 Localisation of head and tail fragments

The localisation of each fragment of FAM21, GFP-FAM21ΔCT and GFP-FAM21ΔNT, expressed in the knockout cell lines of WASH complex subunits was recorded, corresponding to the images in figure 5.4.

In order to do this, we expressed full length GFP-FAM21, GFP-FAM21ΔCT and GFP-FAM21ΔNT in FAM21 null cells, as well as a GFP only control, and purified the proteins from cell lysate using GFP-Trap beads as previously described. The purified proteins and any bound proteins were analysed by mass spectrometry. Unfortunately, the expression of the GFP-FAM21ΔCT construct was too low, even in FAM21 null or Ax2 cells, to allow sufficient protein to be purified for this experiment, therefore only GFP-FAM21ΔNT was available for comparison with the full length protein. The results of the mass spectrometry analysis are shown in table 5.2. In the sample purified using GFP-FAM21, all WASH complex subunits were identified, including both subunits of the capping protein heterodimer, as shown in figure 3.1. The sample purified using GFP-FAM21ΔNT did not contain any of the WASH complex subunits, however again both subunits of capping protein were present.

These results show that the interaction with the WASH complex is through the head domain of FAM21 in *Dictyostelium*, as it is in mammalian cells. This may be the reason the head domain of FAM21 is able to localise without the tail, because it can bind to and use other WASH complex members to localise it correctly to vesicles. They also confirm that the tail of FAM21 is responsible for the interaction with capping protein, most likely through the CPI domain identified within the sequence.

5.6 FAM21ΔCT is dominant negative

Upon expression of GFP-FAM21ΔCT in Ax2 cells, we noticed that the vesicles within the cells seemed larger than normal, reminiscent of the FAM21 null phenotype. In order to look into this more closely, we looked at vesicle swelling on addition of dextran in these cells, and whether their rate of exocytosis was affected by expression of this fragment.

Ax2 cells expressing GFP-FAM21ΔCT were fed 5% unlabelled dextran plus TRITC-dextran overnight. The following day, the cells were imaged on a confocal microscope. These images were used to quantify the size of the vesicles the cells contained. We found that Ax2 cells expressing this fragment had enlarged vesicles similar to those seen in FAM21 nulls (fig. 5.5a). We then performed the

Protein Name	Identified Proteins	Accession Number	Molecular Weight (kDa)	Number of Peptides		
				GFP-FAM21	GFP-FAM21ΔNT	GFP only
FAM21	GFP-FAM21_DICDI Protein FAM21 homolog PROJECT LAP_R06_011209	in|LAP002|LAP002_R06		164	183	51
Strumpellin	|Protein|gene: DDB_G0288569 on chromosome: 5 position 1693802 to 1697605	DDB0234050|DDB_G0288569	135	71	2	2
SWIP	|Protein|gene: DDB_G0283355 on chromosome: 4 position 559359 to 563272	DDB0234041|DDB_G0283355	132	60	0	0
ccdc53	|Protein|gene: DDB_G0267948 on chromosome: 1 position 1170991 to 1172151	DDB0349216|DDB_G0267948	38	3	0	0
WASH	|Protein|gene: DDB_G0292878 on chromosome: 6 position 2194668 to 2196220	DDB0305648|DDB_G0292878	51	18	0	0
Cap34	|Protein|gene: acpB on chromosome: 2 position 1562720 to 1564080	DDB0191243|DDB_G0272104	31	13	15	1
Cap32	|Protein|gene: acpA on chromosome: 1 position 1647348 to 1648410	DDB0191202|DDB_G0267374	31	20	22	2

Table 5.2 Peptide identification for FAM21 immunoprecipitations

A coimmunoprecipitation was performed by purification of GFP-FAM21 or GFP-FAM21ΔNT using GFP-Trap as previously described. Identification of the WASH complex subunits and capping protein subunits by mass spectrometry is shown.

115

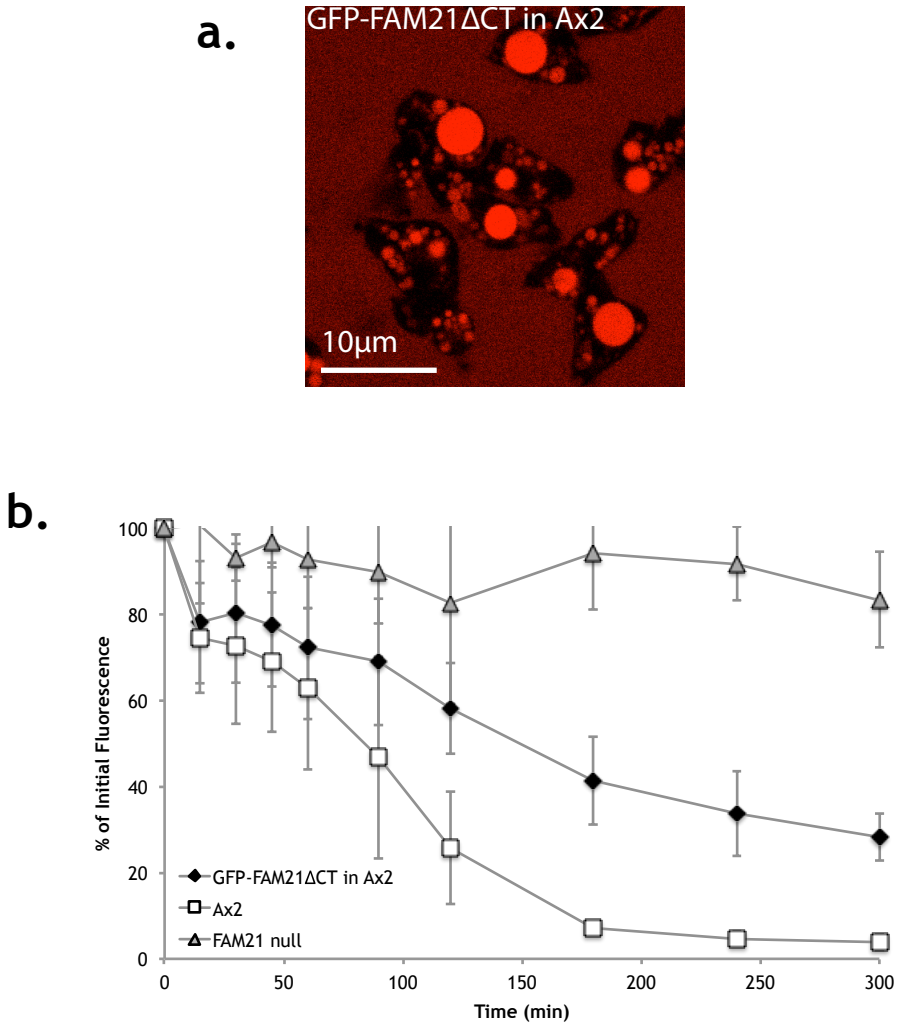


Figure 5.5 Dominant negative FAM21ΔCT

GFP-FAM21ΔCT was expressed in Ax2 cells. (a) Cells were fed unlabelled dextran and TRITC-dextran and imaged on a confocal microscope. (b) The exocytosis assay was performed as previously described, with Ax2 and FAM21 null cells as controls.

exocytosis assay as previously described, and we found that the cells were able to perform exocytosis, but the rate was slower compared to Ax2 cells (fig. 5.5b). It appears that expression of this head region fragment of FAM21 has a dominant negative effect in Ax2 cells. This partial FAM21 null-like phenotype, with enlarged vesicles and a slight delay in exocytosis, are likely due to the incorporation of the head of FAM21 into constructed WASH complexes. The tail region does not bind FAM21 which is why this portion of the protein does not have this effect (data not shown) however the head region may compete for binding with the full length endogenous protein, resulting in some mutant complex formation. These mutant complexes are not fully functional, and hinder the activity of the normal WASH complexes in the cell.

5.7 Lipid binding analysis of the tail

The tail of FAM21 is able to localise independently of the WASH complex in both Dictyostelium cells (table 5.1) and mammalian cells (Gomez and Billadeau, 2009; Harbour et al., 2010). As described, the repeats it contains suggest a binding partner of low complexity, such as a lipid, which may also account for its ability to bind the vesicle membranes independently. To determine whether the ligand of the repeats was a specific lipid, we performed a lipid blot assay, or PIP-array. This works on the same principles as a western blot, whereby an array of known phospholipids is fixed to a membrane surface. The ligand in question is then incubated with the membrane before visualising binding through use of enhanced chemiluminescence (ECL).

To perform this experiment, GFP-FAM21ΔNT was expressed in FAM21 null *Dictyostelium* cells and purified using GFP-Trap beads. GFP alone was used as a negative control, and GFP-CRAC, which has a known interaction with phosphoinositol-3-4-5-trisphosphate ($PI(3,4,5)P_3$; Parent et al., 1998), was used as a positive control. The purified GFP-tagged proteins were then incubated with the PIP-array membrane overnight. The next day, the membrane was incubated with an anti-GFP antibody and then developed. We found that the controls were correct; there were no positive spots for GFP alone, and there were positive spots for GFP-CRAC with $PI(3,4,5)P_3$ and a weaker interaction with $PI(3,4)P_2$. No positive spots were detected for GFP-FAM21ΔNT (fig. 5.6).

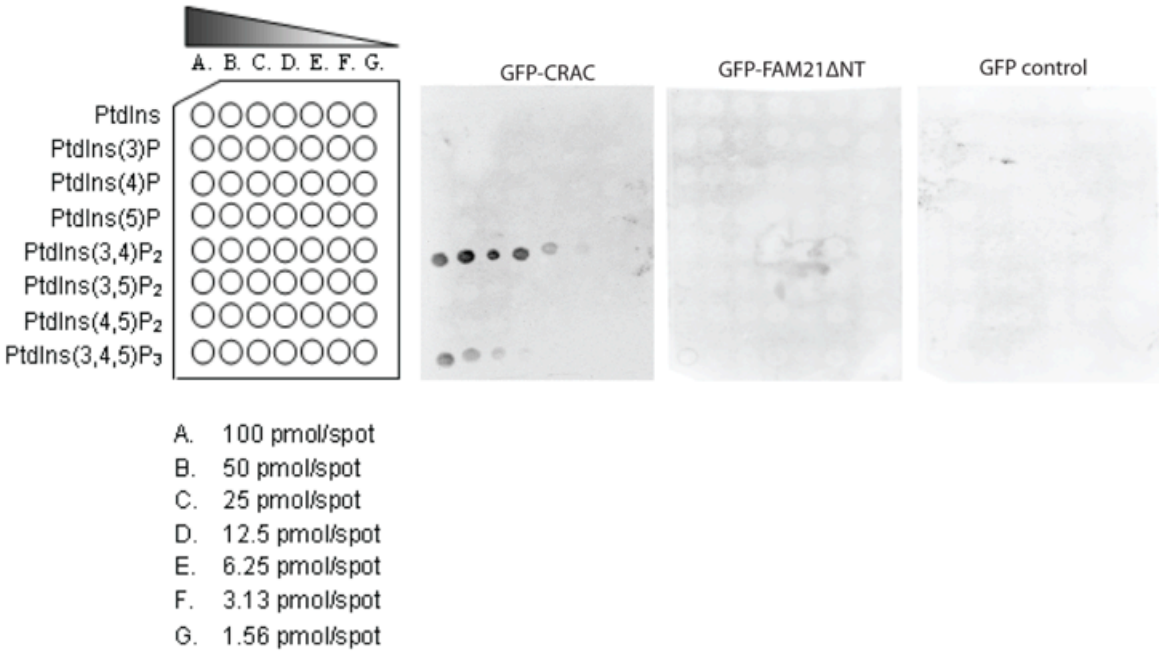


Figure 5.6 Lipid blot assay

A lipid blot was performed using a PIP Array (Echelon) membrane. Listed above are the lipids and concentrations fixed to the membrane. GFP, GFP-FAM21ΔNT and GFP-CRAC were expressed in FAM21 null *Dictyostelium* cells and purified using GFP-Trap beads. The membrane was incubated with the purified proteins overnight and binding was visualized using ECL.

In mammalian cells, a similar experiment using a PIP-array was performed by Jia et al. (2010) with a GST-C terminal fusion of human FAM21. They detected interactions with a number of lipids including PI(3)P and PI(3,5)P2, both known to be involved in the transition between early and late endosomes (van Meer et al., 2008) however they also saw interactions with a number of other lipids such as PI(4)P, which is associated with the Golgi (Audhya et al., 2000; Hama et al., 1999), and PI(5)P, about which little is known. It may be that the tail of FAM21 can bind to lipids, however the correct *in vivo* conditions are required for specificity and efficacy of binding, hence the 'all or nothing' results seen using the PIP-arrays for our data and other groups.

5.8 Endosomal specificity of the tail

We wanted to determine whether expression of the tail region of FAM21 was specific to the same endosomal membranes as the remainder of the WASH complex. We hypothesized that without the head region, it could not be correctly targetted to the WASH complex and may decorate the membranes of other vesicle populations. Coexpression of the tail with another subunit would reveal whether the two were constitutively colocalised, or that the tail region of FAM21 had a more dispersed localisation, present on membranes both with and devoid of the WASH complex.

To begin with, we created a coexpression construct for GFP-FAM21ΔNT and full length RFP-FAM21, however GFP-FAM21ΔNT failed to show any localisation in Ax2 or FAM21 nulls when coexpressed with full length FAM21. We tried switching the fluorescent tags to create a GFP-FAM21/RFP-FAM21ΔNT construct but the result was the same, as was GFP-WASH/RFP-FAM21ΔNT. We found this unusual, because as single constructs, both GFP- and RFP-FAM21ΔNT are expressed and localised normally in cells.

It may be that the tail region of FAM21 is competing with the full length protein for binding sites, therefore overexpression of the full length protein at the same time prevents FAM21ΔNT from localizing. Similarly, when coexpressed with WASH or another member of the complex, the same principle is true; overexpression of the complex leads to less availability of binding sites. Complexes containing full length FAM21 are likely to have a higher affinity for

membrane binding sites as the complex contains several different localisation signals.

5.9 Species specificity of the tail repeats

The repeats found in the tail of FAM21 vary greatly in stringency throughout the individual proteins, and between species. They also vary in length and number. As shown in figure 5.2, one common inter-species pattern is that the repeats consist of 3 or more acidic residues, normally preceded (or in the case of *Dictyostelium*, followed by) by a lysine and a phenylalanine residue. In order to test how specific these repeats are for binding their ligand, we decided to test the efficacy of human FAM21 protein in *Dictyostelium*.

We obtained cDNA of human FAM21C, and inserted it into a *Dictyostelium* GFP-tagged expression vector. This GFP-HsFAM21 construct was then expressed in FAM21 null *Dictyostelium* cells. GFP-HsFAM21 was able to correctly localise in cells (fig. 5.7a), however it was unable to rescue the block in exocytosis (fig. 5.7b). We also expressed the head region of HsFAM21 alone, GFP-HsFAM21ΔCT, in FAM21 nulls. This construct was unable to localise in cells and remained cytoplasmic (fig. 5.7a). The HsFAM21 head region was unable to localise, which is likely to be because it couldn't interact with the *Dictyostelium* WASH complex. This means that the full length human protein must be using the tail region to localise to vesicles, as we have already shown this portion of the protein contains its own localisation signal.

We then engineered a hybrid FAM21 protein, which consisted of the *Dictyostelium* FAM21 head region, and the human FAM21 tail region. This protein in theory should be able to localise through the tail, and bind the WASH complex through the head, therefore giving a fully functional FAM21. We expressed GFP-HyFAM21 in FAM21 nulls and found that it was still able to localise (fig. 5.7a). However, it was not sufficient to rescue the block in exocytosis (fig. 5.7b). This may be because the linking region between the head and the tail is important, and is disrupted with our hybrid protein. Alternatively, it may be that, apart from being able to localise to endosomes, the tail region has another purpose which is more specific, like the binding of the head region to the complex, and this is not conserved between species.

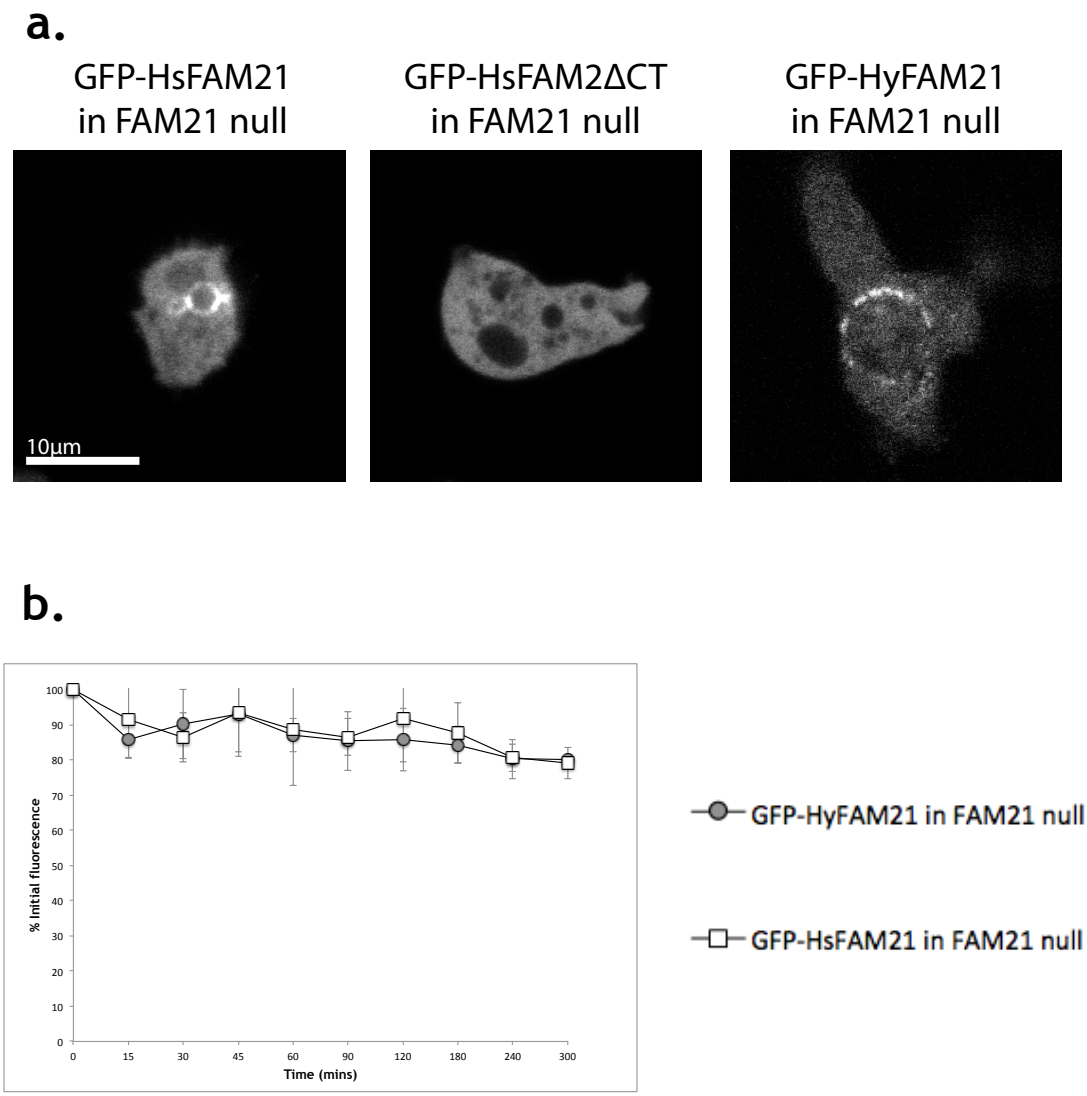


Figure 5.7 Human and hybrid FAM21 proteins

GFP-HsFAM21, GFP-HsFAM2ΔCT and GFP-HyFAM21 were expressed in FAM21 nulls. (a) Cells were incubated in LoFlo with unlabelled dextran before imaging on a confocal microscope. (b) FAM21 nulls expressing GFP-HsFAM21 or GFP-HyFAM21 were used to perform the exocytosis assay as previously described (n=3; error=SD).

5.10 Capping protein binding of FAM21

Our results to this point suggest that the tail of FAM21 has a vital role in function of the protein, as the head region alone is insufficient to rescue FAM21 nulls. Apart from the repeats, there are little or no other recognisable domains within the tail except for the capping protein interaction (CPI) site, originally detected by Hernandez-Valladares et al. (2010). An interaction between capping protein and the WASH complex in mammalian cells has already been confirmed by several groups (Derivery et al., 2009; Gomez and Billadeau, 2009; Jia et al., 2010) and we have identified both CAP34 and CAP32 subunits in coimmunoprecipitations of WASH complex subunits. We therefore wanted to confirm that this interaction is mediated through this specific site in FAM21, as no study as yet has shown this. To do this, we created a CPI mutant of FAM21 to test whether the removal of the CPI abrogates binding of capping protein.

We engineered a FAM21 mutant, FAM21ΔCPI, by removing the 17 amino acid sequence which comprises the CPI from the tail. We then expressed GFP-FAM21ΔCPI or wild type GFP-FAM21 in FAM21 nulls. These were purified from cell lysates using GFP-Trap beads and the purified protein was run by SDS-PAGE followed by a western blot. We detected the CAP32 subunit of capping protein using an anti-CAPZβ antibody (CAPZβ is the human homologue of *Dictyostelium* CAP32) which was present for wild type FAM21, but not present for the mutant (fig. 5.8).

This confirms the ability of FAM21 to bind capping protein specifically through this site in the tail of the protein. It also confirms that the mutant we have created is no longer able to bind capping protein.

5.11 CPI is essential for FAM21 function

To investigate the importance of the interaction between FAM21 and capping protein for both function of FAM21 and of the complex as a whole, we tested whether the FAM21ΔCPI mutant was able to rescue the FAM21 null phenotype and rescue the block in exocytosis.

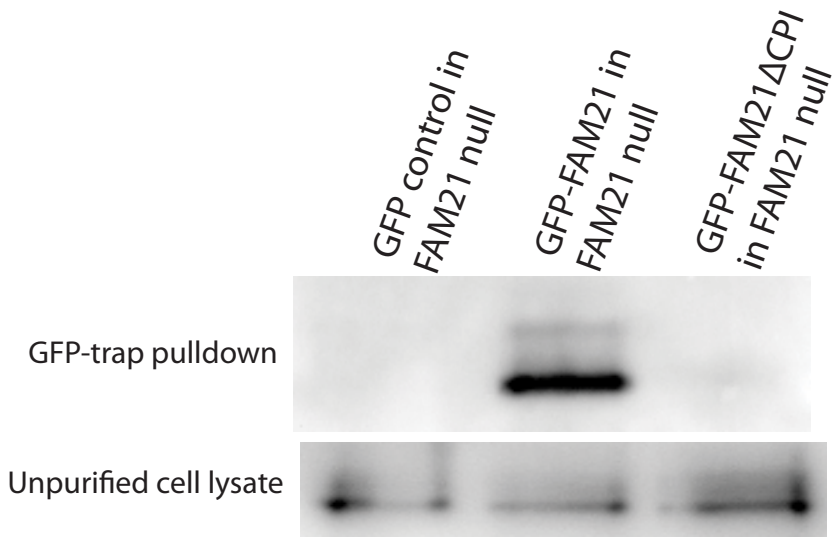


Figure 5.8 Coimmunoprecipitation of capping protein

GFP-FAM21 and GFP-FAM21ΔCPI were expressed and purified from FAM21 null cells using GFP-Trap. GFP alone was used as a control. A western blot was performed and probed using an anti-CAPZß antibody to detect the CAP32 subunit. Unpurified cell lysate was also run to confirm the presence of capping protein before coimmunoprecipitation.

We first expressed GFP-FAM21ΔCPI in FAM21 null cells and found that it was able to localise to vesicles (fig. 5.9a). We then used these cells to perform the exocytosis assay and found that GFP-FAM21ΔCPI is unable to rescue the block in exocytosis of FAM21 nulls (fig. 5.9b). These cells were also fed medium containing 5% unlabelled dextran and TRITC-dextran in order to look at the size of the endosomal compartments, and we found that cells containing GFP-FAM21ΔCPI still contained very enlarged post-lysosomal compartments, whereas those expressing wild type GFP-FAM21 had near normal sized post-lysosomes in nearly every cell (fig. 5.9c). Lastly, we coexpressed GFP-FAM21ΔCPI and RFP-actin in FAM21 nulls, which revealed that the excessive actin comets were still present on the post-lysosomes, although these appeared slightly less exaggerated and deregulated than those in cells completely lacking FAM21 (fig. 5.9d).

It is clear that capping protein is very important for the function of FAM21, as the removal of the CPI prevents the FAM21 protein rescuing the FAM21 null phenotype almost completely. This also shows that capping protein is needed for progression through the endocytic cycle, and is therefore essential for WASH complex function whether it is constitutively bound with the other subunits or not.

5.12 FAM21ΔCPI is dominant negative

We had previously seen that expressing the head region of FAM21 in Ax2 cells had a dominant negative effect. This did not happen with the tail because the tail region does not bind the WASH complex therefore does not appear to disrupt complex function. The FAM21ΔCPI mutant should still bind the WASH complex as the head region is intact, however removal of the CPI prevents it from rescuing the FAM21 null phenotype. In order to see whether removal of this site really did prevent FAM21 from functioning correctly, we wanted to see if this mutant also disrupted WASH complex function in Ax2 cells.

We performed the exocytosis assay, as previously described, using Ax2 cells expressing GFP-FAM21ΔCPI. We found that the cells were still able to perform exocytosis however the rate of exocytosis was much slower (fig. 5.10a). The

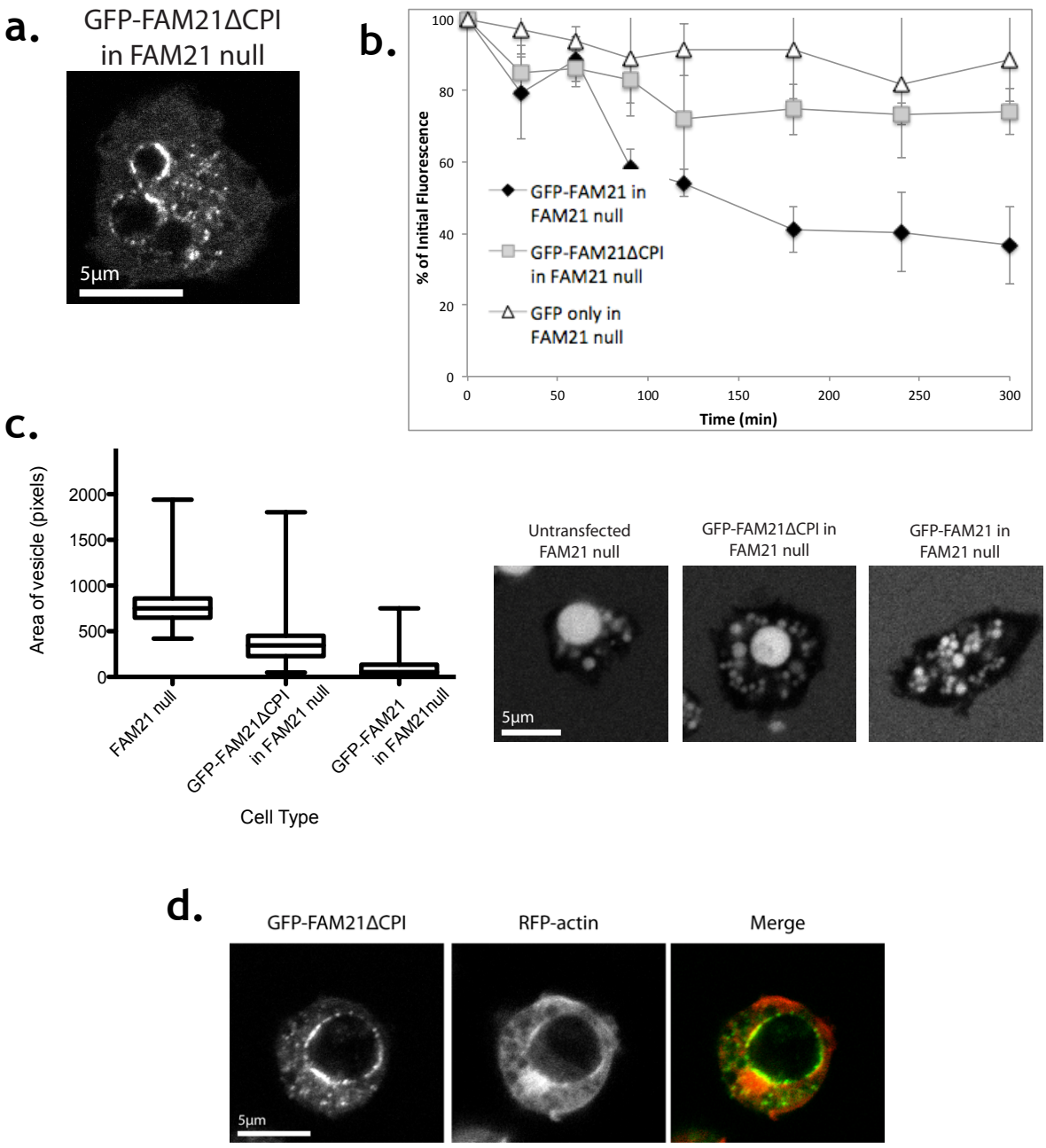


Figure 5.9 GFP-FAM21ΔCPI expression in FAM21 nulls

GFP-FAM21ΔCPI was expressed in FAM21 nulls. (a) Cells were fed LoFlo medium plus unlabelled dextran and imaged on a confocal microscope. (b) Cells were used to perform the exocytosis assay as previously described (n=3; error=SD). (c) GFP-FAM21ΔCPI or GFP-FAM21 were expressed in FAM21 nulls and fed TRITC-dextran and unlabelled dextran. Cells were imaged on a confocal microscope and vesicle size was quantified from the images obtained as previously described. (d) FAM21 nulls coexpressing GFP-FAM21ΔCPI and RFP-actin were fed unlabelled dextran and imaged on a confocal microscope.

cells were also fed unlabelled dextran with TRITC-dextran and imaged to find that they contained enlarged vesicles like those seen in FAM21 nulls (fig. 5.10b).

These results are very similar to those obtained when we expressed GFP-FAM21ΔCT in Ax2 cells, whereby the construct has a dominant negative effect on the cells which develop a partial FAM21 null phenotype with the enlarged post-lysosomes and delay in exocytosis. As before, the reason for this is the incorporation of a non-functional form of FAM21 into WASH complexes, rendering this subset of complexes unable to dissociate from post-lysosomal membranes. This further proves that the CPI is essential for FAM21 function within the WASH complex.

5.13 FAM21 links WASH complex to CP

We hypothesized that the possible function of the CPI and the interaction with capping protein is to recruit this actin regulating protein to the vesicular actin coats created by WASH to contribute to its regulation. To investigate this, we expressed capping protein in both Ax2 and FAM21 null cells to compare the localisation in respect to the intermediate endosomal actin coats in each case.

GFP-WASH and RFP-CAP32 were coexpressed in Ax2 cells. We found that capping protein was colocalised with all GFP-WASH on endosomal vesicles in the cells at the membrane (fig. 5.11a). We also expressed these constructs in FAM21 nulls and found that the localisation in these cells was slightly different. GFP-WASH is always restricted to the membrane of the vesicle, and capping protein was colocalised with WASH here, however capping protein was also localised throughout the actin comet which protrudes from the vesicle membrane (fig. 5.11b).

The complete overlap of capping protein with WASH that we see in Ax2 cells shows that normally, capping protein and the WASH complex are tightly coupled on post-lysosomal membranes. In FAM21 nulls we see that this coupling is lost. Capping protein is still localised to post-lysosomal actin, therefore the purpose of FAM21 is not to recruit capping protein to these actin structures, however it no longer completely colocalises with WASH complex. This indicates that the

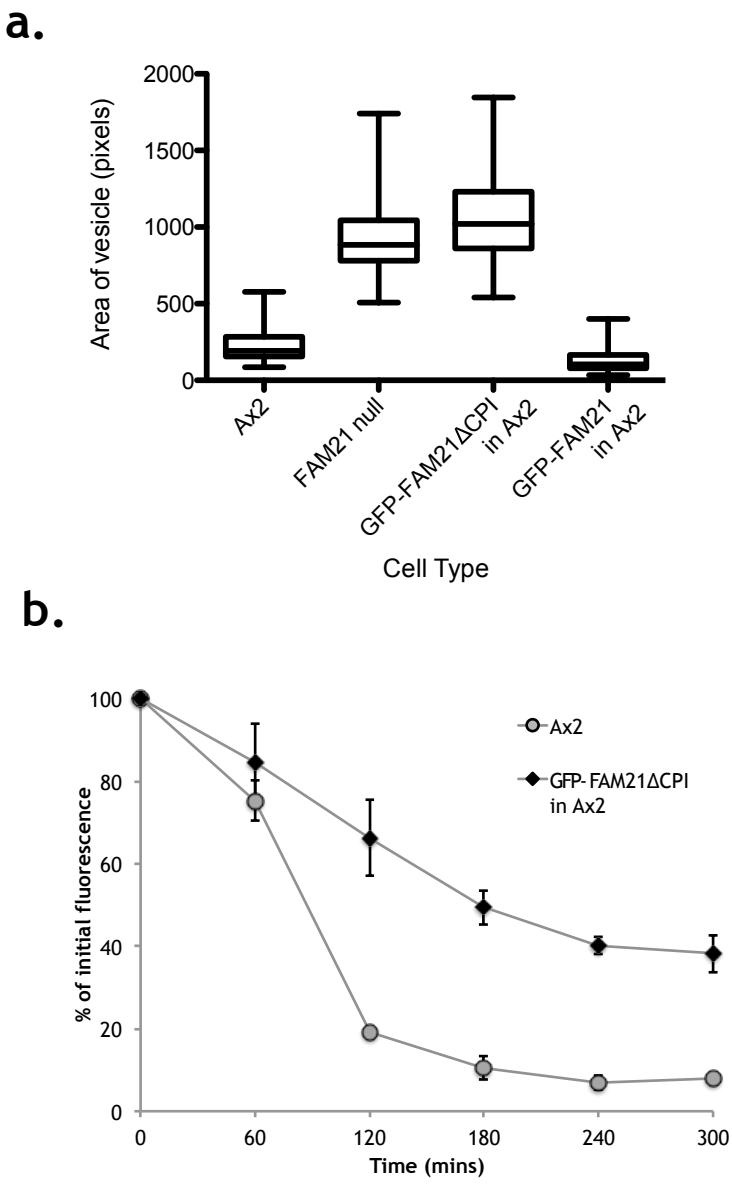


Figure 5.10 GFP-FAM21ΔCPI expression in Ax2 cells

GFP-FAM21ΔCPI was expressed in Ax2 cells. (a) Cells were fed TRITC-dextran and unlabelled dextran. Cells were imaged on a confocal microscope and vesicle size was quantified from the images obtained as previously described. (b) Cells were used to perform the exocytosis assay as previously described (n=3; error=SD).

purpose of FAM21 is to couple the two together, rather than use one to recruit the other in regards to the WASH complex and capping protein.

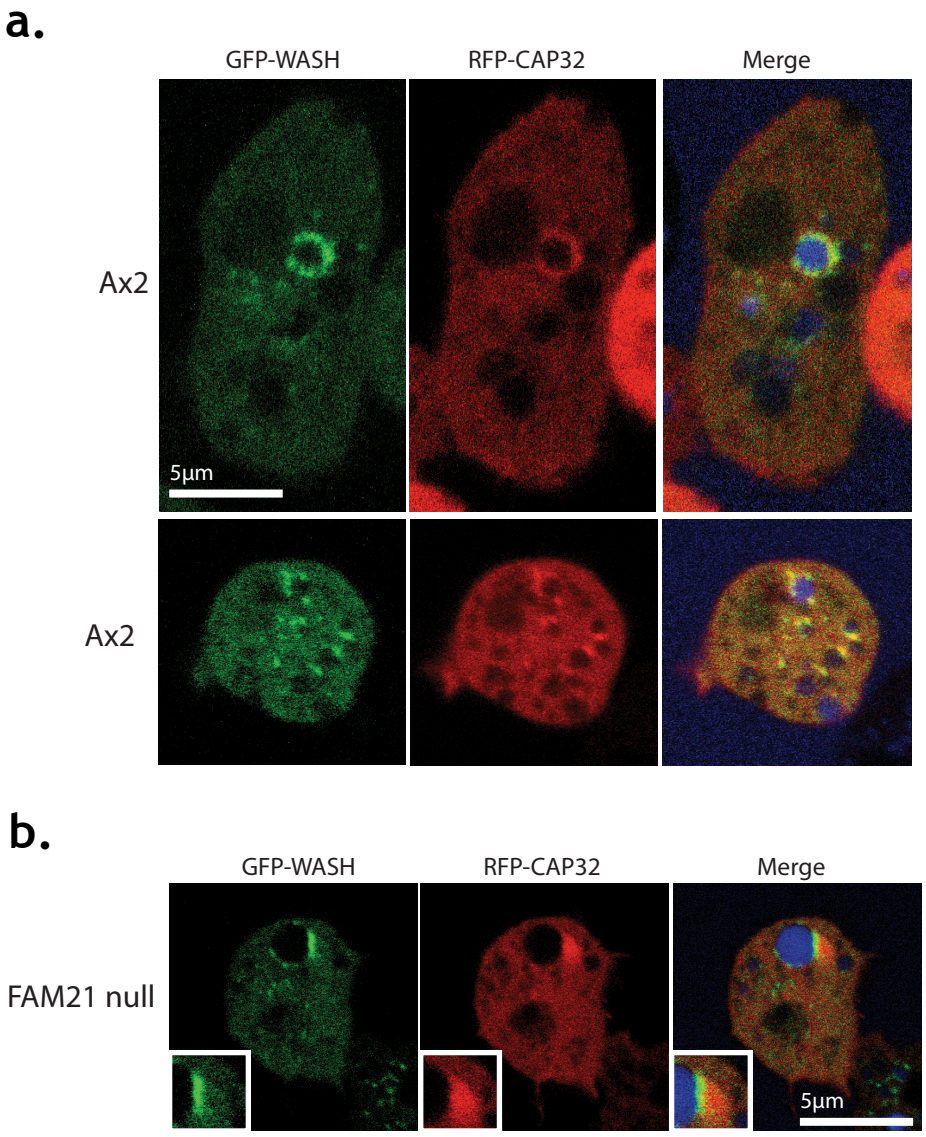


Figure 5.11 Capping protein localisation

GFP-WASH and RFP-CAP32 were coexpressed in (a) Ax2 cells and (b) FAM21 nulls. The cells were fed unlabelled dextran in LoFlo before being imaged on a confocal microscope.

CHAPTER SUMMARY

In this chapter, we have dissected the function of the FAM21 protein. The highly conserved head region of FAM21 is responsible for binding the WASH complex, and mutant forms containing the head region compete with endogenous FAM21 for complex binding sites, causing a partial FAM21 null phenotype in wild type cells.

The tail region of FAM21 contains a number of repeats, the ligand of which remains unknown, but we do know that the tail is able to localise independently to endosomal membranes. The tail also contains the CPI site, which appears to be the only functional part of the tail other than the repeats.

The CPI is essential for FAM21 function; FAM21 lacking the CPI cannot rescue the null phenotype. We have shown that capping protein does not require FAM21 to be recruited to endosomal actin, therefore we can conclude that the purpose of FAM21 must be to couple the complex to capping protein directly. The possible implications of this coupling and how it contributes to regulation and removal of the WASH complex will be discussed in the final chapter.

CHAPTER 6
DISCUSSION

6 Discussion

6.1 Dictyostelium and the WASH complex

Previous use of *Dictyostelium discoideum* to investigate the function of WASH has shown it is a suitable model in which to study the WASH complex. By using immunoprecipitation and mass spectrometry, we confirmed that the five known members identified in mammalian cells, WASH, SWIP, Strumpellin, FAM21 and ccdc53 (Derivery et al., 2009; Gomez and Billadeau, 2009), were expressed and formed a constitutive complex in *D. discoideum* cells. These proteins all have a high homology to their mammalian counterparts, and the functions of other WASP family proteins, such as Scar/WAVE, are already known to be conserved between *Dictyostelium* and mammalian cells. We therefore sought to determine the possible functions of the four regulatory subunits of the complex in a global context.

WASH is an NPF (Linardopoulou et al., 2007) whose regulation is through incorporation into a complex (Derivery et al., 2009; Gomez and Billadeau, 2009). There are parallels between the WASH complex and the Scar/WAVE complex (Jia et al., 2010), showing that this form of regulation is not a unique mechanism. Both are heteropentameric complexes which are evolutionarily highly conserved, and organisms generally tend to contain either none or all of the constituents of each complex within their genome (Veltman and Insall, 2010). One key similarity between these two complexes is the inability of the complex to survive incomplete; for both WASH and Scar/WAVE complexes to remain intact in the cell, all the subunits must be present, with the exception of FAM21 in the WASH complex.

6.2 Strumpellin and Spastic Paraplegia

We found that the loss of either of the Strumpellin and ccdc53 subunits causes a growth defect in cells. Although we decided to focus the study of the project on the FAM21 subunit, it would be interesting for the future to find out the relevance of these two particular subunits in cell growth/division.

As mutations in Strumpellin are a known cause in spastic paraplegia, we looked into the effect of these mutations on WASH complex function. It seems that

these mutations have no noticeable effect on the function of the WASH complex in *Dictyostelium*. In humans, pure forms of spastic paraplegia such as SPG8 only affect a specific cell type; the upper motor neurons which project to the lower limbs (Fink, 1997) that are some of the longest cells in the body. The disease is also adult onset, and characterized by gradual degradation from the ends of the neurons (Valdmanis et al., 2007). A subtle defect caused by these mutations may not affect a single-celled, and relatively short-lived organism such as *Dictyostelium*. Spastin is another protein known to be mutated in spastic paraplegia. It has been shown to play a role in severing of microtubules (Errico et al., 2002; Evans et al., 2005). Links have previously been shown between microtubules and the WASH complex; Derivery et al. (2009) show an interaction between the WASH complex and tubulin, and that treatment of siWASH cells with nocodazole prevented the tubulation phenotype induced by loss of WASH. This raises the possibility that Strumpellin may also be involved in regulation of microtubules. This would be an interesting start point for investigating the exact role of the Strumpellin subunit in more detail, and could explain why it causes a growth defect in *Dictyostelium* cells.

6.3 WASH builds endosomal actin coats

Endosomal actin coats at intermediate stages of the endocytic cycle had previously been suggested to prevent fusion between different compartments within the endocytic cycle (Drengk et al., 2003). Our data disagree with this, as we observed fusion between the neutral vesicles in FAM21 null cells which have excessive actin polymerized on their membrane (fig. 4.7). We also see there must be a degree of regulation between fusion of vesicles in WASH null cells, which are devoid of actin, as the vesicles do not all fuse into one compartment in these cells like in FAM21 nulls.

WASH has been shown to be the NPF responsible for the construction of these intermediate actin coats on endosomal vesicles in both mammalian and *Dictyostelium* cells (Derivery et al., 2009; Gomez and Billadeau, 2009; Carnell et al., 2011). Loss of WASH in all cells results in the absence of these actin coats. Both the processes of endocytosis and exocytosis also require actin, however it has already been shown that WASH is not required for endocytosis (Carnell et

al., 2011), and we have also shown that WASH dissociates from post-lysosomes well before exocytosis.

Carnell et al. (2011) hypothesize that the function of these intermediate actin coats is to cluster V-ATPase complexes into subdomains on lysosomal membranes. It has already been shown that the V-ATPase is removed in small recycling vesicles from lysosomes (Clarke et al., 2010). There are also F-actin binding sites on several of the V-ATPase subunits which have not previously been assigned a function (Holliday et al., 2000; Vitavska et al., 2003). Other work has also shown that clustering into membrane microdomains is reliant on the cell cytoskeleton. Chichili and Rogers (2007) showed that markers associated with lipid raft domains were unable to cluster on disruption of actin using latrunculin B. In contrast, clustering was enhanced and much tighter on addition of the actin stabilizing drug jasplakinolide.

6.4 Regulatory subunits

In *Dictyostelium,* the presence of WASH and actin on endosomes is necessary for the maturation and neutralisation of lysosomes, without which there is no progression to exocytosis. We began by looking at how the regulatory subunits are important for this function, and found that loss of any one of the four regulatory subunits caused the total block in exocytosis seen in WASH nulls. Loss of three of the four, Strumpellin, SWIP and ccdc53, abolished the intermediate endosomal actin coats, and prevented neutralization. This WASH null-like phenotype in these cell lines was due to the fact that the stability of the complex was compromised if any of these three subunits were not present, therefore there was little or no stable WASH protein present in the cells (fig. 3.8).

There is one exception to this; FAM21. The loss of FAM21 does cause a reduction in endogenous levels of WASH, but to far less of a degree than any other subunits. Gomez and Billadeau (2009) claim that in mammalian cells, loss of FAM21 results in loss of WASH. They used siRNA to knockdown FAM21 and show a reduction in WASH protein which they state is detrimental to WASH complex function, however we suggest the level of WASH reduction is relative. In *Dictyostelium*, we found the relatively small reduction in levels of WASH due to

loss of FAM21 does not appear to affect WASH complex function, and demonstrated that sufficient WASH protein remains in FAM21 nulls to effectively remove V-ATPase from lysosomes (fig.). We also showed that the same concept is true in mammalian cells. Because loss of WASH results in a defect in recycling of α5β1 integrin (Gomez et al., 2012; Zech et al., 2011) we used this to investigate the importance of FAM21 in mammalian WASH complex function. We found that knockdown of FAM21 did lead to a decrease in WASH protein level, however this was not substantial enough to affect the integrin recycling. This demonstrates that sufficient WASH protein is present in the cells to fulfil this function. This may be different in mammalian cells if FAM21 is completely absent, as our work was performed using cells treated with siRNA, which therefore contained low levels of FAM21 protein. Knockdown efficiency was high, however we do not know size of the effect that the small amount of residual FAM21 protein left in the cell has, and a total FAM21 knockout would give a far more definitive result.

6.5 Loss of FAM21

We found that FAM21 null cells had several distinctly unique phenotypes among the WASH complex subunit knockouts. Because WASH was present in FAM21 nulls, these cells did contain endocytic actin coats, and V-ATPase was indeed removed from lysosomal membranes. This in turn allowed maturation of lysosomes to neutral post-lysosomes, however exocytosis remained blocked. This suggests that FAM21 functions at a separate point in the endocytic cycle to WASH, probably downstream as the vesicles were able to progress further along the endocytic pathway, and the creation of a double WASH/FAM21 null mutant confirmed this.

The neutral vesicles in FAM21 nulls were greatly enlarged in comparison to those in wild type cells. Limited and controlled vesicle fusion is known to occur at early endosomal stages in wild type cells (Clarke et al., 2002) which is why some fusion does occur between the vesicles in Ax2 and WASH null cells, and causes slight swelling on addition of dextran, especially in WASH nulls which accumulate far more intracellular dextran than an Ax2 cell. Fusion of later, post-lysosomal compartments may happen naturally in Ax2 cells, however once vesicles have matured to post-lysosomes, they are rapidly progressed to

exocytosis (Aubrey et al., 1993). This means there would be little opportunity for the vesicles to fuse and therefore little need for a regulatory mechanism to prevent this fusion occurring. We showed that in FAM21 nulls, the dextran trafficked into these enlarged, neutral compartments failed to progress any further than this, and was trapped indefinitely at this stage. This persistence of the vesicles within the cell increased the likelihood of fusion. The pH of vesicles also contributes to the fusion competency, with acidic vesicles less competent to fuse with one another than neutral vesicles (Lenhard et al., 1992). The result is that in a FAM21 null, any neutral vesicles that form will inevitably fuse to form a single, enlarged vesicle over time, and indeed this fusion event was observed (fig. 4.8).

6.6 Structural analysis of FAM21

To investigate the function of FAM21, we looked in more detail at the structure of the protein. The head region of the protein, constituting the first ~300 amino acids, had already been identified in mammalian cells as the region responsible for the interaction with the WASH complex (Gomez and Billadeau, 2009; Harbour et al., 2010; Jia et al., 2010). We showed that the same is true in *Dictyostelium*, as the head region of FAM21 was necessary and sufficient to coimmunoprecipitate the other core complex members (table 5.2). The tail region of FAM21 is made up of a repeated sequence, interrupted by the only identified functional domain, the capping protein interaction (CPI) site.

Neither region, the head nor tail, of FAM21 is sufficient to rescue any of the phenotypes of FAM21 nulls (fig. 5.3). FAM21 null cells expressing these regions separately were still blocked in exocytosis, contained enlarged vesicles and seemed to accumulate WASH on post-lysosomal membranes. Even so, both regions were still able to localise independently, showing that FAM21 has two separate localisation signals within the sequence. The head region uses the interaction with the WASH complex to localise correctly, whereas the tail must have a different signal of its own which we suggest is through the repeats.

We also showed that the head domain had a slight dominant negative effect on Ax2 cells, which we also saw later when expressing the capping protein binding FAM21 mutant. This shows it is likely that when WASH complexes are formed,

the subunits cannot dissociate or be replaced. The result is that in Ax2 cells containing mutant versions of FAM21, a proportion of complexes are formed containing these mutants or fragments instead of the endogenous copy of the protein, and these complexes are then rendered incapable of full function, leading to a partial FAM21 null-like phenotype.

6.7 The FAM21 tail

Derivery et al. (2009) demonstrated that there are lipid-binding domains within the complex. As we have shown the tail region of FAM21 was able to bind endosomal membranes without interacting with any of the other subunits of the WASH complex, we hypothesize that the repeated sequence present in the FAM21 tail may be able to directly bind lipids. We attempted to test for this interaction using a PIP-array, however we did not identify any positive interactions. It is possible that the *in vitro* conditions were not sufficient for the interaction between the repeats and their target, or that the lipid for which the repeats are specific were not present on the array. Jia et al. (2010) also performed a similar lipid blot with the tail of human FAM21. They identified a number of interactions with various phospholipids, however their results were very unspecific. They saw a large number of positive interactions, including PI_4P, a phospholipid associated with the Golgi, although neither FAM21 nor WASH are seen to localise there. Another group suggested that it is the retromer complex, a complex involved in retrograde transport of receptors from endosomes to the Golgi, which interacts with the FAM21 tail repeats (Harbour et al., 2010). They identified the retromer subunit VPS35 using mass spectrometry by coimmunoprecipitation with the FAM21 tail, although this was only possible using low detergent lysis buffer. When using normal detergent-containing buffer, retromer components were not coimmunoprecipitated with the FAM21 tail, however other proteins such as the capping protein subunits were. This suggests that the interaction between FAM21 and VPS35 may not be direct. In *Dictyostelium*, we immunoprecipitated three separate members of the complex, ccdc53, WASH and FAM21, yet no retromer subunits were identified by mass spectrometry in any instance, despite the high conservation of the retromer component sequences and functions (Dacks and Field, 2007). Overall, the ligand of the tail repeats is currently unknown, and it will require further investigation in order to determine this elusive binding partner.

To further assess the specificity of the repeats of the FAM21 tail, we expressed the human FAM21 protein in *Dictyostelium*. Human FAM21 could not interact with the *Dictyostelium* WASH complex, as the head region alone was unable to localise in cells. In contrast, the tail region of the human protein did localise correctly, although neither region could rescue the FAM21 null phenotype. These results were surprising, as the head region of FAM21 is the most highly conserved. This, in combination with the high conservation of the other WASH subunits, suggested that if any region of the protein would work in a different species, it would be the head region. In fact it is the variable tail region which was able to maintain its localisation which is relatively divergent between species, especially human and *Dictyostelium*. This shows that the tail-binding ligand must be conserved across species, further supporting the idea that it is something of low complexity such as a lipid. An interesting point about the tail of FAM21 is that it appears to bind exclusively to endosomal membranes, and not to other internal membranes or the plasma membrane. This suggests that the FAM21 tail is able to differentiate between the different membranes, perhaps through detection of different lipid composition.

6.8 WASH complex recycling

Although FAM21 nulls are able to neutralize lysosomes, the process takes considerably longer than in Ax2 cells (fig. 4.6). We found that whether the cells contained giant post-lysosomes or not (by pre-feeding the cells unlabelled dextran or not) made no difference to the time it took for neutral vesicles to appear in the cells. This meant we could rule out the presence of the enlarged vesicle itself being the cause of the delay, for example by causing a physical obstruction within the cytoplasm.

When looking into the cause of this delay, we found that the distribution of WASH appeared to be very biased towards the membrane of the enlarged post-lysosomes in FAM21 nulls. This suggested to us that the WASH complex was being trapped on post-lysosome membranes, however as the presence of the enlarged vesicles in the cells was not normal, comparisons between WASH distribution in FAM21 nulls and Ax2 cells was difficult. To investigate further, we used FRAP to show that WASH complex was indeed being sequestered on

post-lysosomes in the absence of FAM21 (figs. 4.10 and 4.11). This would mean FAM21 nulls would need to continuously synthesize new WASH complexes in order to neutralize any nascent acidic lysosomes, rather than recruiting existing complexes recycled from mature post-lysosomes. Therefore the delay in neutralization was likely due to a lack of available WASH complex as a result of this. We also saw the same failure in complex dynamics in mammalian cells. This tells us that the role for FAM21 in instigating removal of the WASH complex from membranes is likely to be conserved.

6.9 FAM21 and capping protein

We have shown that both the head and tail region of FAM21 are essential to its function. The head domain is required for its incorporation into the WASH complex, but there must also be a role for the tail, as the head alone does not compensate for the full length protein. The repetitive and proline-rich sequence of the tail mean it has little secondary structure, and the only defined domain present in the sequence is that of the capping protein interaction site (Derivery et al., 2009; Jia et al., 2010). The CPI is a small sequence, the consensus being only 17 amino acids (Bruck et al., 2006), hidden within a tail consisting of over 1000 amino acids. Despite this, our results show that this site appears to be essential for the function of FAM21, its removal rendering the protein unable to rescue the FAM21 null phenotype (fig. 5.9).

Jia et al. (2010) suggest that the interaction between FAM21 and capping protein is transient, and it is not a constitutive part of the complex unlike the other five members. They do not consistently see it with coimmunoprecipitation of WASH which correlates with our results in *Dictyostelium*. Despite this, in *Dictyostelium* Ax2 cells we see colocalisation of capping protein and the WASH complex on endosomal structures, although capping protein does also localise elsewhere in the cell (Derivery et al., 2009), having an important role in the regulation of many different actin structures in cells (Cooper and Sept, 2008). Phylogenetic analysis also shows that the five constitutive members of the complex are nearly always found together in a species, however capping protein is often found in organisms not containing a WASH complex (Veltman and Insall, 2010). In contrast, the fact that the organisms which contain a WASH complex

nearly always also contain capping protein points to capping protein being vital for function of the WASH complex (Veltman and Insall, 2010).

FAM21 is a vital subunit for regulation of WASH and endosomal actin structures, and we have shown that the interaction between FAM21 and capping protein is an essential part of this regulation. In the absence of FAM21, capping protein is still recruited to endosomal actin structures in cells (fig. 5.11) therefore the role of this interaction must be to bring capping protein into close proximity to the WASH complex itself. This interaction could work in two ways; FAM21 could sequester capping protein away from the actin in the immediate vicinity of the complex, or it could work to bring capping protein into contact with the actin at this site.

An example of a protein which uses a CPI domain to sequester capping protein away from actin is CK2-interacting protein (CKIP). CKIP is a kinase which contains a CPI site of the same consensus as that seen in FAM21. It is known to bind, sequester and phosphorylate capping protein in order to prevent its capping activity at plus ends (Canton et al., 2006; Fujiwara et al., 2010). If FAM21 were to work in this manner, it could increase the turnover of actin at the interface of the actin structure and the membrane, where the WASH complex is located, and thus produce a more dynamic structure. In FAM21 nulls, we see that the comet tails which stream off the endosomal membranes are very dynamic, making this theory seem unlikely as absence of FAM21 in this case should decrease actin polymerization at the membrane, however we do not know the rate of turnover of actin in normal circumstances on endosomes and therefore have no comparison.

The second possibility is that FAM21 brings capping protein into close proximity with the complex. FAM21 appears to negatively regulate the activity of WASH, indicated by the fact that the actin polymerization in the presence of FAM21 seems much more moderate and controlled, producing a thin vesicular coat rather than a large comet. Also, FAM21 appears to work as an ‘off switch’ for the complex, as we have shown it is required for removal of the complex from post-lysosomal membranes once maturation is complete, acting to stop the actin polymerization directed by WASH to allow vesicle progression. By encouraging

the capping of plus ends near the membrane interface, FAM21 may help prevent such a high turnover of filaments as is seen with the comets in FAM21 nulls, and instead may help form a more stable ring structure. An actin ring formed from highly regulated filaments could potentially allow a contractile force around the membrane which may induce the clustering motion hypothesized for the V-ATPase complex. More study needs to be done of the rate of turnover of actin on membranes in both wild type and FAM21 null cells to determine whether this is the case. Also, a more detailed examination of the distribution of V-ATPase molecules through endosomal membranes at different stages of progression needs to be made to substantiate the clustering hypothesis.

One potential model that we propose is that if the removal of V-ATPase from lysosomal membranes is through clustering, potentially the removal of the WASH complex itself could also be through clustering. The interaction between FAM21 and capping protein may be to directly couple the WASH complex to the actin filaments themselves. If an actin filament is then simultaneously coupled to WASH and capped to prevent further extension, it could act as a binding mechanism and off switch for that complex. The bound complex is then clustered through the binding to the actin filament, possibly through a contraction of an actin ring structure, ready for a bud to form and remove it.

6.10 Progression to exocytosis

No matter what the mechanism involving capping protein and FAM21 may be, there must be a trigger which defines the point at which neutralization is complete and the fully mature post-lysosome is ready to be exocytosed. This may be a threshold of the pH, the removal of some or all of the V-ATPase, or may be determined by Rabs which are well known to define different compartments and stages of the endocytic cycle (Gruenberg and Maxfield, 1995; Pfeffer, 2001). This signal could then instigate the removal of the WASH complex from the membrane, which takes place before exocytosis (fig. 1.5). As FAM21 is essential for complex dissociation and may well be directly involved in the mechanism that instigates its removal, it may be that FAM21 is directly regulated by some means such as phosphorylation. Our mass spectrometry data for FAM21 from the coimmunoprecipitations identified several

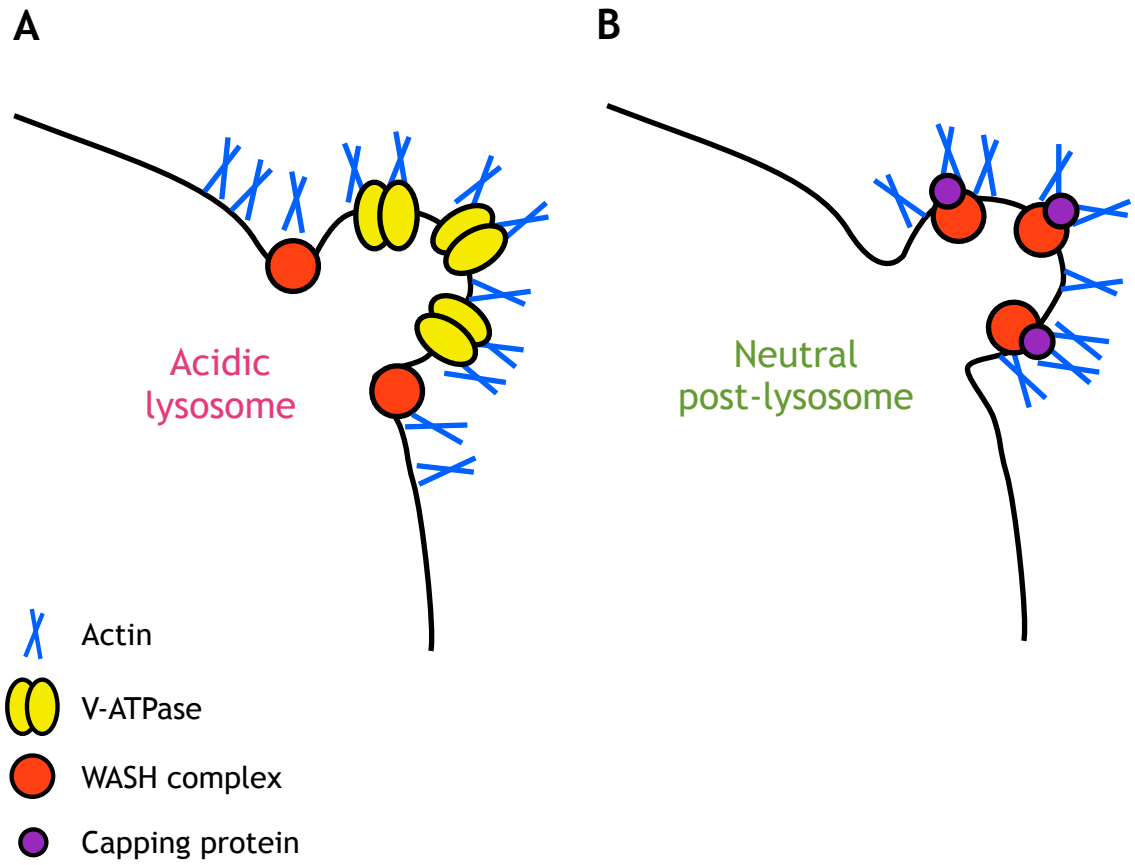


Figure 6.1 Possible model of WASH complex removal

One hypothesis for the mechanism of how the WASH complex is removed from post-lysosomal membranes is illustrated above. Stage A represents the current hypothesis of Carnell et al. (2011); WASH complex is recruited to the acidic lysosomal membrane. WASH builds and actin coat which binds to and clusters the V-ATPase molecules. A bud then forms to remove these from the membrane, allowing neutralization and maturation to post-lysosome. We hypothesize that a similar process occurs at stage B; once neutralization is complete and the post-lysosome is ready to progress to exocytosis, a signal/trigger induces binding of capping protein to FAM21. This couples the WASH complex to the actin coat, causing its own clustering to allow removal through budding.

possible phosphorylation sites, some of which were in close proximity to the CPI domain. It would be interesting to investigate the importance of these sites in FAM21 and WASH complex function, and whether they are conserved in FAM21 proteins of other species, especially in mammals.

6.11 Localisation of the WASH complex

So far, data in mammalian cells suggests that FAM21 is the vital subunit required to localise the WASH complex to endosomal vesicles. Harbour et al. (2010) show that in HeLa cells, the retromer complex is required for localisation of the WASH complex, through a direct interaction with FAM21. Gomez and Billadeau (2009) agree that FAM21 is a key subunit for localisation, but they claim it is totally independent of retromer. Our data do not agree with these findings, as we show that the complex is not only localised but also functional without the FAM21 subunit in both mammalian (fig. 4.5) and *Dictyostelium* cells (fig. 4.3). One point on which all studies agree is that the FAM21 subunit is able to localise independently, regardless of the presence of WASH (Gomez and Billadeau, 2009).

It is likely that the complex uses more than one localisation signal. The one member that we found to be essential for correct vesicular localisation of WASH and Strumpellin is SWIP. Without SWIP, both of these subunits were cytoplasmic. SWIP, cdc53 and FAM21 were all able to localise independently of any other subunits, therefore these proteins clearly have their own localisation signals. SWIP is clearly essential and sufficient for complex recruitment, however ccdc53 may also contribute to complex localisation. It may help strengthen the interaction of the complex with endosomes or help in defining the correct vesicle population.

It would be interesting to investigate further into which of the complex members truly are required for localisation in mammalian cells. So far, no data has been published about what effect the loss of SWIP has on the WASH complex in mammalian cells. WASH complex localisation is more complicated in mammalian cells, because it is distributed on more than one population of vesicles. It has been shown to decorate early endosomes, recycling endosomes and parts of the degradative pathway (Derivery et al., 2009; Gomez and Billadeau, 2009; Zech et

al., 2011). Therefore this system may require a different set of signals to the relatively simple *Dictyostelium* system to direct WASH complex localisation.

6.12 Final summary

The functions of all members of the WASH complex have not yet been completely elucidated, however our focus on FAM21 has substantially increased our knowledge of a potential function and mechanism for this subunit. This will help to further our understanding of NPFs and the signals that feed in to the system of their regulation.

We have shown that the WASH complex requires all four regulatory subunits in order to function completely. Three of these, Strumpellin, ccdc53 and SWIP are essential for WASH function as an NPF. FAM21 is dispensable for the actin polymerizing ability of WASH through Arp2/3, however is still required for completion of the endocytic cycle. We have determined that WASH and FAM21, although constitutively part of the same complex, act at different stages in the same pathway. WASH is responsible for the initial actin polymerization, however FAM21 works downstream, in allowing the WASH complex to be recycled once neutralization is complete. We have shown that the capping protein binding site within the FAM21 tail is essential for its function, and that the direct coupling between the WASH complex and capping protein cannot occur without this subunit. There are several potential mechanisms through which capping protein and FAM21 may work in order to regulate the actin coat built by WASH, and instigate the complex removal from mature post-lysosomes, however further study is required to determine the exact processes which occur at these later stages and the signals which initiate them.

APPENDIX I

CLUSTAL 2.1 MULTIPLE SEQUENCE ALIGNMENT

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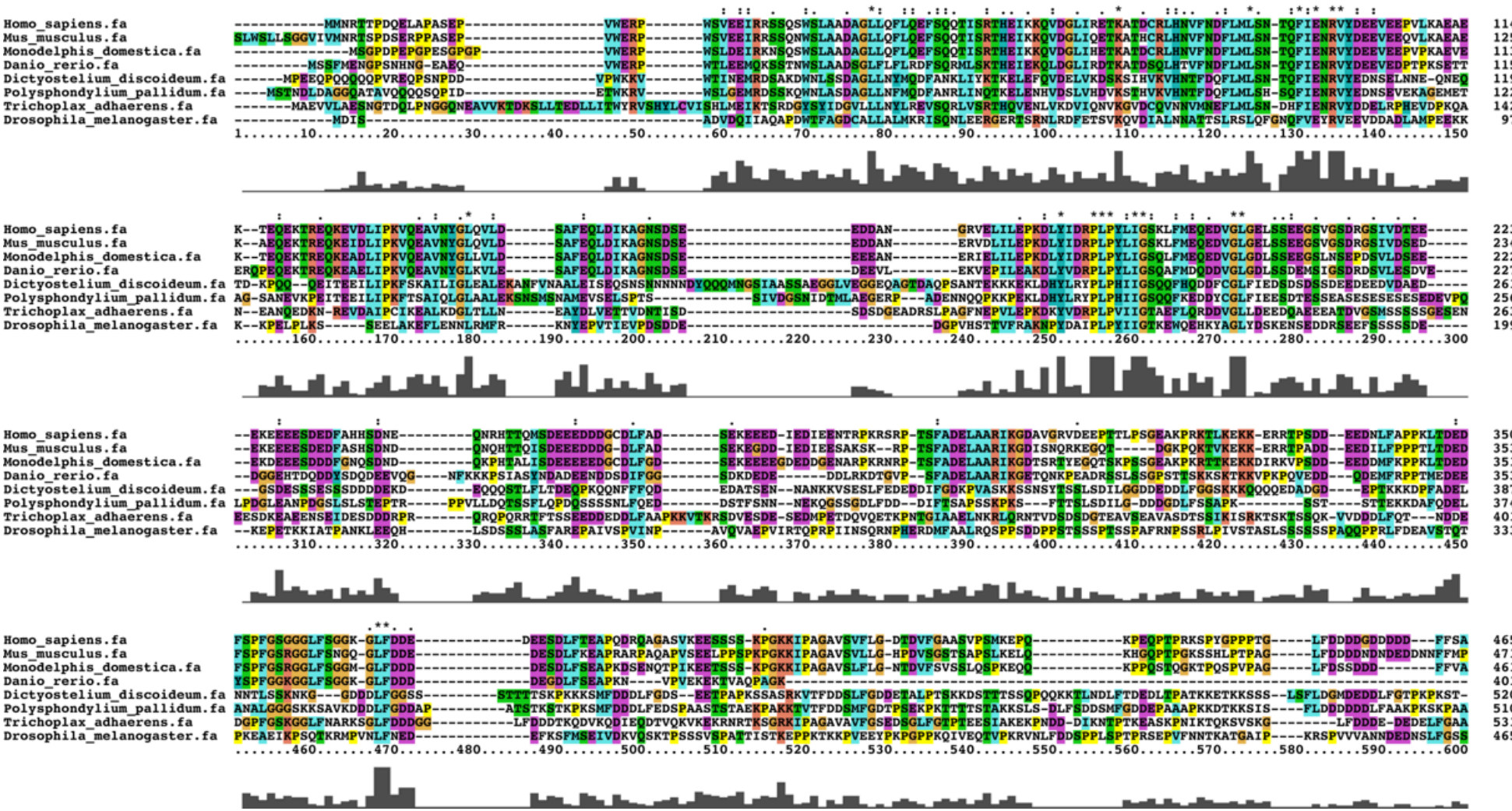

146

CLUSTAL 2.1 MULTIPLE SEQUENCE ALIGNMENT

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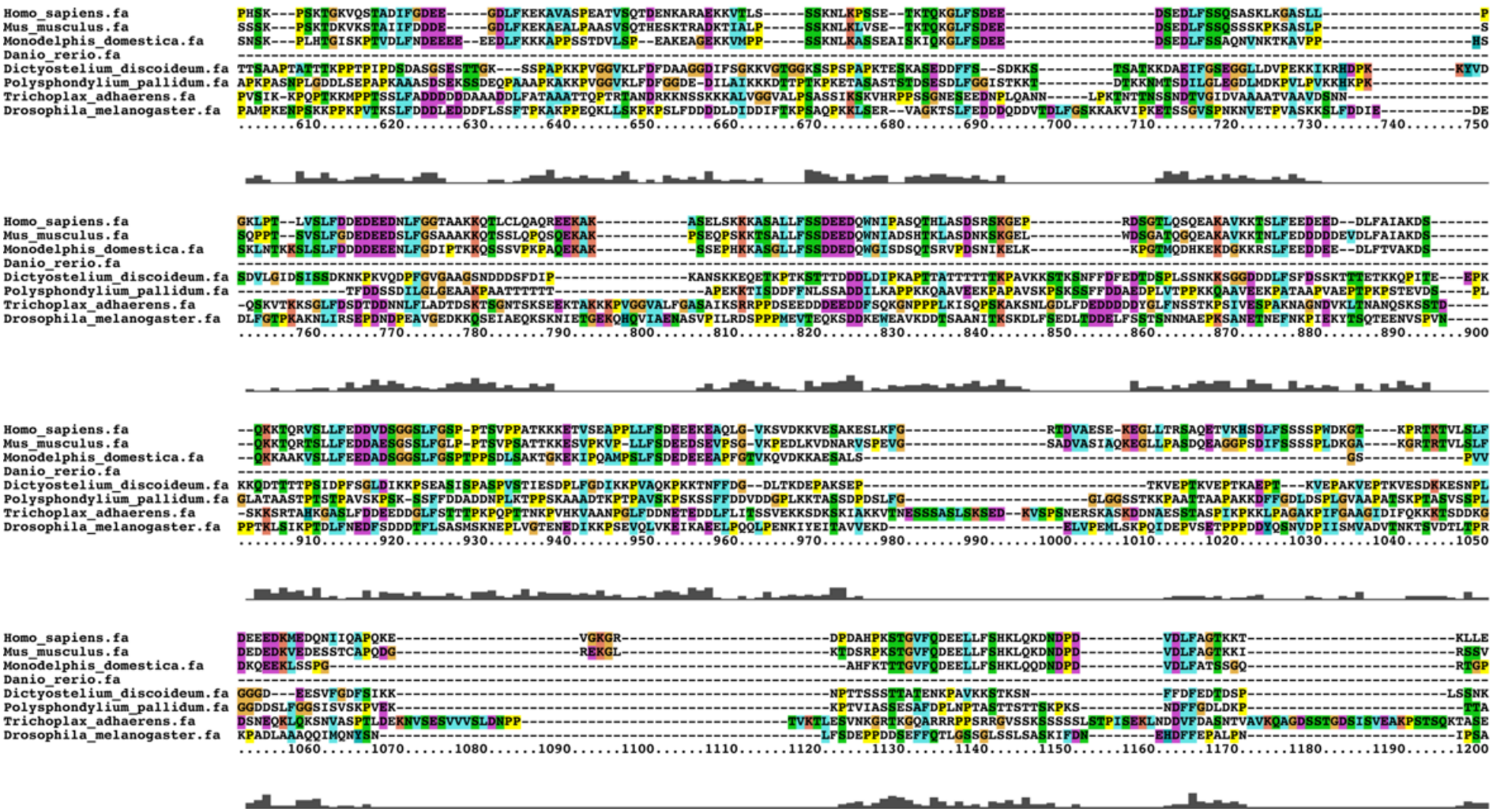

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CLUSTAL 2.1 MULTIPLE SEQUENCE ALIGNMENT

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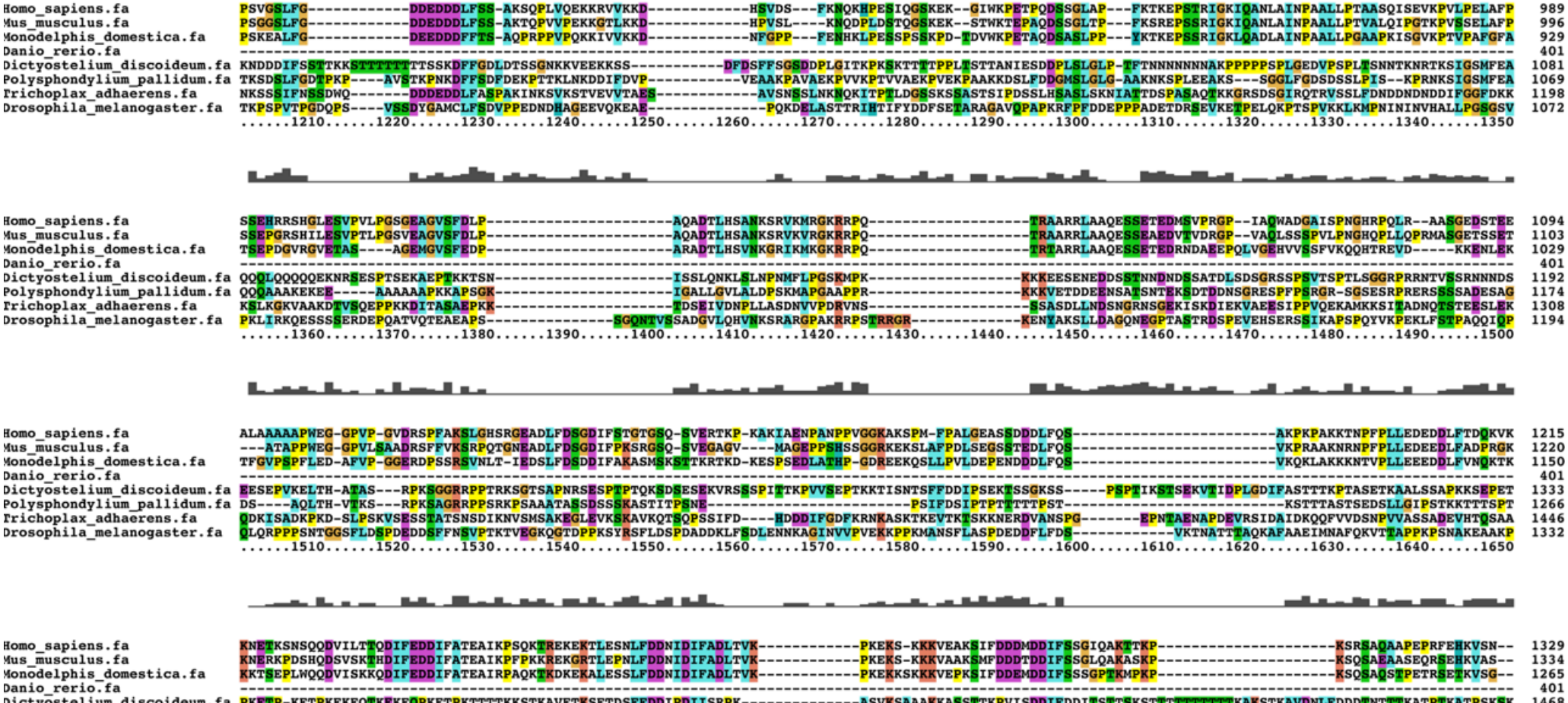

CLUSTAL 2.1 MULTIPLE SEQUENCE ALIGNMENT

File: /Users/lpark/Desktop/FAM21 alignment **Date: Tue Oct 2 11:13:37 2012**

Page 4 of 4

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Mus_musculus.fa              -IFDDPLNAFGSQ----  1346
Monodelphis_domestica.fa     -IFDDPLNALGGQ----  1277
Danio_rerio.fa               ----------------   401
Dictyostelium_discoideum.fa  PKAKNVENLFD------  1479
Polysphondylium_pallidum.fa  KAAANVDSLFD------  1395
Trichoplax_adhaerens.fa      GTFDDPLNAGGN-----  1607
Drosophila_melanogaster.fa   IPSNSGDNPLADLLDFK  1469
                             ......1810.......
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149

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