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Retromer-mediated endosomal protein sorting: all WASHed up!

Matthew N.J. Seaman¹, Alexis Gautreau², and Daniel D. Billadeau³

¹ Cambridge Institute for Medical Research, Wellcome Trust/MRC Building, Addenbrookes Hospital, Cambridge, CB2 0XY, U.K.

² Laboratoire d'Enzymologie et Biochimie Structurales, CNRS UPR3082, Gif-sur- Yvette, France.

³ Department of Immunology and Biochemistry, Division of Oncology Research, Mayo Clinic, Rochester, MN 55905, USA.

Abstract

Endosomal protein sorting governs the fate of many physiologically important proteins involved in a panoply of cellular functions. Recent discoveries have revealed a vital role for endosomally-localised branched actin patches in facilitating protein sorting. The formation of the actin patches has been shown to require the function of the WASH complex – the major endosomal actin polymerisation-promoting complex – that stimulates the activity of the ubiquitously expressed Arp2/3 complex. Another key component of the endosomal protein sorting machinery is the retromer complex. Studies now show that retromer mediates the recruitment of the WASH complex and its regulators to endosomes. In this review, the recent progress in understanding the role of the WASH complex along with retromer in endosomal protein sorting is discussed.

Keywords

Retromer; WASH complex; endosome; sorting; actin; Fam21

The role of endosomal actin patches

The endocytic system is a place where the biosynthetic pathway intersects with proteins and ligands that have been endocytosed from the cell surface. Signalling events initiated at the plasma membrane are processed at endosomes leading to diverse physiological responses. Depending on its respective function, a membrane protein present in an endosome can be directed to three possible destinations; the lysosome for degradation, the trans-Golgi network or the cell surface. The sorting of membrane proteins (often referred to as cargo) to multiple destination requires specialised machinery that can recognise cargo proteins, partition them into discrete domains and generate a suitable transport intermediate for delivery to the appropriate destination (see [1] for review).

The dynamic nature of endosomal protein sorting is underpinned through an equally dynamic set of protein-protein interactions. In this review, recent progress in studies of endosomal sorting that has revealed a key role for branched actin filaments is highlighted. Discrete actin patches may provide a platform for specialised signalling events and through

the rapid elongation of actin filaments can generate localised force that may be involved in production and/or scission of endosomal tubules. Additionally, actin filaments are necessary for myosin motors to mediate short-range movement of proteins within endosomal compartments (see Figure 1). The machinery that generates the localised actin patches that serve to segregate membrane proteins into discrete domains is discussed and the mechanisms through which actin production is regulated and linked to cargo protein sorting explained.

The endosomal WASH complex

The generation of actin filaments on endosomes involves the Arp2/3 complex, which, similar to other sites in the cell, requires activation by nucleation promoting factors (NPFs) (see Text Box and for summary see [2]). At endosomes, the WASH complex is the major Arp2/3 NPF [3,4]. The WASH complex was initially identified through the affinity isolation of tagged WASH1 protein [3,4] and subsequent in-vitro studies have revealed the analogy of the WASH complex with the WAVE complex that activates actin polymerisation at the leading edge of migrating cells [5].

The WASH complex is composed of five proteins: KIAA1033 (also known as SWIP), Strumpellin, FAM21, WASH1 and CCDC53. Biochemical and yeast two-hybrid analyses have delineated the binary interactions that underpin the WASH complex [5,6]. These are summarized diagrammatically in Figure 2A. Similar to the WAVE complex, studies conducted in mammalian cells have demonstrated that RNAi-mediated silencing of individual WASH complex subunits results in instability and degradation of other WASH complex components [3-5]. Moreover, deletion of WASH1 from mouse embryonic fibroblasts (MEFs) leads to a substantial loss of all members of the complex [7]. Taken together, these data strongly support the hypothesis that the proteins operate as an obligate complex.

The WASH complex is ubiquitously expressed in mammalian tissues and is conserved in many eukaryotic taxa including ancient eukaryotes and unicellular organisms [8,9]. Interestingly, many inherited neuropathies stem from mutations in genes encoding endosomal protein sorting machinery [10], and recent studies have demonstrated that mutations in WASH complex components result in neurological pathologies. For example, autosomal dominant mutations in the spastic paraplegia gene, *SPG8*, which encodes Strumpellin, cause hereditary spastic paraplegia (HSP), a length-dependent progressive axonopathy [11,12]. Additionally, recessive mutations of the *KIAA1033* gene result in an inherited intellectual disability whilst some variants of the *KIAA1033* gene have been linked to late-onset Alzheimer disease (AD) [13,14]. Significantly, while the disease-causing point mutation found in the *KIAA1033* protein destabilizes the whole WASH complex, the few mutations identified in Strumpellin had no effect on WASH complex stability [5,15]. Thus the primary defect associated with the Strumpellin mutations remains to be identified.

Cargo proteins and pathways that require the WASH complex

Endosomal protein sorting requires localised actin polymerisation. Drug treatments that inhibit F-actin production cause defects in sorting of multiple membrane proteins and endosomal maturation [16]. The activity of the WASH complex is required for sorting of cargo proteins into at least two distinct endosomal recycling pathways. Initial studies of WASH1 and FAM21 components indicated a role for the WASH complex in the endosome-to-Golgi retrieval of the cation-independent mannose 6-phosphate receptor (CIMPR) in conjunction with the sorting nexin (SNX) component of the retromer complex [4]. As retromer had, in previous studies, been demonstrated to function in endosome-to-Golgi

retrieval [17,18] the identification of a role for WASH complex-mediated actin patch-formation added to the mechanistic understanding of retromer-mediated endosome-to-Golgi retrieval. A parallel study of the WASH complex revealed a role in the endosome-to-cell surface recycling of the transferrin receptor (TfnR) [3].

One way the WASH complex could be important for the sorting of proteins into more than one pathway is through the generation of discrete domains into which specific proteins are directed [19]. The different domains would be maintained through the production of branched actin patches. In line with this idea, WASH1-positive domains were found to coalesce when actin polymerisation is inhibited [20]. Moreover, discrete actin domains generated by the WASH complex are required for endosome to cell surface recycling of the $\alpha 5 \beta 1$ integrin complex [21] and the $\beta 2$ -adrenergic receptor ($\beta 2AR$). The recycling of the $\beta 2AR$ also requires the activity of the sorting nexin protein, SNX27 [22].

A role for the WASH complex in regulating sorting to lysosomes has been revealed in a study that reported WASH complex involvement in epidermal growth factor receptor (EGFR) delivery to lysosomes [23]. It is of interest that the WASH1 protein associates with BLOS2, a component of the biogenesis of lysosomal organelles complex-1 (BLOC-1), hinting that WASH complex mediated actin polymerisation may operate in formation of specialised lysosome-related organelles such as melanosomes [24]. In addition, in *Dictyostelium*, a role for the WASH complex in the retrieval of the Vacuolar (V)-ATPase from post-lysosomal compartments has been reported. Failure of WASH complex mediated V-ATPase retrieval results in an accumulation of undigested phagocytosed material that cannot be excreted by the *Dictyostelium* [25].

Recently, studies of the role of WASH1 in endosomal protein sorting has employed a conditional knockout of WASH1 in transgenic mice whereby the deletion of WASH1 MEFs and T-cells is tightly controlled through cre-mediated excision – a system that has been termed WASHout. The application of the WASHout system results in defects in endosome-to-cell surface recycling of physiologically important proteins including the EGFR in MEFs and the glucose transporter Glut-1, T-cell receptor and CD28 in T-cells [7,26]. Interestingly Glut-1 was also identified as a cargo protein dependent on retromer through a proteomic analysis of membrane proteins that require SNX27 or retromer function cementing the functional interaction between the WASH complex, retromer and SNX27 [27]. Overall, these data are consistent with the notion that the WASH complex promotes local F-actin nucleation to maintain segregation of discrete endomembrane domains to facilitate receptor trafficking.

Loss of endosomal structure following WASH complex disruption

Using the WASHout system, the loss of WASH1 function has also been shown to cause endosomes and lysosomes to aggregate and accumulate in the juxta-nuclear region of the cell consistent with a collapse of the early and late endosomal and lysosomal membrane networks. It is important to note however that the compartments remain distinct [7].

From a morphological perspective, the collapse of the endosomal network following loss of WASH1 expression is similar to that reported after loss of myosin VI expression [28]. The function of myosin VI has been linked to a number of physiological processes including endocytosis, autophagy and secretion and, as the only minus-end directed myosin in eukaryotes, is a prime candidate for utilising the actin filaments generated by the WASH complex for sorting specific proteins. Myosin 1c is another potential candidate motor that could employ the WASH complex-generated actin filaments and is required for the

recycling of proteins often associated with lipid rafts (e.g. caveolin) from recycling endosomes to the cell surface [29].

A key phenotype revealed from the initial studies of the WASH complex was a pronounced tubulation of endosomal membranes following RNAi-mediated silencing of components of the WASH complex [3,4]. The presence of numerous membrane tubules may be the result of defects in membrane scission resulting either from a role for actin in membrane scission or due to inhibition of dynamin activity required to pinch off tubules and Dynamin2 was found to associate with the WASH complex [3]. However, it should be noted that WASHout MEFs do not demonstrate excessive membrane tubulation suggesting that the tubulation observed in RNAi-mediated knockdowns of WASH complex components might be due to incomplete depletion and/or inhibition of WASH1.

The WASH complex and retromer, a critical sorting platform

The apparent requirement for the WASH complex and retromer to mediate protein sorting into at least two distinct pathways (endosome to Golgi or endosome to cell surface) would seem to present a clear biological problem relating to specificity. As the role of the WASH complex is to mediate formation of branched actin patches on endosomal membranes, an involvement in multiple pathways is easy to conceive, but how can retromer also be required for both pathways? The answer to this apparent conundrum is that the retromer cargo-selective complex (CSC) comprising VPS35-VPS29-VPS26 is responsible for recruiting the WASH complex to endosomal membranes [30] (see Figure 2A). Therefore, any cargo protein that is dependent on localised actin patches for its sorting into a specific pathway will, indirectly, also be dependent on retromer. It would naturally follow that a great many membrane proteins require retromer for their localisation and a recent proteomic study has identified greater than 100 proteins that are depleted from the cell surface following loss of retromer function [27]. Specificity for sorting into either the endosome-to-Golgi or endosome-to-cell surface pathways may be provided by retromer or WASH complex associated proteins such as SNX27 that operates in the endosome-to-cell surface pathway [22,27]. However, although WASH complex function is necessary for sorting of the EGFR to lysosomes [23], a direct role for retromer in sorting along the degradation route toward late endosomes and lysosomes has not yet been demonstrated.

The interaction between the WASH complex and the retromer CSC was first demonstrated through native immunoprecipitation of GFP-tagged subunits of the retromer CSC. Direct interactions between the VPS35 protein and both FAM21 and WASH1 were revealed using the yeast two-hybrid system. The RNAi-mediated silencing of components of the retromer CSC causes the WASH complex to dissociate from endosomes but other components of endosomal protein sorting machinery, for example the sorting nexin SNX1 or the RAB5 effector EEA1, are unaffected [30]. The endosomal membrane association of the retromer CSC is itself controlled by the RAB7a and SNX3 proteins that together recruit the retromer CSC to the endosome whilst TBC1D5, a putative rab GTPase activating protein for RAB7a, operates to down regulate recruitment of the retromer CSC [14, 31-34]. Mechanisms that govern recruitment of the retromer CSC will therefore add an additional layer of regulation to the process of WASH complex recruitment to endosomes.

From a structural perspective, FAM21 comprises a globular 'head' domain of approximately 200 amino acids that associates with the KIAA1033 and WASH1 proteins and is integral to WASH complex formation and stabilisation [5,6]. Along with the head domain, FAM21 also comprises a large (~1100 amino acid) unstructured 'tail' domain. The initial characterisation of FAM21 revealed that the unstructured tail of FAM21 is sufficient for FAM21 to be membrane associated [4]. The FAM21-tail contains a short motif that uncaps actin filaments

by binding to actin capping proteins (CP) [5,35,36]. This motif was recently shown to be critical for WASH complex function in *Dictyostelium* [37].

In addition to the CP-binding domain, the unstructured FAM21-tail harbours ~20 repeated motifs comprising Leu-Phe and a number of acidic residues (Asp or Glu) that have been named the LFa motif [38]. Subsequently the FAM21-tail was shown to bind to VPS35 directly [6,38] through multiple LFa motifs spaced along the entire length of the FAM21-tail although the LFa motifs present at the C-terminal end of the FAM21-tail appear to be most important for binding to VPS35 [38,39]. Overexpression of the FAM21-tail can displace the endogenous WASH complex by competing for binding to VPS35 [6,38,39].

Although yeast two-hybrid analysis has shown that VPS35 can bind directly to the FAM21-tail [6], it has recently been demonstrated that, *in vivo*, the binding of the FAM21-tail to VPS35 occurs only when VPS35 is associated with VPS29. This observation indicates that the VPS35-VPS29 interaction can regulate the retromer-WASH complex interaction and may also ensure that the WASH complex interacts only with intact and functionally active retromer complex [39]. The FAM21 protein therefore is critical for the association between the WASH complex and the retromer complex enabling the branched actin patch-forming activity of the WASH complex to be linked to the cargo protein-sorting and membrane-tubulation activity of retromer. It is tempting to speculate that the long unstructured - and hence flexible - nature of the FAM21-tail may provide a means for the retromer and WASH complexes to move relative to each other on the highly dynamic and mobile endosomal membrane whilst remaining associated.

Interestingly, in addition to binding the retromer CSC through the LFa motifs and the CP proteins through a consensus sequence in the C-terminal half of the tail, the FAM21-tail also binds to the FKBP15 protein and two additional proteins, CCDC22 and CCDC93 [6]. The function of these proteins is currently unknown but FKBP15 has been reported to regulate nerve growth-cone collapse and the CCDC22 protein is mutated in X-linked mental retardation (XLID) [40,41]. There is, therefore, a consistent theme of neurological disease associated with mutations in the WASH complex or its interacting proteins.

Conservation and variation of the WASH complex

The WASH complex is evolutionarily ancient being present in eukaryotes such as the amoeboid *Naegleria* but is not conserved in the yeast *Saccharomyces cerevisiae*. However, among the WASH complex members, the FAM21 protein is the most divergent. For example, the human Strumpellin and KIAA1033 proteins share ~90% identity with their homologues in chicken but human and chicken FAM21 are only ~40% identical with the homology most concentrated in the globular head domain [8,30]. This conservation in the head domain is likely very important as it is essential for WASH complex assembly and stabilisation, but this may not be the case in all species. For example, the similarity of FAM21 in *Dictyostelium* to Human FAM21 within the head domain is ~40% but the unstructured C-terminus, although similar in length, displays virtually no similarity with the human protein (see Figure 2B). This divergence of FAM21 through evolution may be reflected in functional differences as well. In fact, a recent study of the WASH complex in *Dictyostelium* revealed that FAM21 is not essential for stabilisation or recruitment of the WASH complex but instead operates to recycle the WASH complex from post-lysosomal organelles to compartments earlier in the phagolysosomal pathway [37].

Indeed loss of FAM21 function in *Dictyostelium* causes an unregulated production of actin on post-lysosomes [37]. Similarly, it was previously shown for the WAVE complex in *Dictyostelium* that without PIR121 (Sra1) WAVE is overactive [42]. These observations are

in contrast to the regulation of WASH and WAVE in complex metazoans where their activity toward Arp2/3 is tightly regulated through assembly into their respective pentameric complexes [3,5,43]. Moreover, as the FAM21-tail has evolved to link the mammalian WASH to retromer, it is likely that distinct actin-dependent sorting pathways are at play in these two evolutionary distant relatives. The implication of the observed differences in WASH complex function between *Dictyostelium* and mammals is that conclusions based on studies in primitive eukaryotes will not necessarily be applicable to complex metazoans such as humans.

Regulation of the WASH complex

The spatial and temporal regulation of F-actin production is key to many cellular processes involving response(s) to external stimuli. Members of the WASP superfamily are kept inactive through inter- and intramolecular interactions that can be disrupted by specific signal inputs leading to release of the inhibited VCA (Verprolin, Connector and Acidic region) domain and subsequent activity toward Arp2/3. As indicated above, the WASH complex has structural similarity to the WAVE complex, and when isolated as a set of recombinant proteins, was also found to be intrinsically inactive [5], whereas the WASH complex isolated from mammalian cells is active [3,44]. Thus, an important question to resolve is the nature of the signals that lead to WASH complex activation.

Toward answering this question, a recent report has demonstrated that WASH complex activity is regulated by the E3 ubiquitin ligase TRIM27. The function of TRIM27 is enhanced by MAGE-L2 that is recruited to endosomes through an interaction with VPS35 [44] (see Figure 2A). Therefore retromer not only recruits the WASH complex [30] but also a major WASH complex regulator thereby connecting the two machineries. Mechanistically, it was demonstrated that WASH1 undergoes K63-linked polyubiquitination at K220. Replacement of endogenous WASH1 with a K220R mutant that cannot be ubiquitinated prevents endosomal Arp2/3-mediated actin polymerisation and leads to mislocalisation of the CIMPR. Similarly, treatment of wildtype WASH complex with AMSH, a K63 deubiquitinase, abrogates its ability to activate Arp2/3. Interestingly, K220 is localized within the putative 'meander' region of WASH1 that is predicted to regulate VCA exposure. Even though ubiquitination of WASH1 appears to be a critical regulatory mechanism, it is important to point out that both K220 and TRIM27 do not appear to be conserved in all eukaryotes that possess WASH complex homologues and therefore its regulatory role may be restricted only to higher eukaryotes.

Concluding remarks

The WASH complex is a relatively new component of the endosomal protein sorting machinery. By associating with retromer, the WASH complex is recruited to endosomes where it can mediate formation of discrete patches of branched actin filaments and thereby contribute to the efficient sorting of many proteins that traverse the endocytic system. Additionally, the localized production of actin may provide mechanical force to assist in segregating distinct endosomal domains, either directly by the action of the polymerising actin, or indirectly through the activity of myosin motors. Furthermore, the WASH complex may contribute to the scission of endosomal tubules either through the production of actin or by its association with the Dynamin2 protein. Mutations to components of the WASH complex are causative in neurological diseases such as hereditary spastic paraplegia and autosomal dominant intellectual disability underscoring the key role that the WASH complex plays in neurological function.

Studies of the WASH complex and how it contributes to membrane protein sorting may assist in the elucidation of the roles of other actin NPFs that operate at different sites in cells, for example the WHAMM protein that is required for transport from the endoplasmic reticulum to the Golgi [45], by providing a benchmark against which other actin NPFs may be compared. Additionally, the dynamic production of actin by the WASH complex and its linkage to retromer-mediated protein sorting to direct proteins to multiple destinations may help illuminate how similar protein sorting mechanisms operate, for example at the trans-Golgi network where actin contributes to sorting towards multiple destinations [46].

There is much to learn yet regarding the regulation of the WASH complex and there is potential for additional post-translational modifications (e.g. phosphorylation) beyond the ubiquitination of WASH1 that may prove to be important. What role the WASH complex may play in the biogenesis of specialized organelles (e.g. melanosomes or other lysosome-related organelles) is an interesting question. Additionally, learning how WASH complex-mediated F-actin production interfaces with other proteins important for domain organisation on endosomal membranes (e.g. RAB GTPases or microtubule motors) will generate new insights into the mechanisms that govern endosomal protein sorting – watch (or WASH) this space!

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Highlights

Localised actin patches on endosomes define discrete domains to facilitate protein sorting

Formation of branched actin on endosomes requires the WASH complex

The retromer complex recruits the WASH complex to endosomes

The WASH complex along with retromer sorts endosomal membrane proteins to multiple destinations

Actin Nucleation Promoting Factors – NPFs

The rapid polymerisation of actin to create patches of actin filaments occurs at multiple sites in the cell often in response to specific stimuli. The Arp2/3 complex is critical to the process of actin polymerisation but it does not act alone. For Arp2/3 to mediate actin polymerisation, the activity of an actin nucleation promoting factor (NPF) is necessary. The prototypical actin NPF is the Wiskott-Aldrich syndrome protein (WASp) that functions in actin polymerisation at the plasma membrane. Several actin NPFs that share homology with WASp have now been identified including WASH1, WAVE and WHAMM (see [48] for review). The WASp family of NPFs are defined by the presence of a VCA (Verprolin, Connector and Acidic region) at the C-terminal end of the protein that is critical to the process of Arp2/3-mediated actin polymerisation. Conformational changes in the actin NPF regulate access of the VCA domain to Arp2/3 and thereby control the activity of the respective actin NPF. For WASp, binding to the small GTPase, Cdc42, targets WASp to the membrane and activates it by releasing the VCA domain. The WAVE and WASH1 actin NPFs both associate with a number of proteins to form structurally similar complexes that provide additional regulation of the activity of the NPF [5] (Figure I). For WAVE and WASH1, the accessory proteins provide the means to target the respective actin NPF to their site of action. In the case of the WAVE complex, it is the Rac GTPase binding to the Sra1 component that targets WAVE to the protruding plasma membrane, whilst for the WASH complex, targeting to endosomes occurs through the Fam21 component binding to the retromer protein, Vps35.

Figure 1. The role of actin patches on endosomes

Schematic diagram of a sorting/recycling endosome where retromer and the WASH complex operate. Branched actin patches formed by the action of the WASH complex demarcate discrete domains into which specific proteins are sorted for transport to their respective destinations, for example, sorting for recycling to the cell surface of proteins such as the β 2-adrenergic receptor requires the WASH complex along with SNX27 and retromer. The FERM domain of SNX27 can bind to Ras potentially linking sorting to signalling [47]. Actin patches close to sites where membrane tubules are formed may contribute to scission of tubules. Additionally, myosin motors could employ the actin filaments to facilitate short-range movement and possibly tubule elongation.

Figure 2. The key role of the Fam21 protein in linking the WASH complex to retromer

A. Cartoon diagram of the retromer and WASH complexes. The retromer cargo-selective complex (CSC) comprising VPS35, VPS29 and VPS26 function as a unit that is recruited to endosomes by RAB7a and SNX3 [14]. The TBC1D5 protein acts antagonistically to RAB7a to downregulate recruitment of the retromer CSC [32]. Through an interaction between VPS35 and FAM21 mediated by numerous LFa motifs within the long unstructured tail of FAM21, the retromer CSC recruits the WASH complex to endosomes to drive formation of branched actin patches. Additionally, MAGE-L2 connects VPS35 to TRIM27 which polyubiquitinates WASH1 and activates its NPF activity toward Arp2/3. **B.** Alignment of

human and chicken FAM21 sequences and also human and *Dictyostelium* (Dicty.) FAM21 sequences showing the position of the globular head and unstructured tail domains which contain the CP-binding region and the LFa motifs (black triangles). This graphically demonstrates the evolutionary divergence of the FAM21 protein resulting in a lack of homology between human and *Dictyostelium* FAM21 proteins. The alignment was performed using the SIM alignment tool (<http://web.expasy.org/sim/>).