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By

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ABSTRACT

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Activators of the Arp2/3 complex, termed nucleation-promoting factors (NPFs), are required for the proper spatial and temporal control of actin assembly in cells. Mammalian cells express several NPFs, each of which serve distinct functions in specific cellular processes, including N-WASP in phagocytosis and endocytosis, WAVE and JMY in cell migration, and WHAMM in ER-to-Golgi transport. Although another NPF termed WASH was recently identified, the cellular function and activity of this protein are not well defined. We demonstrate that human WASH potently activates the Arp2/3 complex in vitro and in cells. Furthermore, we show that WASH localizes to early/sorting endosomes and recycling endosomes. These WASH-positive compartments are often associated with actin networks. Silencing of WASH or Arp2/3 complex expression by RNAi, or disruption of actin function by drug treatments, leads to enlargement and elongation of endocytic compartments. Moreover, disruption of actin dynamics or WASH depletion delays EGF transport to LAMP1-positive late endosomes. These observations indicate that actin polymerization downstream of WASH influences the shape and maturation of endosomes, and shed light on a previously unrecognized role for WASH and the Arp2/3 complex in endocytic trafficking.

WASH and actin dynamics have now been implicated in multiple endocytic trafficking pathways including receptor recycling, cargo degradation, and retromer-mediated receptor retrieval. All of these trafficking pathways converge at the early/sorting endosome, raising the question whether WASH plays a general role in sorting and trafficking cargo through this compartment or if there is a requirement for WASH in trafficking specific cargos. To answer this question, we must characterize the influence of WASH on trafficking cellular cargos that utilize distinct routes through

endocytic pathway. Here, we investigate the influence of WASH and Arp2/3 complex activity during integrin recycling, cell adhesion and migration. We observed that subdomains of early/sorting endosomes associate with dynamic WASH and filamentous actin (F-actin), and that α5-integrins traffic through this population of endosomes. Depletion of WASH causes accumulation of α5-integrins in intracellular compartments, reduction of α5-integrin localization at adhesive structures, and reduction in focal adhesion number. Recycling of α5-integrins from internal endocytic structures to focal adhesions is disrupted upon WASH depletion or Arp2/3 complex inhibition. Furthermore, WASH-depleted cells display greatly reduced affinity for specific ECM proteins including fibronectin. Interestingly, the reduced adhesion capacity of WASH-depleted cells results in more rapid migration of these cells in wound healing assays. These results implicate WASH, the Arp2/3 complex, and actin in the specialized trafficking of integrins. Our findings highlight a role for actin dynamics influencing cell adhesion and migration via endocytic trafficking of integrins, in addition to the well-established role of actin in plasma membrane dynamics and contractility.

DEDICATION

To all my family and friends without whom I would not be where I am today.

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ABBREVIATIONS

A: acidic region

Arp: actin related protein

C: connector

CI-MPR: calcium-independent mannose-6-phosphate receptor

(E)GFP: (enhanced) green fluorescent protein

F-actin: filamentous actin

JMY: Junction-meidating and regulatory protein

NPF: nucleation-promoting factor

N-WASP: neuronal Wiskott-Aldrich Syndrome protein

P: polyproline region

RNAi: RNA interference

siRNA: small interfering RNA

siWASH: siRNA targeting WASH

siNS: non-specific control siRNA

TBR: tubulin-binding region

WAHD1: WASH homology domain 1

WASH: WASP and Scar homolog

WASP: Wiscott-Aldrich Syndrome protein

WAVE: WASP and verprolin homolog

WHAMM: WASP homolog associated with actin, membranes and microtubules

WH2/W: WASP homology 2 domain

WMD: WHAMM membrane interaction domain

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CHAPTER 1

INTRODUCTION

I. Introduction to endocytic trafficking

The ability of cells to sense and respond to the external environment is critical for maintaining homeostasis in all living organisms. Equally important in multicellular organisms is the ability of cells to communicate with neighboring cells and tissues. Transmembrane receptors interact with signaling molecules in the extracellular milieu to facilitate communication with the surrounding environment. These receptors transduce extracellular signals to the intracellular environment thus allowing a pertinent cellular response to distinct external stimuli (Platta and Stenmark, 2011). Additionally, transmembrane surface proteins mediate cell-to-cell adhesions and adhesion to substrata (Margadant et al., 2011). These interactions are critical for cell movements during embryonic development and immune function, as well as maintaining tissue integrity. Therefore, understanding how cells regulate and maintain their repertoire of surface proteins is a fundamental question in biology.

Cells utilize the secretory machinery to transport transmembrane proteins from the endoplasmic reticulum where they are translated to the plasma membrane where they contact the extracellular environment. Endocytic trafficking pathways are used to maintain or change the repertoire of proteins present at the cell's plasma membrane (Sigismund et al., 2012). Endocytic internalization of receptors allows a cell to decrease surface levels of a protein, consequently desensitizing a cell to ligands of the downregulated receptor (Platta and Stenmark, 2011). In contrast, endocytic recycling and exocytosis are used to increase the surface levels of protein receptors, thus allowing a cell to increase sensitivity to a particular stimulus (Scita and Di Fiore, 2010). These endocytic trafficking pathways allow cells to rapidly change and adapt to their extracellular environment.

Endocytosis of lipids, cell surface proteins, and their ligands continuously occurs at the plasma membrane of eukaryotic cells. Internalization can occur by several different routes including clathrin-coated pits and vesicles (McMahon and Boucrot, 2011), caveolae (Mayor and Pagano, 2007), clathrin-independent carriers (Kirkham et al., 2005), or GPI-enriched endocytic patches (Sabharanjak et al., 2002). These distinct internalization pathways converge at the common early endosome where proteins are sorted to particular cellular destinations (Gruenberg, 2001; Mayor and Pagano, 2007). Evidence suggests that early endosomes are divided into different regions or microdomains to facilitate sorting of proteins destined for specific cellular compartments (Gruenberg, 2001; Sadowski et al., 2009). While many factors involved in protein sorting have been identified, the precise mechanisms that mediate sorting and subsequent trafficking for many cellular cargos remain to be characterized.

Protein receptors, such as the transferrin receptor, are separated from soluble proteins in the early endosome and recycled back to the cell surface by bulk membrane flow; a process that does not actively sort receptors, but instead allows unsorted receptors to follow membrane components in a default pathway back to the plasma membrane (Mayor et al., 1993). Narrow recycling tubules, which have a high ratio of membrane surface area to volume, facilitate recycling of these transmembrane

receptors via the bulk flow of membrane materials back to the cell surface (Mayor et al., 1993). Ligands are uncoupled from their transmembrane receptors in the mildy acidic early endosomal compartment (Gruenberg, 2001). These narrow tubules provide a geometric basis for concentrating transmembrane nutrient receptors for recycling while leaving soluble nutrients behind to be utilized in the lysosome (Dunn and Maxfield, 1992; Maxfield and McGraw, 2004; Mayor et al., 1993).

Receptors not recycled back to plasma membrane may enter the degradative pathway. Endocytic trafficking in the degradative pathway mediates downregulation of signaling receptors, such as activated epidermal growth factor receptor (EGFR) (Platta and Stenmark, 2011). After internalization, these receptors are targeted by ubiquitination to late endosomes and lysosomes to be degraded. The endosomal sorting complex required for transport-I (ESCRT-I) was shown to engage ubiquitinated cargo at the endosome and mediate sorting of this cargo into multi-vesicular bodies (MVBs) (Katzmann et al., 2001). In addition to mediating the sorting of ubiquitinated cargo, ESCRT proteins appear to deform the endosomal limiting membrane inward, generating intraluminal vesicles enriched with proteins tagged for degradation (Henne et al., 2011). This process, in essence, converts the membrane proteins targeted for degradation into part of the soluble contents of the endosome that will be degraded in late endosomal and lysosomal compartments (Piper and Katzmann, 2007).

Several membrane receptors have been shown to escape the bulk recycling and degradation pathways. Instead, these proteins are recycled in a regulated manner (Hanyaloglu and von Zastrow, 2008; Yudowski et al., 2009). For some receptors, this process is thought to be mediated by a cis-acting sorting sequence on the cytoplasmic surface of the membrane receptors (Cao et al., 1999; Hanyaloglu and von Zastrow, 2008). However, these sorting sequences exhibit wide diversity, making it difficult to predict sorting sequences without direct experimental evidence (Hanyaloglu and von Zastrow, 2008). Additionally, proteins that interact with these sequences and mediate their sorting and regulated recycling are not well defined. Elucidating the machinery that recognizes these sequences and sorts receptors away from the degradative pathway and other recycling routes remains an important goal in the field.

Another essential intracellular trafficking pathway involves the retromer complex. The retromer is a peripheral membrane protein complex that regulates recycling of late Golgi proteins (Collins, 2008). More specifically, the retromer complex mediates endosome-to-Golgi transport of cation-independent mannose-6-phosphate receptor (CI-MPR). Retrograde recycling of CI-MPR from endocytic compartments to the trans-Golgi network is essential for efficient anterograde transport of soluble acid hydrolases required for cellular metabolism, and for the retrieval of mislocalized resident Golgi proteins (Seaman, 2005).

Central to all of these trafficking pathways are mechanisms for reorganizing and deforming cellular membranes. Multiple mechanisms for the shaping biological membranes have been characterized. These include coat proteins, BIN/Amphiphysin/Rvs (BAR) family proteins, dynamin, and cytoskeletal elements that

often act in concert with motor proteins. These mechanisms can be conceptualized as occurring in several distinct but temporally coordinated steps to achieve the appropriate membrane topology of a cell (Brett and Traub, 2006; Kishimoto et al., 2011). The following paragraphs explore some of these remarkable membrane shaping proteins.

Coat proteins have been implicated in numerous cellular trafficking pathways (Bonifacino and Lippincott-Schwartz, 2003; Brett and Traub, 2006). Whether a membrane is associated with clathrin (at the plasma membrane or trans-Golgi network), COPI (at the Golgi network), or COPII (at endoplasmic reticulum exit sites), coat proteins are crucial for generating membrane vesicles, and in some cases coat proteins are involved in targeting transport vesicles to distinct cellular destinations (Cai et al., 2007; Kirchhausen, 2007; Zanetti et al., 2011). The polymerization of membrane coats both concentrates specific proteins into specialized patches and contributes to the deformation of membrane surrounding these specialized patches by acting as an exoskeleton to induce membrane bending (Bonifacino and Lippincott-Schwartz, 2003; McMahon and Gallop, 2005). As we continue to identify components involved in cellular trafficking, coat proteins are proving to be a common theme in the formation of membrane vesicles.

The BAR domain superfamily is another class of membrane shaping proteins. BAR proteins assemble into curved 'banana-like' structures that sense and/or create membrane curvature during cellular trafficking (Qualmann et al., 2011). The intrinsic curvature of the lipid-binding interface of BAR proteins either induces bending, or in the case of curvature-sensing, selectively interacts with specific curved membrane topologies (McMahon and Gallop, 2005; Zimmerberg and Kozlov, 2006). BAR proteins also rely on insertion of hydrophobic or amphipathic protein domains to induce or sense sharply bent membrane topologies (McMahon and Gallop, 2005; Qualmann et al., 2011). In addition to their direct role in membrane deformation, BAR domain superfamily proteins have been shown to recruit other factors important for shaping membranes during endocytic trafficking (Qualmann et al., 2011).

One prominent interacting partner of several BAR family proteins is dynamin. Dynamin is a large GTPase that self-assembles into ring-like structures at the neck of clathrin-coated pits during endocytosis at the plasma membrane or vesicle budding at the trans-Golgi network (Ferguson and De Camilli, 2012; Traub, 2005). Constriction of the collar-like dynamin structures is thought to generate force contributing to membrane fission (Mettlen et al., 2009; Schmid and Frolov, 2011). In two recent studies, membrane fission has been reconstituted using purified dynamin demonstrating the importance of this GTPase during vesicle formation (Bashkirov et al., 2008; Pucadyil and Schmid, 2008). Despite this strong evidence, it is clear that numerous other factors contribute to membrane fission during cellular trafficking in vivo.

Live imaging studies of endocytosis using total internal reflection fluorescence (TIRF) microscopy have revealed many of the players involved in vesicle internalization at the plasma membrane, and importantly, the temporal order of action of these proteins (Kaksonen et al., 2003; Merrifield et al., 2002; Perrais and Merrifield, 2005; Taylor et al.,

2011). Intriguingly, these studies identify a burst of actin polymerization coincident with internalization of clathrin-coated pits at the plasma membrane (Kaksonen et al., 2003; Merrifield et al., 2002; Taylor et al., 2011). In yeast cells, the polymerization of actin at endocytic sites along with the activity of type I myosins provide force required for scission of endocytic invaginations at the plasma membrane (Kaksonen et al., 2006). During clathrin-mediated endocytosis in mammalian cells, actin dynamics and BAR proteins act in concert to tubulate membranes upstream of dynamin. Dynamin activity is then required for vesicle scission of these membrane tubules (Ferguson et al., 2009).

While distinct machinery for reshaping membranes during cellular trafficking have been identified, understanding how these mechanisms are coordinated in vivo during myriad membrane trafficking processes presents an immense task. Additional factors involved in these trafficking pathways are continually being identified. Defining the contributions of these new factors will require precise measurements of protein dynamics in vivo and characterization of the biochemical properties of these proteins in vitro. Another important question is why specific cargos and pathways require certain machinery to sort and organize membranes, while others do not. Addressing this question will require a more complete picture of the biochemical and biophysical properties of distinct lipid membranes and further characterization of the membrane shaping factors involved in each pathway. The remainder of this discussion will focus on the contribution of actin dynamics to membrane organization with a focus on cellular trafficking events.

II. Introduction to the actin cytoskeleton

While the functions of actin polymerization at the plasma membrane have been intensely studied over the past decade, less is known about actin polymerization on internal cellular membranes. Due to recent advances in microscopy we are beginning to appreciate these more subtle dynamic actin events that occur within the cell. Spinning disc confocal microscopy and improved fluorescent markers have allowed us to visualize dynamic actin on internal membranes without our view being obscured by abundant cortical actin structures. We now know that actin networks polymerize on intracellular membranes, including the cis-Golgi and endocytic compartments (Campellone and Welch, 2010; Firat-Karalar and Welch, 2011; Rottner et al., 2010). Dynamic actin has been implicated in reshaping intracellular membranes during protein secretion and endocytic trafficking (Campellone et al., 2008; Derivery et al., 2009b; Duleh and Welch, 2010; Morel et al., 2009; Puthenveedu et al., 2010). To better understand the role of dynamic actin during intracellular trafficking, we must first explore what is known about cellular actin dynamics.

Actin is a highly conserved and abundant intracellular protein found in eukaryotes. Dynamic cycles of actin assembly into filaments (F-actin) and disassembly into monomers (G-actin) are highly regulated in cells. Inside the cell, these polymerization and depolymerization events are utilized to generate force, create scaffolds, and provide tracks for motor proteins. These functions are often coupled to cellular processes that require membrane reorganization such as morphogenesis, migration, trafficking, and cytokinesis (Campellone and Welch, 2010).

The polymerization of new actin filaments in cells is a tightly regulated process (Campellone and Welch, 2010; Welch and Mullins, 2002). The rate-limiting step for de novo actin polymerization is the formation of an actin multimer from monomers (Pollard, 2007). This reaction is termed nucleation. Three distinct nucleation reactions have been identified in cells (Firat-Karalar and Welch, 2011). Tandem actin monomer binding proteins (Renault et al., 2008), such as Spir (Quinlan et al., 2005), Cordon-bleu (Ahuja et al., 2007), and Leiomodin (Chereau et al., 2008), bind multiple actin monomers and arrange them into a nucleus to generate new actin filaments. Formin family members exhibit another mechanism of actin nucleation. Formin proteins dimerize to stabilize an actin nucleus and, unlike other known actin nucleators, formins processively associate with barbed end of the actin filament during the elongation phase (Chesarone et al., 2010; Pollard, 2007). Lastly, the Arp2/3 complex drives actin assembly by mimicking the barbed end of an actin filament thus serving as a template for filament nucleation (Goley and Welch, 2006; Pollard, 2007).

The Arp2/3 complex is unique among other actin nucleation factors because it binds to the side of pre-existing actin filaments and nucleates new filaments that remain connected to the side of the original filament, generating branched actin networks (Goley and Welch, 2006; Pollard, 2007). Numerous cellular processes rely on Arp2/3-mediated actin assembly including cell migration, cellular trafficking, cell-cell communication, and endocytosis (Goley and Welch, 2006; Kaksonen et al., 2006; Le

Clainche and Carlier, 2008; Pollard and Cooper, 2009). Efficient nucleation by the Arp2/3 complex requires a class of proteins termed nucleation-promoting factors (NPFs). NPFs bind to the Arp2/3 complex and actin monomers, together with a preexisting actin filament, these factors initiate the nucleation reaction for daughter filament formation (Goley and Welch, 2006; Pollard, 2007).

The Wiscott-Aldrich syndrome protein (WASP) and WASP family and verprolin homologous (WAVE) protein families are the best characterized NPFs to date. Interestingly, both of these NPF families direct actin assembly that is important for plasma membrane dynamics. The WASP family is comprised of WASP, which is primarily expressed in hematopoietic cells, and its ubiquitously expressed homology neuronal-WASP (N-WASP). Mutations in WASP result in defective cell migration, phagocytosis, and T-cell signaling (Bosticardo et al., 2009). Disruption of N-WASP function impairs filopodia formation (Bu et al., 2009; Snapper et al., 2001) and under certain conditions endocytosis (Benesch et al., 2005; Bu et al., 2009; Innocenti et al., 2005). Mammalian cells contain three WAVE isoforms (WAVE1-3). Dorsal membrane ruffling and migration through extracellular matrix are impaired in the absence of WAVE1 (Suetsugu et al., 2003). The most abundant and well-studied of these WAVE isoforms, WAVE2, is also involved in cell migration, as cells deficient for WAVE2 exhibit defects in peripheral membrane ruffling, lamellipodia dynamics, and cell motility (Innocenti et al., 2005; Yamazaki et al., 2003; Yan et al., 2003). WAVE3 knockout mice exhibit sensorimotor defects and developmental abnormalities indicating a function in central nervous system development (Dahl et al., 2003). However, the molecular function of WAVE3 is not well characterized. While we are still uncovering the functions of the WASP and WAVE family proteins, additional factors that regulate Arp2/3 activity and actin assembly continue to be identified.

Exciting recent work resulted in the identification of three additional NPFs: junction-mediating and regulatory protein (JMY) (Zuchero et al., 2009), WASP homolog associated with actin, membranes and microtubules (WHAMM) (Campellone et al., 2008), and WASP and Scar homolog (WASH) (Linardopoulou et al., 2007). Intriguingly, JMY drives both Arp2/3-mediated and Arp2/3-independent actin assembly. JMY has been implicated in cell migration and as a negative regulator of neurite formation (Firat-Karalar et al., 2011; Zuchero et al., 2009). Studying the regulation of JMY nucleating and NPF activity will provide insight into the relative contribution of each nucleation mechanism to these cellular processes.

Compared to the well-known roles of N-WASP and WAVE2 at the plasma membrane, the function of NPFs and the Arp2/3 complex at internal cellular organelles and membranes is not well understood. Both WHAMM and WASH direct actin assembly to reorganize intracellular membranes (Campellone et al., 2008; Derivery et al., 2009b; Duleh and Welch, 2010; Puthenveedu et al., 2010). Moreover, WHAMM and WASH have both been shown to interact with microtubules (Campellone et al., 2008; Gomez and Billadeau, 2009; Liu et al., 2009b), suggesting these NPFs may help coordinate membrane organization using both the actin and microtubule networks in cells. While similarities exist between WASH and WHAMM, each protein influences

distinct trafficking pathways. WHAMM is involved in membrane tubulation events at the cis-Golgi that are important for protein secretion (Campellone et al., 2008). In contrast, absence of WASH results in increased endosomal tubulation in cells, suggesting WASH may be involved in the scission of these structures (Derivery et al., 2009b; Duleh and Welch, 2010).

Interestingly, WASH contributes to several endocytic trafficking pathways in mammalian cells including transferrin recycling (Derivery et al., 2009b; Zech et al., 2011), transport of EGFR from early to late endosomes (Duleh and Welch, 2010), retromer-mediated retrieval of CI-MPR (Gomez and Billadeau, 2009), and sorting and recycling of the β2-adrenergic receptor (Puthenveedu et al., 2010; Temkin et al., 2011). It is not yet clear whether WASH serves a specific function in each of these pathways or if WASH plays a more general role organizing endosomal membranes and trafficking cargo.

As we continue to identify additional factors that regulate actin dynamics and membrane trafficking pathways, a significant overlap of factors involved in these cellular processes is emerging. While actin polymerization has been implicated in multiple steps of endocytic trafficking and protein secretion, it appears that actin cooperates with distinct machinery and serves different functions in several of these pathways. Continued study of factors that regulate cytoskeletal dynamics will provide useful insight into how actin networks contribute to distinct membrane trafficking pathways in cells.

The focus of my dissertation research has been to understand the cellular function of WASH. We began this work five years ago with a bioinformatic search that identified an uncharacterized open reading frame encoding a putative actin and Arp2/3 binding region. The road of discovery has been an exciting journey that has explored the fields of actin dynamics, intracellular trafficking, cell adhesion, and cell migration. While our work and the contributions of many others have provided important information about WASH function that influences these cellular processes, there will undoubtedly be many more fascinating findings to come in the years ahead.

CHAPTER 2

WASH and the Arp2/3 complex regulate endosome shape and trafficking

Note: The majority of information presented in this chapter was included in the publication:

Duleh S.N. and Welch M.D. (2010) WASH and the Arp2/3 complex regulate endosome shape and trafficking. Cytoskeleton.

Introduction

The actin cytoskeleton plays an important role in cellular behaviors such as migration and division (Barr and Gruneberg, 2007; Pollard and Borisy, 2003), as well as in intracellular processes including endocytosis and vesicle trafficking (Engqvist-Goldstein and Drubin, 2003; Kaksonen et al., 2006; Robertson et al., 2009). Proper functioning of the actin cytoskeleton requires precise regulation of the polymerization and organization of actin filaments. One of the principal actin polymerizing and organizing factors in the cell is the Arp2/3 complex, a protein complex that nucleates new filaments from the sides of existing ones and cross-links filaments into Y-branched networks (Goley and Welch, 2006). However, purified Arp2/3 complex does not display potent nucleating and Y-branching activity unless it is engaged by a class of proteins called nucleation-promoting factors (NPFs) (Welch and Mullins, 2002). Mammalian cells express a diverse array of NPFs, each of which coordinates Arp2/3 complex activity during distinct cellular behaviors or processes.

The WASP and Scar homolog (WASH) protein was recently identified as an NPF (Linardopoulou et al., 2007) that belongs to a group called Class I. This group also includes the well-studied WASP, N-WASP, WAVE/Scar NPFs (Stradal et al., 2004; Takenawa and Suetsugu, 2007), as well as the recently discovered WHAMM (Campellone et al., 2008), and JMY (Zuchero et al., 2009) proteins. All Class I NPFs share a common C-terminal WCA domain that includes a WASP-homology-2 (WH2 or W) element that binds to actin monomers, a connector (C) region that binds to both the Arp2/3 complex and actin monomers, and an acidic (A) region that binds to the Arp2/3 complex (Marchand et al., 2001). The WCA region of WASH, like that of other Class I NPFs, is sufficient to activate the Arp2/3 complex in vitro (Linardopoulou et al., 2007; Liu et al., 2009b).

In contrast to their conserved C-terminal WCA domains, the N-terminal domains of Class I NPFs differ considerably. WASH contains two distinctive N-terminal domains, termed WASH homology domain 1 (WAHD1) and tubulin-binding region (TBR) (Gomez and Billadeau, 2009), that are not present in other NPFs (Linardopoulou et al., 2007). The exact contribution of these domains to the function and regulation of WASH has not yet been defined. However, the various N-terminal sequences of other Class I NPFs are known to confer each protein with a distinct cellular function and mode of regulation. For example, under resting conditions WASP and N-WASP are autoinhibited by an intramolecular interaction between a central GTPase-binding domain and the WCA region (Kim et al., 2000; Miki et al., 1998; Prehoda et al., 2000), and are activated by Rho family GTPases like Cdc42 to promote endocytosis, phagocytosis, and filopodia formation (Stradal et al., 2004; Takenawa and Suetsugu, 2007). In contrast, WAVEs are inhibited by association with a complex of interacting proteins (Derivery et al., 2009a; Eden et al., 2002; Ismail et al., 2009), and can be activated by the Rho family GTPase Rac (Eden et al., 2002; Ismail et al., 2009; Miki et al., 2000) to promote lamellipodia protrusion (Stradal et al., 2004; Takenawa and Suetsugu, 2007). WHAMM has also been proposed to be regulated in a manner similar to the WAVE proteins

(Campellone et al., 2008). The mechanism of WASH regulation, in contrast, is not well understood.

Nevertheless, recent studies have provided some insight into the function and regulation of WASH in cells. An initial study reported that GFP-tagged WASH localizes to lamellipodia and filopodia, suggesting that it may be involved in plasma membrane dynamics (Linardopoulou et al., 2007). Additionally, genetic and biochemical studies in *Drosophila melanogaster* indicate that WASH functions downstream of the Rho family GTPase Rho1 and interacts with the actin nucleators Spire and Cappuccino (Liu et al., 2009b). These data suggest that WASH may regulate formation of both branched-actin networks with the Arp2/3 complex and unbranched assemblies with Spire and Cappuccino in cells (Liu et al., 2009b). Moreover, WASH displays both F-actin and microtubule bundling activity in vitro (Liu et al., 2009b), although the cellular functions of these activities are not yet defined. Finally, WASH was recently implicated in the internalization of the bacterial pathogen *Salmonella typhimurium* into host cells (Hanisch et al., 2009), raising the possibility that WASH functions in the endocytic pathway.

One of the central functions that have emerged for the Arp2/3 complex and NPFs is to facilitate the process of endocytosis (Engqvist-Goldstein and Drubin, 2003; Kaksonen et al., 2006; Perrais and Merrifield, 2005; Robertson et al., 2009). In mammalian cells, N-WASP and the Arp2/3 complex have been implicated in coordinating actin assembly to facilitate endocytic vesicle formation at the plasma membrane. For example, N-WASP and the Arp2/3 complex are found at sites of clathrin-mediated endocytosis (Merrifield et al., 2004; Rodal et al., 2005; Yarar et al., 2005), and cells lacking functional N-WASP exhibit reduced internalization kinetics of epidermal growth factor receptor (EGFR) (Benesch et al., 2005; Innocenti et al., 2005). In addition to a role in endocytic vesicle formation, evidence is accumulating that actin plays a role at later stages of the endocytic pathway (Soldati and Schliwa, 2006). For instance, N-WASP is required for the actin-dependent propulsion of endosomes (Benesch et al., 2002; Rozelle et al., 2000; Taunton et al., 2000). Actin patches have also been observed on early endosomes, and are thought to be involved in the membrane remodeling events that accompany endosome biogenesis (Morel et al., 2009). However, the exact role of the Arp2/3 complex and NPFs in this process is not clear.

Here we investigated the function of WASH in mammalian cells, and discovered a role for WASH in the endocytic pathway. We found that WASH localized to early endosomes, and together with the Arp2/3 complex and actin, was critical for maintaining the shape of this compartment. We also showed that WASH and actin polymerization play an important functional role in trafficking of epidermal growth factor (EGF) to late endosomes. These data demonstrate a previously unappreciated role for WASH and the Arp2/3 complex during trafficking of cargo in the degradative endocytic pathway.

Results

Human WASH is a potent Arp2/3 complex nucleation-promoting factor in vitro

WASH contains two distinct N-terminal domains, termed WAHD1 (WASH homology domain 1) and TBR (tubulin-binding region) (Gomez and Billadeau, 2009), as well as a C-terminal WCA domain that is conserved among mammalian Class I NPFs (Figure 2.1A). Previous studies demonstrated that a truncated variant of human WASH consisting of the WCA domain (WASH-WCA) (Linardopoulou et al., 2007), as well as full-length *D. melanogaster* WASH or its WCA domain (Liu et al., 2009b), activate the Arp2/3 complex to promote actin nucleation in vitro. However, the activity of full-length human WASH has not been evaluated. To assess the relative activity of full-length human WASH compared with the WASH-WCA domain, we purified His-tagged full-length recombinant WASH (His-WASH) (Figure 2.1B) and His-WASH-WCA from *E. coli* (Figure 2.1C), and tested their activity in pyrene-actin polymerization assays. In the presence of the Arp2/3 complex, increasing concentrations of both His-WASH and His-WASH-WCA resulted in a dose-dependent acceleration of actin assembly, although neither WASH nor the Arp2/3 complex alone were able to efficiently nucleate actin polymerization (Figure 2.1D). Full-length His-WASH was as potent at promoting Arp2/3 complex-mediated actin nucleation as WASH-WCA (Figure 2.1D). This observation suggests that recombinant WASH is not autoinhibited, a behavior that is similar to the NPFs WHAMM (Campellone et al., 2008) and WAVE (Machesky and Insall, 1998), but different from N-WASP (Rohatgi et al., 1999).

To compare the activity of WASH to that of other mammalian Class I NPFs, we purified recombinant human His-N-WASP-WCA, His-WAVE2-WCA and His-WHAMM-WCA from *E. coli* (Figure 2.1B) and again measured their activities in pyrene-actin assembly assays. WASH-WCA displayed comparable activity to N-WASP-WCA, and greater activity than either WAVE2-WCA or WHAMM-WCA (Figure 2.1E). Thus, WASH is a potent NPF like N-WASP and is constitutively active in vitro similar to WHAMM and WAVE2.

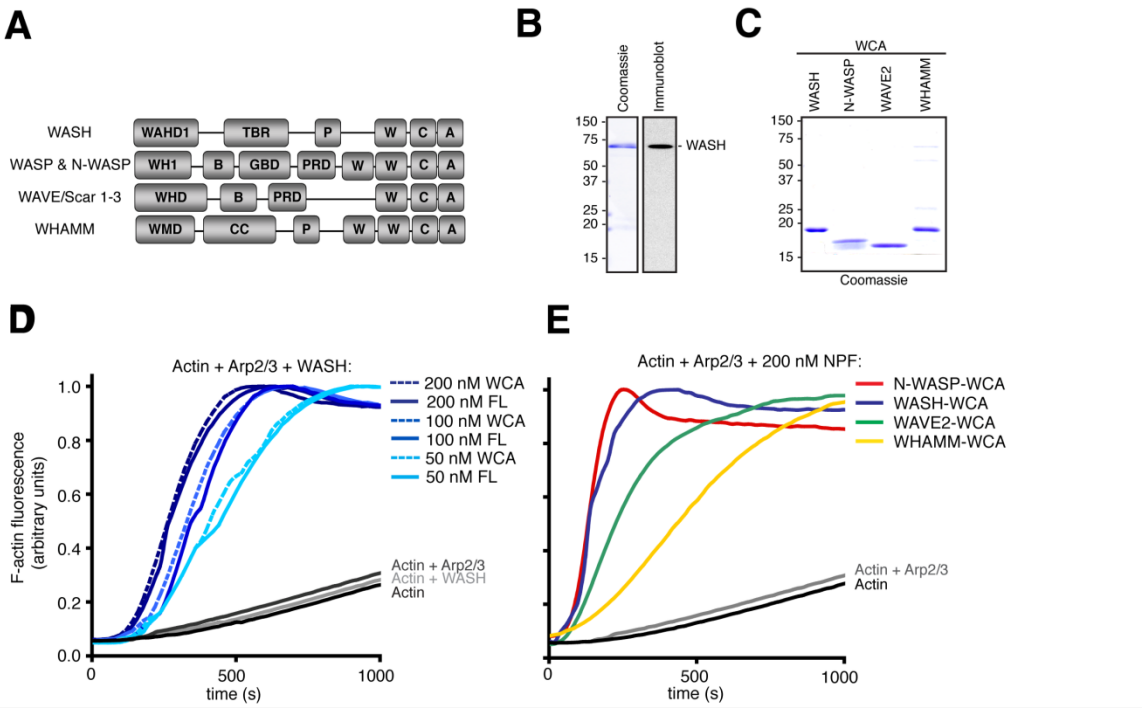

Figure 2.1. WASH is a potent Arp2/3 complex nucleation-promoting factor in vitro. (A) Schematic diagrams showing the domain organization of mammalian NPFs. (B) Purified recombinant full-length His-WASH was subjected to SDS-PAGE and stained with Coomassie blue or detected by immunoblotting with anti-WASH antibodies. (C) Purified His-WCA domains were separated by SDS-PAGE and stained with Coomassie blue. (D) Pyrene-actin assembly assays with 3 µM actin polymerized in the presence of 20 nM Arp2/3 complex and 50-200 nM full-length His-WASH (FL) or His-WASH-WCA (WCA). (E) Pyrene-actin assembly assays as in (D), but with 200 nM of the indicated NPF-WCA.

WASH is widely expressed in mammalian tissues and cell lines and fractionates with both cytosol and membranes

To investigate the expression pattern of WASH in mammalian tissues and cells, we generated antibodies against the full-length WASH protein and used these to detect WASH in extracts from mouse tissues and mammalian cultured cell lines by immunoblotting. These antibodies recognized purified recombinant WASH (Figure 2.1B), as well as one or a small number of WASH species in mammalian tissue or cell extracts (Figure 2.2). We found that WASH was expressed in most mouse tissues examined, with the highest expression levels observed in cardiac and renal tissue (Figure 2.2A). Additionally, WASH was expressed in all cultured cell lines that we tested, including mouse NIH-3T3, monkey COS7, and human HeLa cells (Figure 2.2B). We conclude that WASH is expressed widely in mammalian tissues and cells.

Given that other NPFs activate Arp2/3-mediated actin assembly adjacent to cellular membranes, we next assessed whether WASH associated with cellular membranes by biochemical fractionation. NIH-3T3 cell lysates were fractionated into membrane and cytosolic components. The fidelity of the fractionation was confirmed by immunoblotting for the endoplasmic reticulum marker calnexin, which was found exclusively in the membrane fraction, and tubulin, which was only detected in the cytosolic fraction (Figure 2.2C). Interestingly, WASH was slightly enriched in the membrane fraction, although a significant portion was also observed in the cytosolic fraction (Figure 2.2C). The distribution of WASH was similar to that previously observed for WAVE2 (Suetsugu et al., 2006), but different from that of N-WASP and WHAMM which are more heavily enriched on membranes (Campellone et al., 2008). Thus, although WASH associates with cellular membranes, a significant pool of WASH is either free in the cytoplasm or associates with small vesicles that do not pellet in our assay.

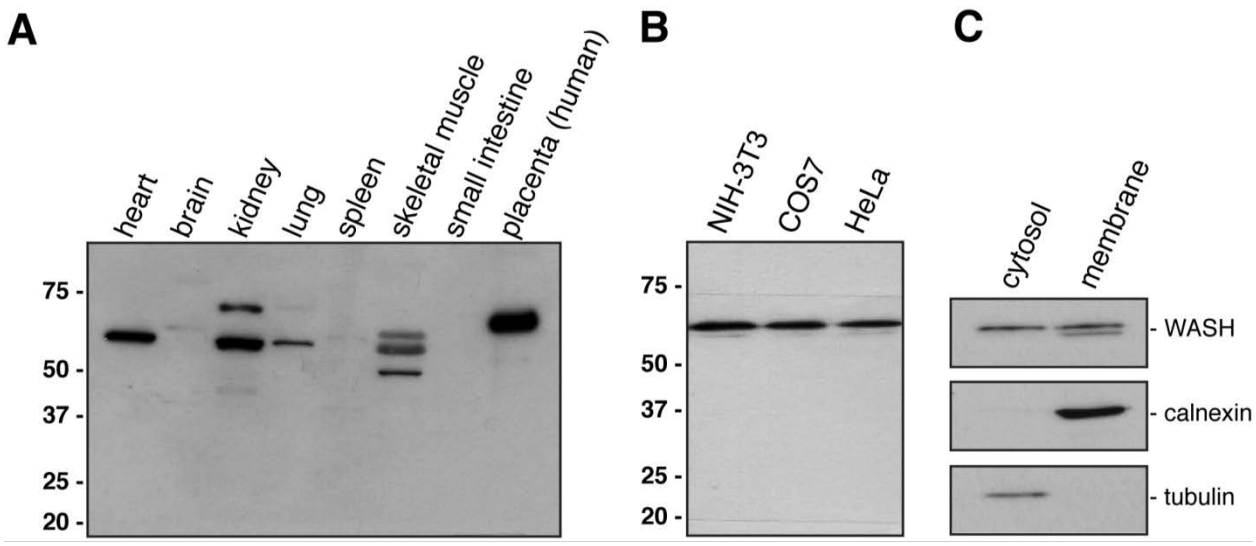

Figure 2.2. WASH is widely expressed in mammalian tissues and cell lines and fractionates with both cytosol and membranes. (A) Cell extracts from mouse tissues or (B) from mammalian cultured cell lines were separated by SDS-PAGE and immunoblotted with anti-WASH antibodies. (C) NIH-3T3 cells were fractionated into membrane and cytosolic fractions by centrifugation and then separated by SDS-PAGE and immunoblotted with anti-WASH antibodies, antibodies against tubulin (cytosolic fraction marker), or antibodies recognizing calnexin (membrane fraction marker).

WASH associates with perinuclear vesicles enriched with F-actin

Previously published work suggested that WASH-GFP colocalizes with actin in cellular filopodia and lamellipodia (Linardopoulou et al., 2007). To assess the subcellular localization of endogenous WASH, we used polyclonal antibodies raised against full-length human WASH for immunofluorescence microscopy. Surprisingly, we did not observe WASH staining at the cell periphery in lamellipodia or filopodia in COS7 cells. Instead, WASH was almost exclusively associated with perinuclear punctae that overlapped with a region enriched in F-actin (Figure 2.3A). To further verify this localization pattern, we generated an N-terminally EGFP-tagged variant of WASH (GFP-WASH) and observed its localization in COS7 cells. Importantly, GFP-WASH accumulated in the perinuclear region in a pattern similar to endogenous WASH (Figure 2.3B). Together with the fractionation data (Figure 2.1), the immunofluorescence staining and GFP-WASH localization data indicate that WASH primarily localizes to a perinuclear membrane-bound compartment.

Notably, GFP-WASH expression resulted in a striking accumulation of F-actin in the perinuclear region of transfected cells (Figure 2.3B). A similar accumulation of F-actin was observed after overexpressing WHAMM and WAVE2, but not N-WASP (Campellone et al., 2008). This result is consistent with the notion that WASH is not autoinhibited in cells, similar to the behavior of the recombinant WASH protein in vitro (Figure 2.1).

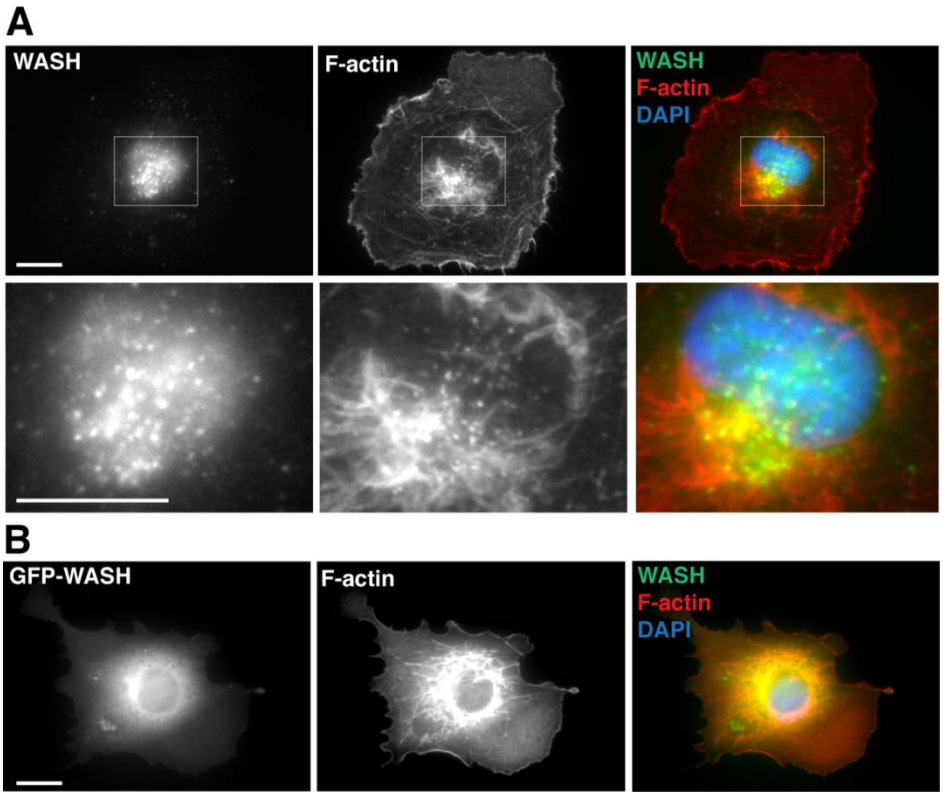

Figure 2.3. WASH associates with perinuclear vesicles enriched with F-actin. (A) WASH visualized by immunofluorescence (green) colocalizes with perinuclear F-actin visualized by Alexa Fluor 568-phalloidin (red) in COS7 cells. (B) GFP-WASH (green), F-actin stained with Alexa Fluor 568-phalloidin (red), and DNA stained with DAPI (blue) in COS7 cells. Scale bars, 10 μm.

WASH is asymmetrically distributed on early endosomes

To determine which organelle WASH associates with, we sought to colocalize WASH with markers of perinuclear membrane-bound compartments by immunofluorescence staining together with deconvolution microscopy. We found that WASH associates with several proteins of compartments in the endocytic pathway, including the early endosome markers early endosome antigen 1 (EEA1) and Rab5, the recycling endosome marker Rab11, and the fast recycling vesicle marker Rab4 (Stenmark, 2009) (Figure 2.4). We quantified the percentage of WASH-containing punctae that colocalized with each of these proteins. We found that WASH most extensively colocalized with EEA1- and Rab5-positive early endosomes, and less frequently colocalized with Rab11 recycling endosomes and Rab4-positive vesicles (Figure 2.4C).

The EEA1/Rab5-positive early endosomes with which WASH colocalizes represent a crossroads of the endocytic pathway that sort and move materials to recycling endosomes, late endosomes, and the Golgi apparatus (Maxfield and McGraw, 2004; Perret et al., 2005). Recent studies have shown that annexin A2 organizes into cholesterol-rich platforms on early endosomes (Mayran et al., 2003), suggesting that other proteins may be organized into subdomains on these vesicles. To determine if WASH is uniformly distributed on endosomes or is concentrated in subdomains, we closely examined the relative distributions of WASH and EEA1 on early endosomes using deconvolution microscopy and 3D reconstruction (Figure 2.4B). Interestingly, WASH staining did not completely overlap with EEA1, but instead appeared asymmetrically distributed relative to EEA1 staining, suggesting that WASH is targeted to a subdomain of the early endosome.

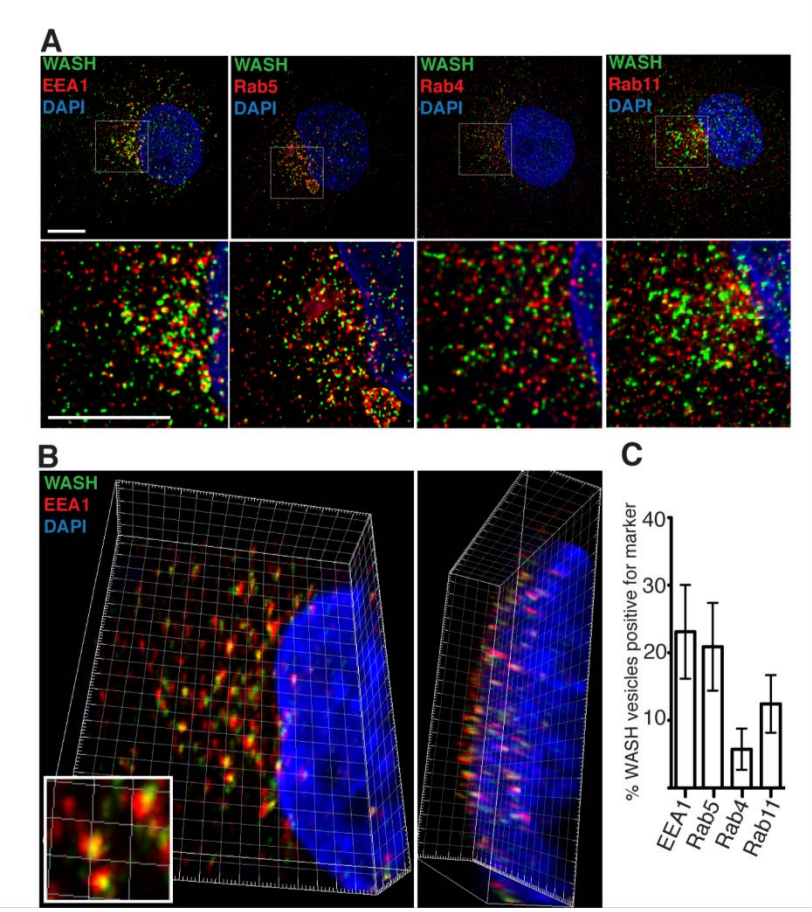

Figure 2.4. WASH is asymmetrically distributed on endosomes. (A) Maximum intensity projections from 3D deconvolved images of COS7 cells in which WASH (green) and various endosome markers (red) were visualized by immunofluorescence, and DNA was stained with DAPI (blue). Scale bars, 10 μm. (B) 3D reconstruction of WASH (green) and EEA1 (red) visualized by immunofluorescence. Inset show the asymmetric distribution of WASH on EEA1-positive vesicles. Gridlines are 1 µm apart. (C) The percentage of WASH structures that colocalized with each endosomal marker expressed as the mean ± SD. 10 images from 3 independent experiments were counted.

The actin cytoskeleton controls the morphology of WASH-containing compartments and EEA1-positive endosomes

The observations that WASH acts as an actin nucleation-promoting factor and localizes to endosomes suggested that actin assembly might influence the physical characteristics of endosomes. To investigate the role of actin polymerization in determining the shape of WASH-containing compartments, we treated COS7 cells with cytochalasin D, a drug that binds to actin filament barbed ends and blocks polymerization, or latrunculin A, a toxin that sequesters actin monomers and leads to rapid filament disassembly. Treated cells or untreated controls were stained for WASH or EEA1 by immunofluorescence and for actin by fluorescent phalloidin, and then imaged by super-resolution structured illumination microscopy (Figure 2.5). In control cells, we often observed F-actin staining colocalizing with WASH vesicles. Treatment with cytochalasin D resulted in partial actin disassembly and the formation of enlarged and elongated WASH structures compared to untreated controls. Moreover, treatment with latrunculin A resulted in more complete actin disassembly and very enlarged WASH-positive compartments consisting of long interconnected tubules. Additionally, treatment with both drugs resulted in enlargement and lengthening of some EEA1-positive early endosomes, although the effect was not as pronounced as it was for WASH-positive compartments. These observations indicate that actin polymerization plays an important role in maintaining the size and shape of WASH- and EEA-positive compartments.

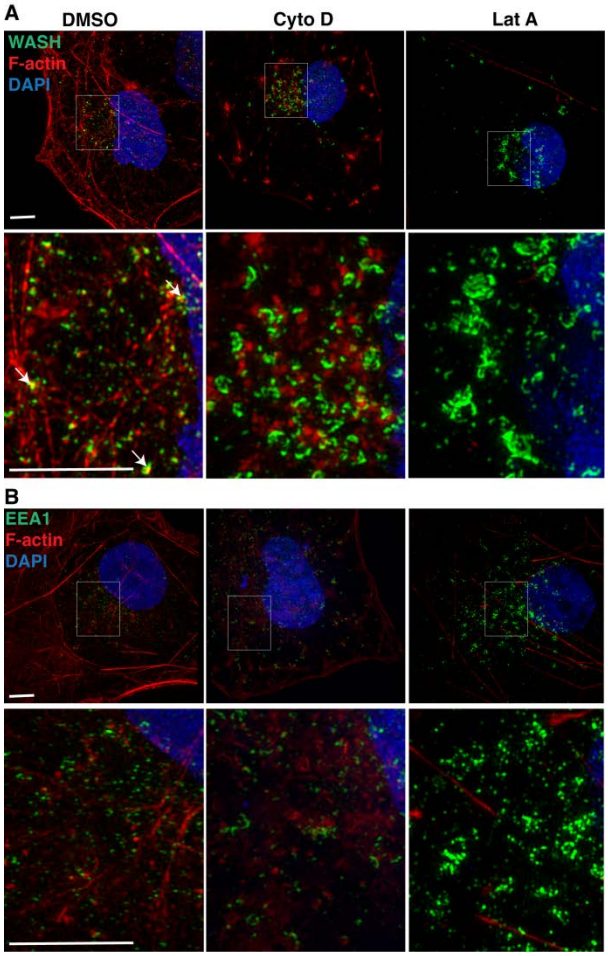

Figure 2.5. Disruption of the actin cytoskeleton results in an enlarged and elongated WASH-containing and EEA1-positive endosomes. (A) WASH or (B) EEA1 visualized by immunofluorescence (green), F-actin stained with Alexa Fluor 568-phalloidin (red) and DNA stained with DAPI in COS7 cells treated with DMSO alone, or DMSO with 10 µM cytochalasin D (Cyto D), or 10 µM latrunculin A (Lat A). Imaging was by 3D structured illumination microscopy. Each panel is a representative maximum intensity projection of the 3D images. Actin colocalized with WASH structures (see arrows) in control (DMSO) treatment. Scale bars, 5 μm.

WASH and the Arp2/3 complex regulate endosome shape

Because actin assembly appears to be crucial for determining the shape of WASH-positive compartments and early endosomes, the actin nucleating activity of WASH and the Arp2/3 complex might also be important for regulating endosome shape. To assess the role of the Arp2/3 complex, we transfected mouse fibroblast-like cells (FLCs) with siRNAs targeting the Arp3 and ARPC3 subunits of the complex, and confirmed depletion of the complex by immunoblotting for the Arp3 subunit (Figure 2.6A). The effect on the morphology of WASH-associated compartments was then examined by immunofluorescence. Interestingly, WASH-containing structures were enlarged and extended when compared with similar compartments in FLCs treated with control siRNAs (Figure 2.6B). We conclude that the Arp2/3 complex, like actin, is critical for determining shape of WASH compartments on endosomes.

Next, we sought to ascertain if WASH also plays a role in endosome shape determination. Therefore, we transfected FLCs with siRNA targeting WASH, which resulted in substantial depletion of WASH protein levels (Figure 2.6A), and visualized early endosomes by immunofluorescence staining for EEA1 (Figure 2.6C). In contrast to cells transfected with control siRNAs, which had small, round EEA1-positive vesicles, WASH-depleted cells contained many larger and elongated EEA1-positive structures. Collectively, these data demonstrate that actin assembly by WASH and the Arp2/3 complex is important for maintenance of endosome geometry.

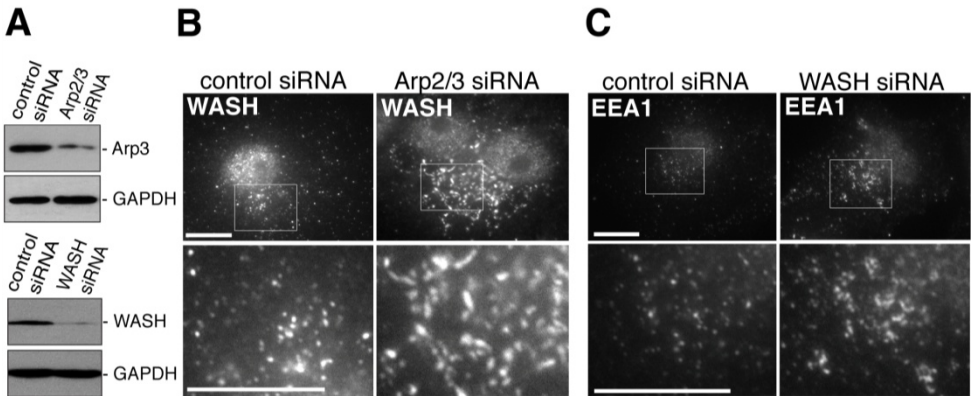

Figure 2.6. WASH and the Arp2/3 complex control endosome shape. (A) Extracts from fibroblast-like cells (FLCs) treated with control non-specific siRNAs, siRNAs targeting the Arp3 and ARPC3 subunits of the Arp2/3 complex, or WASH siRNAs were separated by SDS-PAGE and immunoblotted with anti-Arp3, anti-WASH, or anti-GAPDH (loading control) antibodies. (B) RNAi silencing of Arp2/3 complex expression in FLCs lead to enlarged WASH-positive compartments visualized by immunofluorescence. (C) RNAi silencing of WASH in FLCs lead to enlarged EEA1-positive endosomes visualized by immunofluorescence. Scale bars, 10 μm.

WASH depletion does not affect the kinetics of transferrin uptake or recycling

To determine if the ability of WASH to influence endosome shape is correlated with a role in modulating endocytic trafficking, we first examined the effect of WASH depletion on the kinetics of internalization and recycling of the endocytic cargo protein transferrin. Mouse NIH-3T3 cells were transfected with control or WASH siRNAs, and depletion of WASH was confirmed by immunoblotting (Figure 2.7A). To measure a single round of transferrin internalization and recycling, biotinylated transferrin (B-SS-Tfn) was prebound to cell surface receptors at 4 °C, then chased at 31 °C for various times, and the percentage of B-SS-Tfn internalized was determined by ELISA for each time point (Figure 2.7B). Unexpectedly, WASH depletion had no effect on the kinetics of transferrin internalization or trafficking. Thus, the defect in endosome morphology observed in WASH-depleted cells does not perturb transferrin trafficking (Figure 2.7B), consistent with previous data showing that severe disruption of endosome geometry is not always predictive of transferrin trafficking defects (Ceresa et al., 2001).

Previous studies have shown that the NPF N-WASP also associates with endosomes and is important for vesicle rocketing (Chang et al., 2003; Taunton et al., 2000), raising the possibility that the failure to observe a defect in transferrin trafficking in WASH-depleted cells may be due to functional redundancy between N-WASP and WASH. To test this hypothesis, we measured the kinetics of transferrin uptake and recycling, as described above, in cells lacking both N-WASP and WASH. N-WASP$^{+/+}$ and N-WASP$^{-/-}$ FLCs (Snapper et al., 2001) were treated with control siRNAs or siRNAs targeting WASH, and depletion of WASH was confirmed by immunoblotting (Figure 2.7A). Surprisingly, both N-WASP $^{-/-}$ cells treated with control siRNAs and N-WASP$^{-/-}$ cells depleted for WASH displayed transferrin uptake and recycling kinetics that were indistinguishable from control cells (Figure 2.7B). These results indicate that neither WASH nor N-WASP plays a discernible role in transferrin trafficking in this cell type.

To further investigate whether WASH and N-WASP might perform a redundant function in cells, we examined whether the two proteins colocalized in cells by immunofluorescence staining. To this end, we generated an anti-N-WASP antibody that recognized a single band in N-WASP$^{+/+}$ FLCs, but not N-WASP$^{-/-}$ FLCs. Notably, we did not observe any colocalization between the two proteins, in contrast to the colocalization between WASH and endosomal marker proteins (Figure 2.7C). These data indicate that N-WASP and WASH reside in different compartments and suggest that they perform distinct cellular functions.

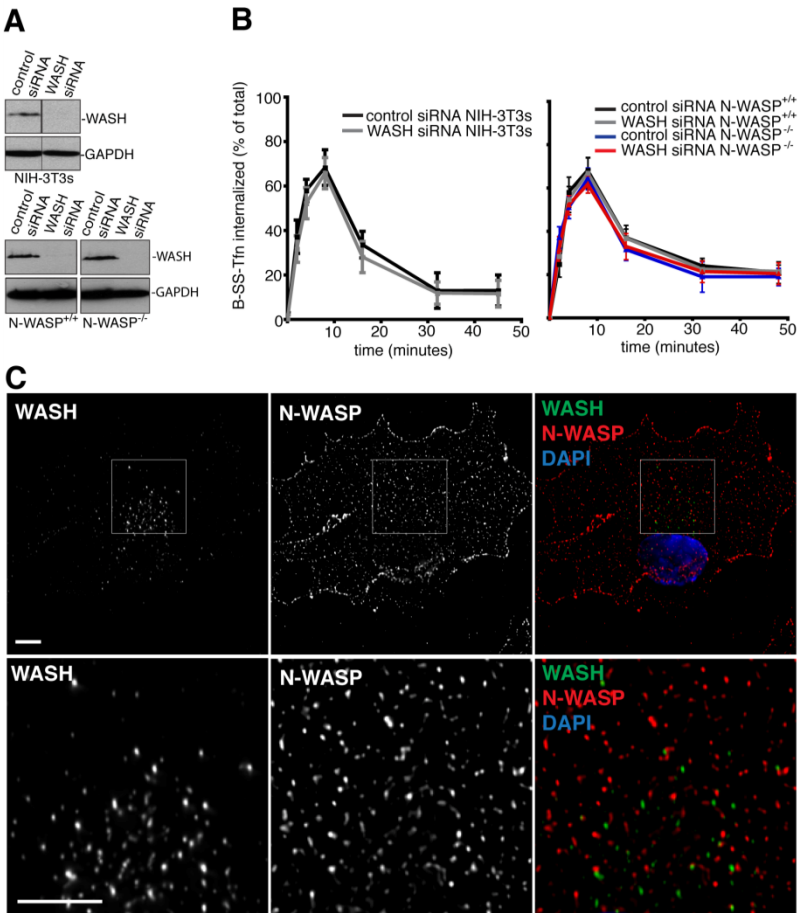

Figure 2.7. WASH depletion does not affect the kinetics of transferrin uptake or recycling in the presence or absence of N-WASP. (A) Extracts from NIH-3T3s or fibroblast-like cells (FLCs) transfected with control or WASH siRNAs were separated by SDS-PAGE and immunoblotted with anti-WASH or anti-GAPDH antibodies. (B) Single-round kinetics of uptake and recycling of prebound biotinylated transferrin (B-SS-Tfn) in NIH-3T3 cells, N-WASP$^{+/+}$ FLCs, and N-WASP$^{-/-}$ FLCs. Graph shows percent of total B-SS-Tfn that is internalized (mean of 3 independent experiments ± SD) versus time. (C) WASH (green) and N-WASP (red) stained by immunofluorescence and DNA (blue) stained by DAPI were visualized in COS7 cells by deconvolution microscopy. Scale bar, 5 μm.

WASH depletion or actin cytoskeleton disruption delays EGF transport to late endosomes

Because WASH silencing did not affect transferrin internalization or recycling, we next investigated whether WASH and/or actin played a role in trafficking of EGF in the degradative pathway. Previous studies have shown that EGFR and EGF could be internalized either by a clathrin-dependent pathway, which resulted in recycling back to the cell surface (Sigismund et al., 2008), or a clathrin-independent pathway, which leads to degradation in lysosomes (Orth et al., 2006; Sigismund et al., 2008; Sigismund et al., 2005). In our experiments, FLCs were treated with high concentrations of EGF to enhance the degradative pathway, as described previously (Sigismund et al., 2008). To measure the transport of EGF to LAMP1-positive late endosomes, FLCs were pulsed with fluorescent EGF for 10 min to allow internalization, chased with unlabeled EGF for 30 min to allow transport to late endosomes, and then fixed and stained for the late endosome marker LAMP1, and imaged by deconvolution microscopy (Figure 2.8A). The percentage of fluorescent EGF that was transported to late endosomes was then quantified (Figure 2.8C).

In this assay, disrupting actin polymerization by treating cells with cytochalasin D, or depolymerizing actin with latrunculin A, dramatically reduced the transport of fluorescent EGF to LAMP1-positive vesicles compared to control cells (Figure 2.8A, C). Importantly, silencing WASH expression by RNAi (Figure 2.8B) also caused a significant reduction in the percentage of EGF reaching LAMP1-positive structures (Figure 2.8C). Intriguingly, WASH depletion and actin disruption also resulted in a redistribution of LAMP1-positive structures from the perinuclear region to more peripheral regions in the cell (Figure 2.8A). However, there was no statistically significant difference in the total number of LAMP1 compartments after either treatment (data not shown). Together these results suggest that WASH and F-actin play an important role in both transport to late endosomes and positioning these compartments in the cell.

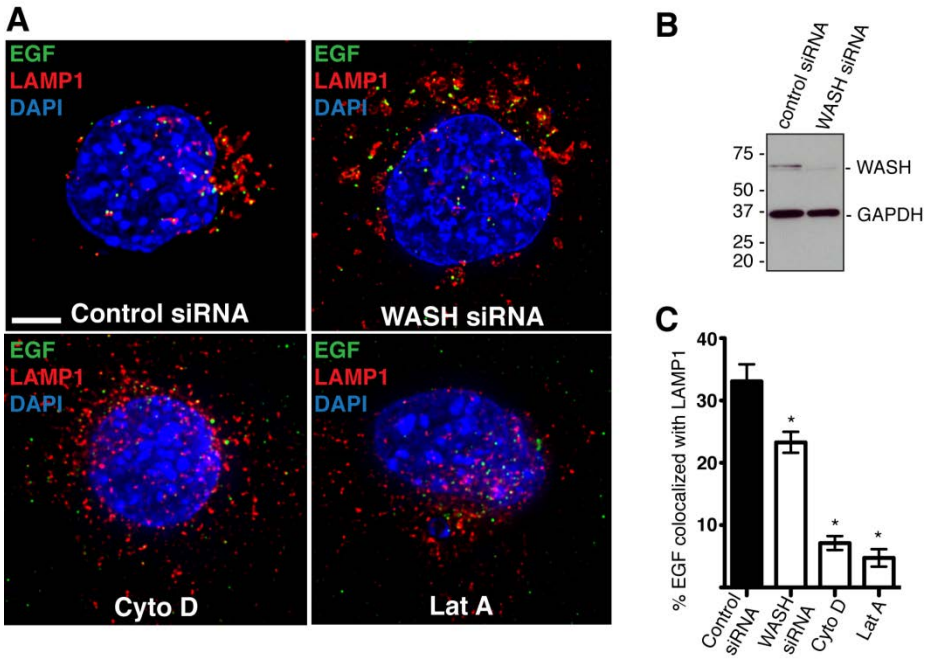

Figure 2.8. WASH depletion or disruption of the actin cytoskeleton delays EGF transport to LAMP1-positive late endosomes. (A) Fibroblast-like cells (FLCs) were transfected with nonspecific siRNA (control) or WASH siRNA, or treated with latrunculin A (Lat A) or cytochalasin D (Cyto D). After Alexa Fluor 488 EGF complex (green) was internalized for 10 min at 37 °C and chased for 30 min at 37 °C, cells were fixed, and LAMP1 (red) was stained by immunofluorescence and DNA (blue) was stained with DAPI. Scale bar, 5 µm. (B) Extracts from FLCs transfected with control or WASH siRNAs were separated by SDS-PAGE and immunoblotted with anti-WASH or anti-GAPDH antibodies. (C) The amount of fluorescent EGF colocalized with LAMP1-positive vesicles as a percentage of the total fluorescent EGF is shown for each treatment. 10 cells were counted for each condition, and data are mean ± s.d. of three independent experiments. Asterisk (*) indicates a p-value < 0.05.

Discussion

Nucleation-promoting factors regulate the Arp2/3 complex to coordinate actin assembly during diverse cellular behaviors. Here we show that the NPF WASH and the Arp2/3 complex initiate actin assembly on endosomes. Moreover, WASH, the Arp2/3 complex, and actin dynamics are important for determining endosome morphology and trafficking to late endosomes.

Our results demonstrate that human WASH is a potent NPF in vitro. The nucleation-promoting activity of WASH-WCA is comparable to that of N-WASP-WCA, the most potent of the mammalian NPFs, and greater than that of WAVE2-WCA or WHAMM-WCA. The relative potency of the human protein is consistent with the activity of *D. melanogaster* WASH-WCA, which has also been reported to be as active as fly WASP-WCA and more potent than WAVE/Scar-WCA (Liu et al., 2009b). Thus, WASH activity appears to be well-conserved among metazoans.

Our results also suggest a potential mechanism for WASH regulation. We observe that the NPF activities of full-length WASH and WASH-WCA are comparable in vitro. Similarly, it has been reported that full-length *D. melanogaster* WASH is slightly more active than WASH-WCA (Liu et al., 2009b). These results imply that WASH may not be regulated by an autoinhibitory mechanism like that of N-WASP. Furthermore, we find that overexpression of GFP-WASH in cells results in ectopic actin assembly. This activity of WASH in cells is shared with several other NPFs including the WAVEs (Machesky and Insall, 1998) and WHAMM, but not N-WASP (Campellone et al., 2008). It is now well-established that the activity of native WAVE2 is suppressed in cells by its association with a complex of interacting proteins (Derivery et al., 2009a; Eden et al., 2002; Ismail et al., 2009). Therefore, WASH NPF activity is likely to be similarly regulated *in trans* by interacting proteins to tightly control actin polymerization. In support of this idea, the results of two recent studies suggest that WASH exists in a stable complex with other proteins in cells (Derivery et al., 2009b; Gomez and Billadeau, 2009).

In contrast to a previous report that a C-terminally GFP-tagged WASH derivative localizes to filopodia and lamellipodia (Linardopoulou et al., 2007), we find that endogenous WASH and an N-terminally GFP-tagged variant localize to a subpopulation of endosomes. This discrepancy could be due to the location of the GFP tag (N- or C-terminal) or differences in expression levels of these constructs. Nevertheless, the fact that we observe similar localization patterns for endogenous WASH and the N-terminally GFP-tagged protein supports our observations. Using deconvolution microscopy we show that endogenous WASH colocalizes with markers of several endosomal compartments, including the early endosome markers EEA1 and Rab5, the recycling endosome marker Rab11, and to a lesser extent, the fast recycling pathway marker Rab4. Our observations are in broad agreement with two recent studies that also concluded that WASH colocalizes with markers of early endosomal compartments (Derivery et al., 2009b; Gomez and Billadeau, 2009). Nevertheless, some differences in the localization patterns have been reported, most notably the observation of either

strong (Derivery et al., 2009b) or weak (our study) association with Rab4-positive fast recycling endosomes. Such differences might be attributed to the distinct cell types, experimental conditions, and antibodies used in each study. In our study, WASH is particularly enriched on early endosomes, suggesting that it functions during the early stages of endocytic trafficking.

Early endosomes are the sorting station for most internalized cargo and are thought to be organized into multiple structural and functional domains (Gruenberg, 2001) to facilitate the efficient trafficking of cargo destined for degradation, recycling back to the plasma membrane, or delivery to the *trans*-Golgi network (Maxfield and McGraw, 2004; Perret et al., 2005). Interestingly, we find that WASH is not uniformly distributed on early endosomes. Instead, it is asymmetrically distributed relative to EEA1, suggesting that it localizes to specific subdomains of endosomes. Two recent studies also documented the asymmetric localization of WASH relative to endosomal membranes (Derivery et al., 2009b; Gomez and Billadeau, 2009). How these specialized WASH-associated domains are organized remains an open question. One possibility is suggested by the observations that the actin-binding protein annexin A2 interacts specifically with cholesterol-rich membrane platforms on early endosomes (Harder et al., 1997; Mayran et al., 2003), and that these domains are associated with F-actin (Morel et al., 2009). Thus, WASH may also be associated with cholesterol-rich subdomains, since WASH colocalizes with F-actin on endosomes. It is also possible that actin itself plays a role in organizing these subdomains. We propose that WASH localization and actin polymerization on early endosomes coordinates localized membrane remodeling.

Consistent with a role for WASH in membrane remodeling, we show that WASH, the Arp2/3 complex, and actin are critical for controlling early endosome morphology in cells. Depletion of WASH or the Arp2/3 complex by RNAi, or disruption of actin assembly by drug treatments, results in the appearance of enlarged WASH- or EEA1-positive endosomes that exhibit an exaggerated tubular morphology. This result is similar to what was recently reported for WASH-depleted cells (Derivery et al., 2009b; Gomez and Billadeau, 2009), and to the recent observation that actin disassembly by drug treatment causes elongated tubules to emanate from early endosomes as visualized by electron microscopy (Morel et al., 2009). Our results complement these studies and further support a direct role for WASH and the Arp2/3 complex in modulating endosome shape and tubulation.

Despite the disruption of early endosome shape in WASH-depleted cells, we do not observe any defect in the uptake or recycling of transferrin, even if WASH is depleted in cells genetically lacking N-WASP. Two recent studies also examined the role of WASH in transferrin trafficking (Derivery et al., 2009b; Gomez and Billadeau, 2009). Consistent with our results, one group observed that surface transferrin levels were unaffected in WASH-depleted cells (Gomez and Billadeau, 2009). In contrast, the second group observed that transferrin recycling was impaired in cells silenced for WASH expression (Derivery et al., 2009b). It is noteworthy that all three studies examined transferrin trafficking using different assays. Moreover, our study examined

transferrin recycling kinetics in NIH-3T3 cells, whereas HeLa cells were used in the study that observed an effect of WASH depletion (Derivery et al., 2009b). Therefore, it is likely that differences in experimental procedures and/or cell type contributed to any apparent discrepancies.

Although we do not detect a role for WASH in transferrin uptake and recycling, we find that WASH is important for endocytic trafficking of EGF. WASH depletion by RNAi or actin disassembly by drug treatment impairs EGF trafficking to late endosomes. In agreement with our observations, it has recently been shown that disrupting the actin cytoskeleton inhibits EGFR degradation (Morel et al., 2009). These observed defects in EGF trafficking upon WASH depletion or actin disruption, together with the appearance of enlarged and tubulated endosomes (this study; (Morel et al., 2009), raise the possibility that that tubular extensions observed on early endosomes represent exaggerated intermediates in the endosomal maturation process. A second possibility arises from the recent observation that WASH regulates retromer-mediated retrograde transport of CI-MPR (Gomez and Billadeau, 2009). In light of this finding, it is also possible that the tubular extensions we observe represent arrested retromer traffic or recycling cargo, which delays the transition from early-to-late endosomes in our assay. Future work will be required to distinguish between possibilities.

Our results suggest a model in which WASH, the Arp2/3 complex, and actin dynamics are involved in membrane remodeling events that are required for the transition from early-to-late endosomes. Consistent with this notion, annexin A2, Spire1, and the Arp2/3 complex were recently proposed to contribute to actin assembly and remodeling or severing of early endosomes (Morel et al., 2009). Our work expands on this idea by implicating WASH in the activation of the Arp2/3 complex on endosomes. WASH may also work cooperatively with other actin nucleators such as Spire and formins, as has been suggested to occur during *D. melanogaster* oogenesis (Liu et al., 2009b). We propose that WASH localization to subdomains of early endosomes may reflect an association with emerging membrane tubules on this compartment. Because interfering with WASH, the Arp2/3 complex, or actin function results in elongated endosome tubules, we speculate that actin assembly by WASH and the Arp2/3 complex is involved in severing these nascent tubules. While the precise mechanism of membrane severing by WASH, the Arp2/3 complex, and actin is not clear, this process may be analogous to how N-WASP and branched actin networks function during endocytic vesicle scission at the plasma membrane (Kaksonen et al., 2006; Perrais and Merrifield, 2005).

Although our study sheds light on the cellular function of WASH and actin in endocytic trafficking, numerous questions remain. One question centers on the regulation of WASH activity in cells. Most other mammalian NPFs are regulated by small GTPases (Stradal and Scita, 2006), and it has been proposed that WASH is regulated by the Rho family GTPase Rho1 in *D. melanogaster* (Liu et al., 2009b). Mammalian WASH may also be regulated by Rho, or alternatively by one of the many Rab-family GTPases that coordinate endocytic trafficking events. A second question centers on the mechanism by which actin assembly contributes to endosome

morphogenesis and trafficking. The contribution of actin filaments may be to act as a scaffold for the recruitment of other factors, or to generate force by polymer assembly or via the activity of motor proteins. Furthermore, microtubules may also play a role in this process, and WASH may contribute to coordinating the activity of multiple cytoskeletal systems, as *D. melanogaster* WASH displays both actin filament and microtubule bundling activities in vitro (Liu et al., 2009b) and mammalian WASH has been shown to interact with tubulin (Gomez and Billadeau, 2009). Future experiments will reveal how WASH acts in concert with other actin assembly factors and different cytoskeletal elements to coordinate membrane remodeling in the endocytic pathway.

Conclusions

A general consensus is emerging that WASH localizes to endosomes and is involved in endocytic trafficking. However, WASH has now been implicated in three separate endocytic pathways: transferrin recycling (Derivery et al., 2009b), retromer-mediated retrograde CI-MPR trafficking (Gomez and Billadeau, 2009), and EGF trafficking (this study). As early endosomes serve as a crossroads for each of these processes, it is possible that WASH activity directly or indirectly influences all of these trafficking pathways. It is also possible that WASH acts in distinct pathways for different endocytic cargoes or in diverse cell types. We are clearly only beginning to understand the role of actin in endocytic trafficking in mammalian cells, and the next few years are likely to bring exciting developments as we learn more details about the role of NPFs and the Arp2/3 complex in controlling actin and its interacting proteins to shape and reorganize intracellular membranes during membrane trafficking processes.

Experimental Procedures

Plasmids, Bacteria, and Cells

The genes encoding full-length WASH or WASH-WCA (nucleotides 912-1404) were amplified by PCR from a human cDNA clone (accession BC048328, Open Biosystems). PCR products were digested with KpnI and NotI (New England Biolabs) and ligated into pKC-ET16b for bacterial expression or pKC-EGFP-C1 for mammalian expression. Plasmids used to express N-WASP-WCA (pET-N-WASP-WCA), WAVE2-WCA (pET-WAVE2-WCA) and WHAMM-WCA (pET-WHAMM-WCA) were described previously (Campellone et al., 2008). Plasmids were maintained in *E. coli* XL-1 Blue (Stratagene), whereas *E. coli* BL21-Rosetta (EMD Biosciences) was used for recombinant protein expression. N-WASP$^{+/+}$ and N-WASP$^{-/-}$ fibroblast-like cells (FLCs) were a kind gift from S. Snapper (Snapper et al., 2001). COS7 cells, NIH-3T3 cells, and FLCs were cultured in DMEM (Invitrogen) supplemented with 10% Fetal Bovine Serum (FBS; JR Scientific) at 37 °C in 5% CO_2.

Protein Expression and Purification

E. coli transformed with pET16b-WASH were grown to OD_{600} = 0.6 and induced with 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) for 12 h at 16 °C. Following expression, bacteria were resuspended in lysis buffer (8 M urea, 10 mM Tris pH 8.0, 250 mM KCl, 100 mM NaH_2PO_4, 10 μg/ml each of aprotonin, leupeptin, pepstatin, and 1 mM PMSF). Ni-NTA affinity purification was carried out for His-tagged protein purification under denaturing conditions (QIAGEN). Elutions were performed by lowering the lysis buffer pH to 4.5 with HCl. Protein refolding was performed by slowly dialyzing against 250 mM KCl, phosphate buffered saline (PBS) pH 7.4 with 10% glycerol. Gel filtration chromatography on a Superdex 75 (GE Healthcare) equilibrated with 250 mM KCl, PBS pH 7.4 with 10% glycerol was used to further purify the recombinant protein.

Full-length bovine N-WASP tagged at its N-terminus with 6xHis and FLAG epitopes was expressed in Sf9 insect cells using the Bac-to-Bac baculovirus expression system (Invitrogen). 72 h post-infection, cells were harvested by centrifugation at 500 x *g* for 10 min at 25 °C, resuspended in lysis buffer (50 mM Tris pH 8.0, 250 mM NaCl, 100 mM KCl, 20 mM imidazole, 1% NP-40 with protease inhibitors) and frozen in liquid N_2. To prepare the lysate, cells were thawed and centrifuged at 200,000 x *g*, 4 °C, 20 min. The supernatant was incubated with Ni-NTA agarose (QIAGEN) to isolate His-N-WASP. His-N-WASP was eluted in 50 mM Tris pH 8.0, 250 mM NaCl, 100 mM KCl with 300 mM imidazole. His-tagged WCA domains were purified as described previously (Campellone et al., 2008).

Antibodies

Anti-WASH antibodies were generated by immunizing rabbits with full-length recombinant human WASH (Covance Inc.). Anti-N-WASP antibodies were generated

by immunizing guinea pigs with full-length recombinant bovine N-WASP (Pocono Rabbit Farms). Polyclonal antibodies were affinity purified on WASH or N-WASP affinity columns as described previously (Welch et al., 1997). Antibodies recognizing the Arp2/3 subunits were also described previously (Welch et al., 1997). Antibodies raised against EEA1, Rab4, Rab5, and Rab11 (BD Biosciences), LAMP1 (Santa Cruz Biotechnology), GAPDH (Ambion), calnexin (Stressgen), and tubulin (Developmental Studies Hybridoma Bank at the University of Iowa) were purchased from the indicated vendors. Secondary antibodies conjugated to HRP (Jackson Immunoresearch) were used for immunoblotting. Alexa Fluor 488 and 568 conjugated secondary antibodies from Invitrogen were used for immunofluorescence.

Pyrene-Actin Polymerization Assays

Full-length recombinant WASH and WASH-WCA, N-WASP-WCA, WAVE2-WCA and WHAMM-WCA were dialyzed into 20 mM MOPS pH 7.0, 200 mM KCl, 2 mM $MgCl_2$, 5 mM EGTA, 1 mM EDTA and 10% glycerol prior to assembly reactions. His-tagged Arp2/3 complex, rabbit skeletal muscle actin, and pyrene-actin were prepared as described previously (Goley et al., 2004). Actin assembly reactions were allowed to reach steady state, and the curves were normalized to differences in steady state fluorescence.

Cell/Tissue Extracts, Fractionation, and Immunoblotting

Cell extracts were prepared by lysing cells in 50 mM Tris pH 7.6, 150 mM NaCl, 1% Triton X-100, and protease inhibitors. Membrane and cytosol components were fractionated as previously described (Campellone et al., 2008). To determine tissue-specific expression of WASH, a mouse tissue blot (BioChain) was used. For immunoblotting, nitrocellulose membranes were blocked with 5% milk in PBS, probed with primary and secondary antibodies, and visualized by chemiluminescence (GE Healthcare).

Drug Treatments and Preparation for Fluorescence Microscopy

Cells grown on coverslips were treated with 10 μM latrunculin A or 10 μM cytochalasin D (EMD Bioscience), or DMSO (Sigma) for 10 min. Cells were fixed for 20 min in 2.5% paraformaldehyde (EMD Biosciences) at 37 °C. Immediately following fixation, cells were permeablized with 0.1% Triton X-100 for 5 min, incubated with primary antibodies diluted in PBS with 1% BSA (Sigma) and 5% normal goat serum (Invitrogen), washed in PBS, treated with Alexa Fluor-conjugated secondary antibodies, 1 μg/ml DAPI, and/or 4 U/ml Alexa Fluor 568-phalloidin (Invitrogen). Coverslips were mounted on glass slides using ProLong Gold anti-fade reagent (Invitrogen).

Image Acquisition and Analysis

Wide-field fluorescence images were taken on an Olympus IX71 inverted microscope with a 100X (1.35 NA) PlanApo objective lens equipped with a photometrics Coolsnap

HQ camera. Images were captured using Metamorph software (Universal Imaging), converted to 8-bit tiff files and processed using ImageJ software (National Institutes of Health). Deconvolution images were taken using an Applied Precision DeltaVision 4 Spectris microscope with a 100X (1.4 NA) PlanApo objective equipped with a Photometrics CH350 CCD camera. Images were captured using SoftWoRx v3.3.6 software (Applied Precision), and were deconvolved with Huygens Professional v3.1.0p0 software (Scientific Volume Imaging). 3D reconstruction animations were made using Imaris 5.2 software (Bitplane). Areas of colocalization were quantified using ImageJ and the JACoP plugin (Bolte and Cordelieres, 2006). 10 images from 3 independent experiments were quantified. The mean percentage of WASH structures that colocalized with each marker was determined with Prism software. Structured illumination microscopy images were captured on a DeltaVision OMX microscope (Applied Precision) with a 100X (1.4 NA) PlanApo objective equipped with a Sony ICX285 20 MHz CCD camera. SIM images were processed as described above for standard deconvolution images.

DNA and siRNA Transfections

To visualize WASH localization, COS7 cells were transfected with 250 ng of pEGFP-C1-WASH in a 6-well plate with Lipofectamine2000 (Invitrogen). For WASH silencing by RNAi, NIH-3T3 or FLCs were transfected twice, first at 0 h on day 1 when cells were 30-40% confluent and at 24 h on day 2. Transfections were carried out using a 20 nM final concentration of siRNA with RNAiMAX (Invitrogen). Either of two siRNAs (J-054931-09 and J-054931-12 from Dharmacon) were used independently with identical results for WASH depletions. Two siRNAs (I.D. numbers: 74834 and 77135 from Ambion) were used to target the ARPC3 and Arp3 subunits of the Arp2/3 complex. A 10 nM final concentration of each siRNA was used for each transfection. Transfections were performed as described above for WASH depletion. Endocytosis assays were performed and/or cells were fixed for microscopic analysis on day 3 (72 h after the first siRNA transfection).

Transferrin Uptake and Recycling Assays

Uptake and recycling of biotinylated transferrin (B-SS-Tfn) was performed as described previously (Osborne et al., 2005) with the following modifications. FLCs or NIH-3T3 cells were serum-starved for 1 h in DMEM at 37 °C. Cells were then incubated with 1 μg/ml B-SS-Tfn in PBS + 0.2% BSA for 30 min at 4 °C, washed 3X with PBS + BSA, and chased with 100 μg/ml unlabeled transferrin in PBS/BSA at 31 °C for 0, 2, 4, 8, 16, 32, 48, or 64 min. The amount of internalized B-SS-Tfn at each time point was measured by ELISA and expressed as a percentage of the total surface-bound B-SS-Tfn prior to internalization.

EGF Transport Assays

Wild type FLCs were grown to 70-80% confluency and serum-starved for 1 h prior to internalization. Drug treatments were carried out using 1 μM latrunculin A, 1 μM

cytochalasin D, or DMSO. Cells were allowed to internalize 20 ng/ml Alexa Fluor 488-EGF complex (Invitrogen) for 10 min at 37 °C, were washed 6X with PBS to remove surface bound EGF, and were chased for 30 min in serum-free DMEM with or without drug treatment. Cells were fixed, processed, and imaged as described above. The percentage of fluorescent EGF localized to LAMP1 structures was determined using ImageJ and JACoP. 10 images from 3 independent experiments were quantified for each treatment. The data were analyzed using Prism and experimental treatments were compared pairwise with control treatments using a one-way ANOVA. Differences with p-values < 0.05 were designated statistically significant.

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CHAPTER 3

Regulation of integrin trafficking, cell adhesion and cell migration by WASH and the Arp2/3 Complex

Introduction

The actin cytoskeleton plays a fundamental role in processes including cell migration and intracellular trafficking (Firat-Karalar and Welch, 2011; Pollard and Cooper, 2009). During cell migration, the polymerization of actin monomers (G-actin) into actin filaments (F-actin) at the plasma membrane drives the protrusion of lamellipodia and filopodia at the leading edge, and actin assembly in contractile stress fibers provides traction by linking the cytoskeleton to the extracellular matrix through transmembrane integrin receptors (Caswell and Norman, 2008; Caswell and Norman, 2006). During intracellular trafficking, actin polymerization enables membrane remodeling in endocytic internalization, endocytic recycling and degradation, as well as exocytic transport (Anitei and Hoflack, 2011; Kaksonen et al., 2006; Taylor et al., 2011). Although actin acts in both migration and intracellular transport, the molecular pathways that connect actin function in these processes remain poorly understood.

A key actin nucleating factor that functions in both cell migration and intracellular trafficking is the Arp2/3 complex, a multiprotein complex that promotes the assembly of branched F-actin networks (Goley and Welch, 2006). Activation of the Arp2/3 complex requires proteins called nucleation-promoting factors (NPFs), of which there are several families in mammalian cells, each participating in distinct subcellular processes (Campellone and Welch, 2010; Firat-Karalar and Welch, 2011). A recently identified NPF termed WASH (Linardopoulou et al., 2007) is part of a large multiprotein complex (Derivery et al., 2009b; Gomez and Billadeau, 2009; Jia et al., 2010) and has been implicated in endocytic trafficking. WASH localizes to early and recycling endosomes in mammalian cells, regulates the shape of endocytic compartments, and influences endocytic trafficking events, including recycling of transferrin and β2 adrenergic receptors, retromer-mediated trafficking of CI-MPR to the trans-Golgi network, and EGF transport to late endosomes (Derivery et al., 2009b; Duleh and Welch, 2010; Gomez and Billadeau, 2009; Puthenveedu et al., 2010; Temkin et al., 2011). Interestingly, WASH was also among a set of proteins found to be specifically present in organisms that undergo amoeboid-like cell motility (Fritz-Laylin et al., 2010), suggesting that WASH may function in the trafficking of proteins involved in cell migration. It has remained unclear, however, which specific cargos are trafficked by a WASH-dependent mechanism, and how WASH activities in endocytic trafficking and cell migration might be connected.

Integrins are one class of cargos that are trafficked through early and recycling endosomes and are central to the process of cell migration. The α5β1 integrin heterodimer is of particular importance in migration because in many cell types it is the major receptor for the extracellular matrix (ECM) protein fibronectin, and it plays a crucial role in cell adhesion and migration, as well as cancer cell invasion (Caswell and Norman, 2008; Jones et al., 2006; Pellinen and Ivaska, 2006). The α5β1 integrin is internalized by clathrin-dependent (Pellinen et al., 2008) and calveolar-mediated (Shi and Sottile, 2008) pathways, both of which converge at the early endosome (Naslavsky et al., 2003). It is then recycled back to the plasma membrane through a Rab11-dependent mechanism (Powelka et al., 2004; Roberts et al., 2004), where the integrins

cluster to form focal adhesions (Caswell and Norman, 2008; Jones et al., 2006; Pellinen and Ivaska, 2006). Because WASH has been implicated in trafficking at early and recycling endosomes, we investigated its role in transport of α5β1 integrin.

Here we show that actin nucleation by WASH and the Arp2/3 complex plays an important role in transport of α5-integrin from intracellular compartments to ventral adhesive structures in fibroblast cells. Furthermore, WASH is crucial for maintaining focal adhesion number and promoting adherence to specific ECM proteins. Surprisingly, due to its role in modulating adherence, WASH potentiates the rate of cell migration in two-dimensional wound healing assays. Our work complements a recent study that implicated WASH in integrin trafficking during invasive cell migration (Zech et al., 2011). These data highlight a role for WASH and Arp2/3 activity in regulating integrin trafficking important for cell adhesion and migration.

Results

WASH and F-actin colocalize on subdomains of endosomes that also contain α5-integrin

Previous observations indicate that WASH and F-actin localize to subdomains of early and recycling endosomes (Derivery et al., 2009b; Duleh and Welch, 2010; Gomez and Billadeau, 2009), which are compartments through which integrins are trafficked (Caswell et al., 2009; Pellinen and Ivaska, 2006). Therefore, we sought to determine whether WASH, F-actin, and integrins are present in the same population of endosomes. To better observe endosomes in NIH3T3 fibroblast cells, we expressed a constitutively active Rab5 mutant, pDsRed-Rab5-Q79L, which promotes early endosome fusion, resulting in enlarged endosomes that are easily visualized by fluorescence microscopy in live cells (Rab5-Q79L enlarged endosomes have been shown to exhibit efficient receptor and membrane recycling (Ceresa et al., 2001)). These cells were also engineered to express GFP-WASH, and Lifeact-BFP to visualize actin filaments. Both GFP-WASH and F-actin colocalized at subdomains on these enlarged endosomes (Figure 3.1A,B). Moreover, live cell imaging revealed dynamic F-actin polymerization at endosomal WASH subdomains. Thus, WASH frequently colocalizes with dynamic F-actin on subdomains of early endosomes.

To determine if α5-integrin is trafficked through these same enlarged endosomes, cells transfected with pDsRed-Rab5-Q79L and pLifeact-BFP were stained for α5-integrin by immunofluorescence (Figure 3.1C,D). Although α5-integrin was present in many enlarged endosomes, its localization only partially overlapped with domains containing F-actin (Figure 3.1C,D). Moreover, much of the α5-integrin appeared internal within endosomes. This may represent multiple subpopulations of integrin in endocytic compartments, including integrin tagged for degradation, unsorted integrins, and integrins sorted to subdomains to enable recycling back to the plasma membrane (Caswell et al., 2009; Lobert et al., 2010; Margadant et al., 2011). Taken together, these observations suggest that α5-integrin traffics through compartments that contain WASH and dynamic F-actin.

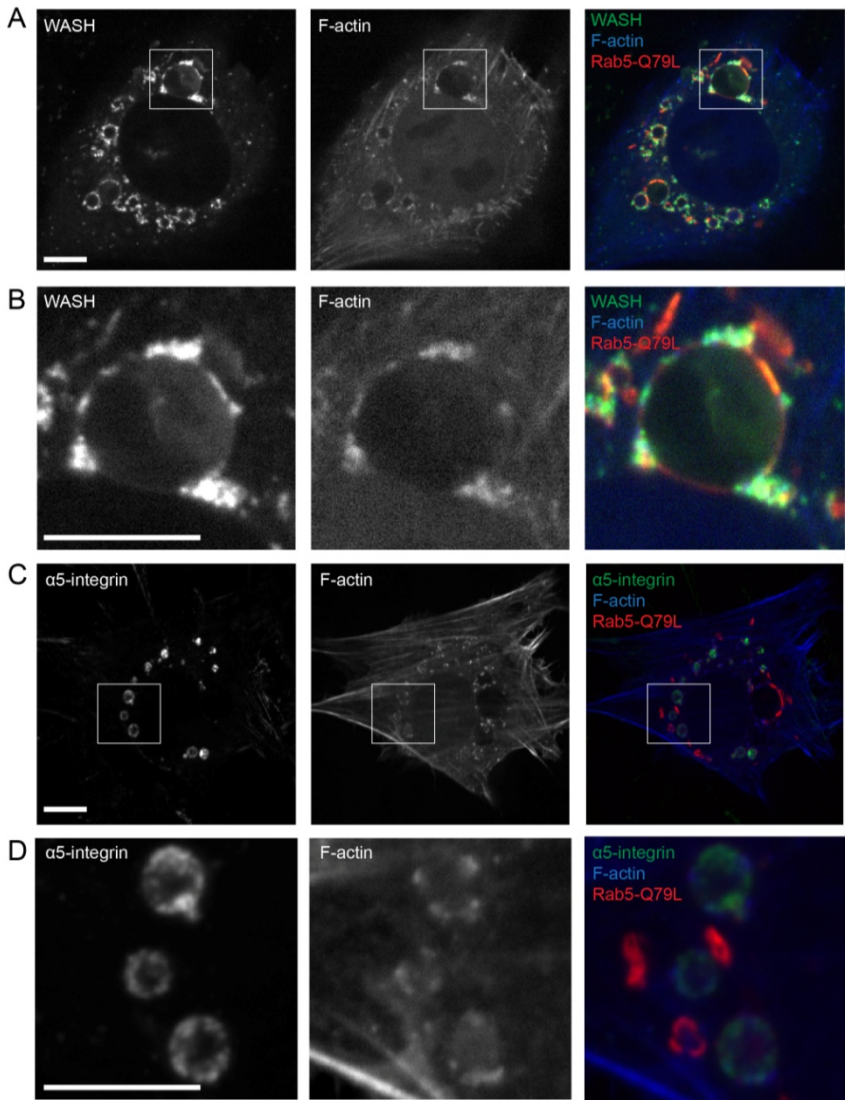

Figure 3.1. WASH and F-actin colocalize on subdomains of enlarged endosomes containing α5-integrin. (A) NIH3T3 cells transfected with pGFP-WASH (green), pLifeact-BFP (blue) to mark F-actin, and pDsRed-Rab5-Q79L (red). **(B)** Enlarged insets from (A). **(C)** NIH3T3 cells expressing Lifeact-BFP (blue) to mark F-actin, and DsRed-Rab5-Q79L (red) were stained for α5-integrin (green) by immunofluorescence. **(D)** Enlarged insets from (C). Scale bars, 10 μm.

WASH is important for α5-integrin localization to focal adhesions and for maintaining focal adhesion number

The observation that α5-integrin traffics through compartments that contain F-actin raised the question of whether WASH plays a functional role in integrin trafficking. To address this, we first investigated the importance of WASH in α5-integrin localization by silencing WASH expression in NIH3T3 cells with either of two distinct siRNAs targeting WASH (or with a non-specific control siRNA). Treatment with either WASH siRNA resulted in ~80% reduction in WASH protein levels. In cells treated with the control siRNA, α5-integrin was primarily localized to focal adhesions (Figure 3.2A-C). In contrast, WASH-silenced cells exhibited aberrant α5-integrin localization in large internal punctae (Figure 3.2A) and significantly less α5-integrin colocalized with vinculin-positive focal adhesions (Figure 3.2B,C). Interestingly, WASH deficient cells also displayed significantly fewer focal adhesions than control cells (Figure 3.2D). These data implicate WASH in regulating α5-integrin localization to adhesive structures, and in controlling the overall number of focal adhesions in fibroblast cells.

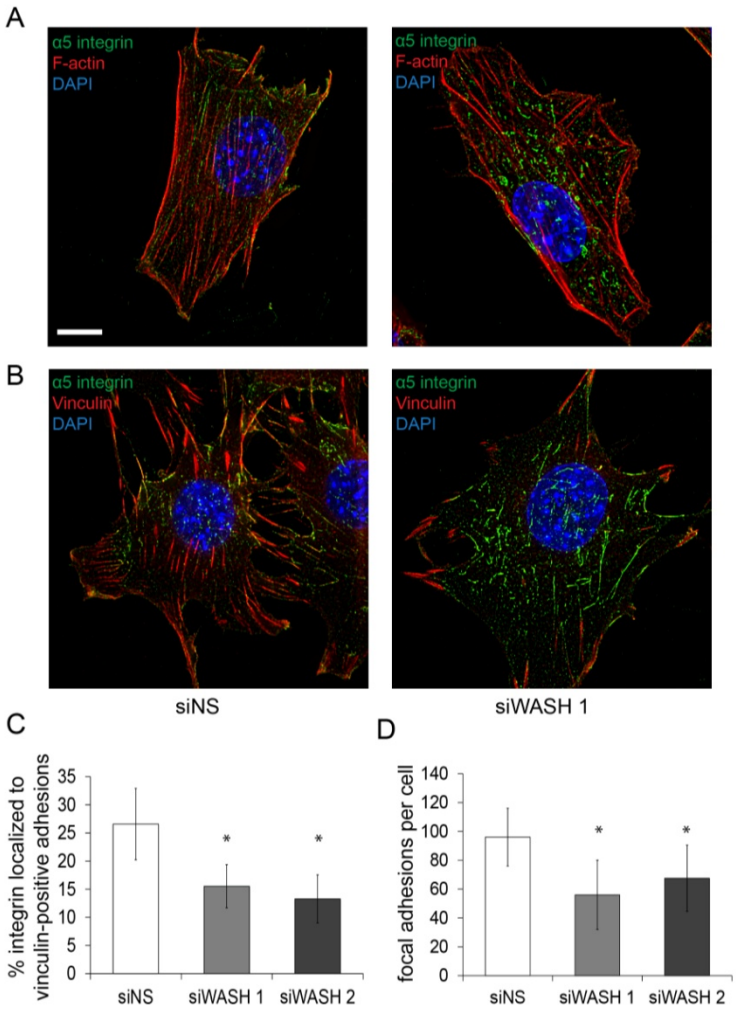

Figure 3.2. WASH depletion disrupts α5-integrin localization to focal adhesions and decreases focal adhesion number. (A) α5-integrin (green) visualized by immunofluorescence, F-actin (red) stained with Alexa Fluor 568 phalloidin, and DNA (blue) stained with DAPI in NIH3T3 cells treated with non-specific siRNA (siNS; left) or WASH siRNA (siWASH 1; right). **(B)** α5-integrin (green) and vinculin (red) visualized by immunofluorescence and DNA (blue) stained with DAPI in NIH3T3 cells treated with siNS (left) or siWASH 1 (right). **(C)** The percentage of α5-integrin associated with vinculin-positive focal adhesions in siNS, siWASH 1, or siWASH 2 treated cells. **(D)** The total number of focal adhesions per cell in NIH3T3 cells treated with siNS, siWASH 1, or siWASH 2. At least 10 cells were counted in three independent experiments. Error bars indicate the s.d. Asterisk (*) indicates a p-value < 0.05 by the student's t-test. Scale bar, 10 μm.

WASH and the Arp2/3 complex play an important role in α5-integrin transport to adhesion sites

Next, we investigated whether WASH and its downstream effector the Arp2/3 complex play a role in α5-integrin transport to plasma membrane adhesion sites. NIH3T3 cells were serum starved to bias integrin localization to endocytic compartments, and serum was then added to stimulate transport to the plasma membrane. To quantify transport, we imaged individual cells using both total internal reflection fluorescence (TIRF) microscopy to measure ventral α5-integrin at adhesion sites, and epifluorescence microscopy to measure the total cellular α5-integrin. The ratio of TIRF intensity to epifluorescence intensity (TIRF:EPI ratio), which is a measure of the fraction of cellular α5-integrin at adhesion sites, was followed over time to examine the kinetics of integrin transport to ventral adhesive structures (Figure 3.3). At all time points, cells silenced for WASH (with either of the two siRNAs used) exhibited an approximately 25% reduction in the TIRF:EPI ratio compared with cells treated with the non-targeting siRNAs, indicating that WASH silencing reduces the fraction of α5-integrin at ventral adhesion sites (Figure 3.3A,B,C). However, we did not observe a significant kinetic delay in α5-integrin trafficking to adhesion sites following serum stimulation in WASH-silenced versus control cells.

To investigate the role of the Arp2/3 complex in α5-integrin transport, we used CK666, a chemical inhibitor of Arp2/3 (Nolen et al., 2009). In experiments similar to those described above, cells treated with CK666 exhibited significantly reduced α5-integrin levels at adhesive sites compared with cells treated with the inactive control drug CK689 (Figure 3.3D). The effect was apparent at all time points, and Arp2/3 complex inhibition did not affect the overall kinetics of transport following serum stimulation. Thus, actin polymerization by WASH and the Arp2/3 complex influenced the steady state levels of α5-integrin at ventral adhesive sites in fibroblast cells, but did not affect the kinetics of α5-integrin transport in our experiments.

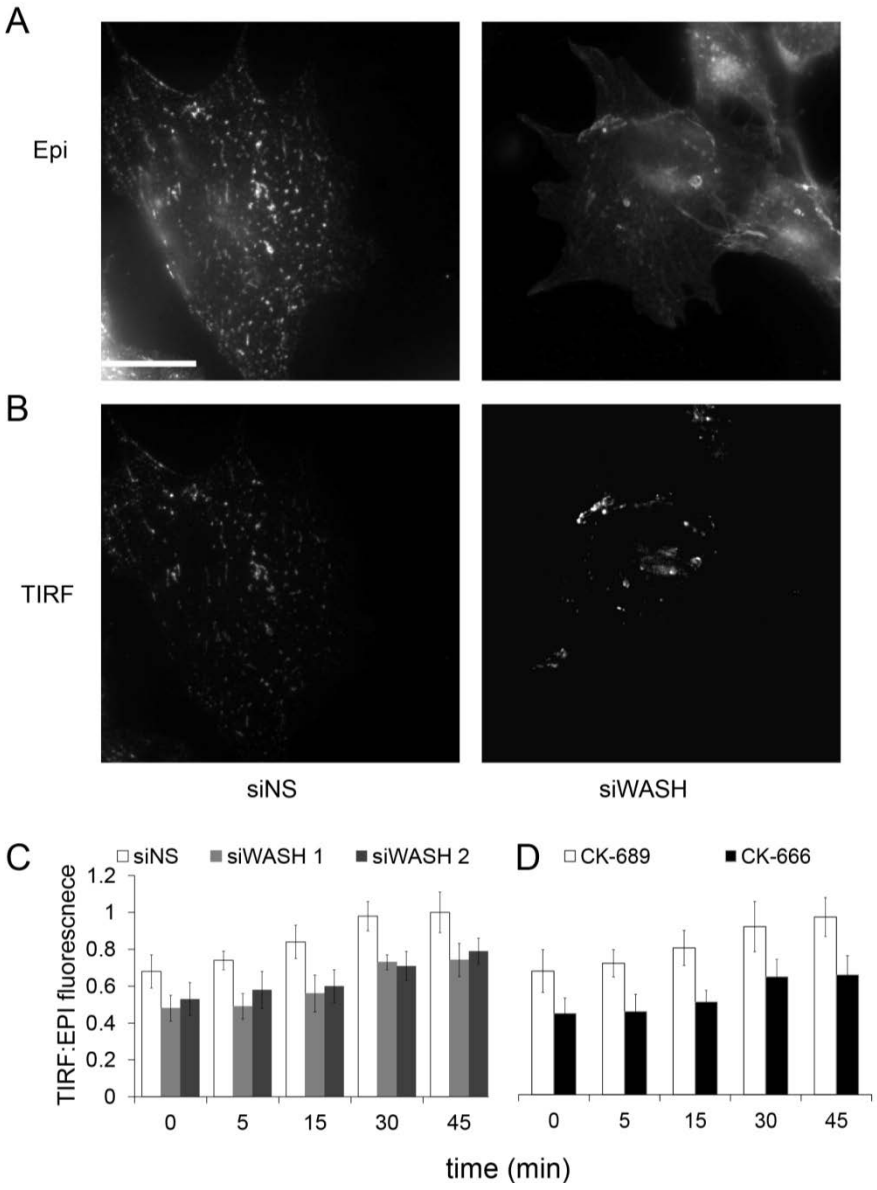

Figure 3.3. α5-integrin transport to adhesion sites is disrupted in WASH-silenced cells. (A) Epifluorescence images of α5-integrin stained by immunofluorescence in cells treated with non-specific siRNA (siNS; left) or WASH siRNA (siWASH 1; right). **(B)** TIRF images of the cells shown in (A). **(C)** Kinetics of α5-integrin transport to the ventral surface of the cells following serum treatment in siRNA- or drug-treated cells. Data represent the mean TIRF:Epifluorescence ratio for each treatment. At least 10 cells were quantified from 3 independent experiments. Error bars represent the s.d. A significant difference between cells treated with control siNS versus WASH siRNAs (siWASH 1 or siWASH 2), or **(D)** cells treated with the inactive control compound (CK689) versus the Arp2/3 complex inhibitor (CK666), was observed at each time point.

Asterisk (*) indicates a p-value < 0.05 as determined by a student t-test. Scale bar, 10 μm.

WASH is important for adhesion to specific extracellular matrix molecules

It is well known that integrins mediate attachment between cells and ECM molecules that include fibronectin, vitronectin, tenascin, laminin, and various collagen isoforms. Because WASH influences α5-integrin localization, we tested the role of WASH in the adhesion of NIH3T3 cells to a panel of extracellular matrix proteins. Cells treated with a non-specific control siRNA or either of two siRNAs targeting WASH were allowed to adhere to a surface coated with specific ECM proteins, and after gentle agitation to detach non-adherent cells, the remaining adherent cells in each well were quantified. We observed a low affinity of cells for BSA and multiple collagen isoforms. However, the cells exhibited a strong affinity for fibronectin and an intermediate affinity for vitronectin and laminin (Figure 3.4). Interestingly, the affinity for fibronectin was reduced by 40% in WASH-silenced cells compared to control cells (Figure 3.4). Moreover, adherence to laminin and vitronectin was decreased by 15-20% in cells treated with WASH siRNAs (Figure 3.4). The specific effect of WASH depletion on adhesion to fibronectin, vitronectin and laminin is consistent with our previous results that demonstrate that WASH influences the trafficking of the α5-integrin, part of the α5β1 integrin heterodimer that is the primary fibronectin-binding integrin in these cells.

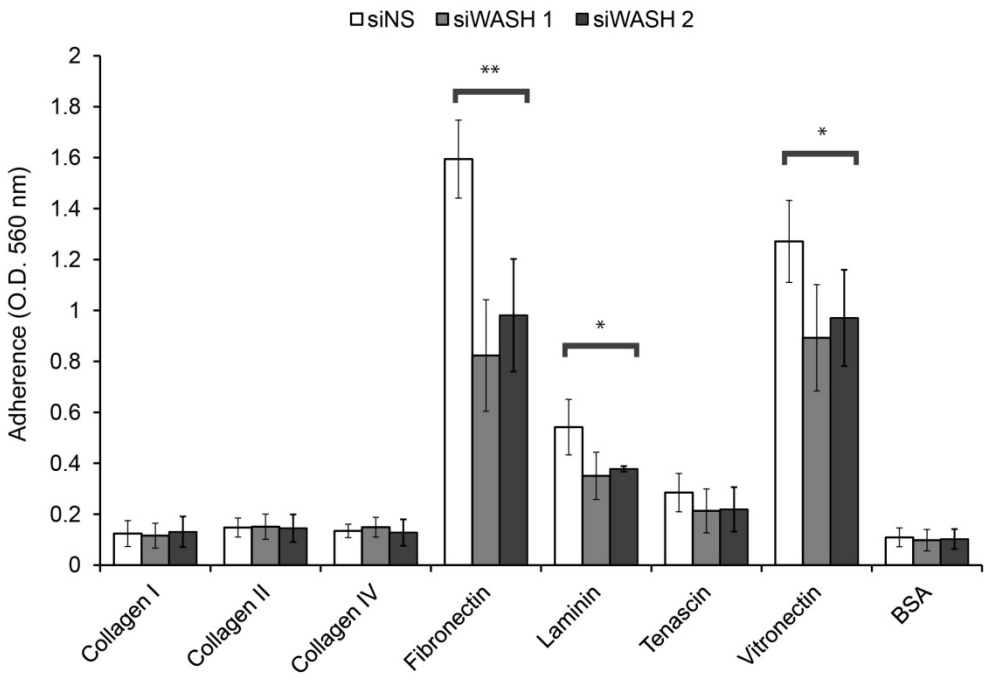

Figure 3.4. Adhesion to fibronectin, laminin, and vitronectin is decreased in WASH-depleted cells. NIH3T3 cells treated with siNS, siWASH 1, or siWASH 2 were incubated for 2 h in wells coated with the indicated ECM proteins or BSA as a negative control. Unbound cells were removed by gentle agitation. Adherent cells were fixed, stained with Cell Stain Solution, and quantified by measuring the absorbance at 560 nm. Data represent the mean of 3 independent experiments, and error bars represent the s.d. Asterisk (*) indicates a p-value < 0.05 as determined by a student's t-test.

WASH silencing increases the rate of directional cell migration in mouse embryonic fibroblasts

Because WASH affects integrin transport and cell-substrate adhesion, and adhesion is critical for cell migration, we asked whether WASH also influences cell migration. To examine the role of WASH in cell migration, we performed scratch wound healing assays using confluent layers of mouse embryonic fibroblasts (MEFs) treated with a non-specific siRNA or either of two distinct siRNAs targeting WASH. In these experiments, WASH-silenced MEFs exhibited significantly faster migration rates than control cells (Figure 3.5). However, no defects were observed for sub-confluent layers of MEFs treated with WASH siRNAs compared with controls. Lamellipodia behavior, including the total number of lamellipodia per cell, protrusion velocity, retraction rate, and persistence lifetime were similar for WASH-silenced and control cells. Therefore, we propose that WASH influences cell migration by regulating cell adhesion, and not the protrusion functions of lamellipodia.

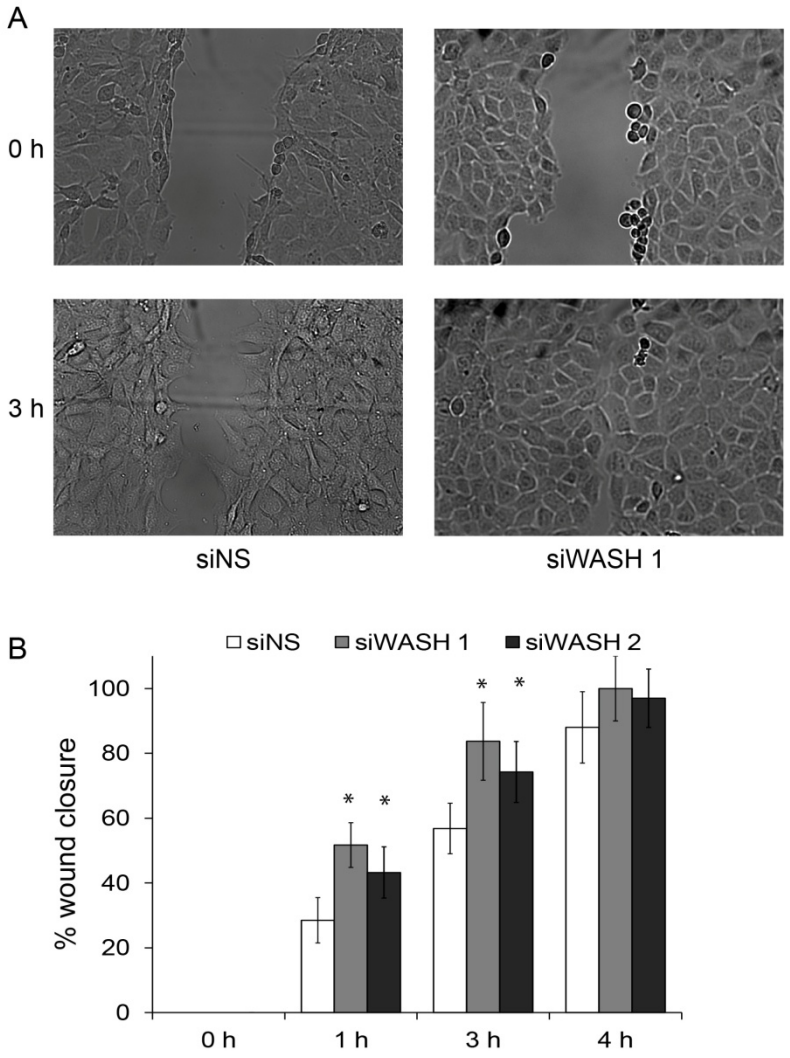

Figure 3.5. WASH silencing increases the rate of directional cell migration in mouse embryonic fibroblasts. (A) Brightfield images of wound closure at 0 h and 3 h time points in MEF cells treated with siNS or siWASH 1. **(B)** Quantification of wound closure over a 4 h time course in MEF cells treated with siNS, siWASH 1 or siWASH 2. Ten wounds were counted in 3 independent experiments. Data represent mean percent of wound area closed at each time point, and error bars represent the s.d. Asterisk (*) indicates a p-value < 0.05 by a student's t-test.

Discussion

The molecular pathways that connect actin function in cell migration and membrane trafficking remain poorly understood. Here we implicate the endocytic trafficking protein WASH in cell adhesion and migration via its role in the endocytic recycling of α5-integrin. We find that WASH and F-actin form dynamic subdomains on endosomes that contain α5-integrin, and that WASH is important for trafficking α5-integrin to ventral adhesive structures. WASH is also important for maintaining focal adhesion number and enabling adhesion to specific ECM proteins, including fibronectin. Surprisingly, WASH negatively regulates 2D migration of fibroblast cells. These results demonstrate that actin polymerization by WASH and the Arp2/3 complex plays a critical role in regulating membrane trafficking pathways that impact cell adhesion and migration.

WASH was previously shown to localize with F-actin on early and recycling endosomes (Derivery et al., 2009b; Duleh and Welch, 2010; Gomez and Billadeau, 2009; Puthenveedu et al., 2010). Here we show that WASH also colocalizes with dynamic F-actin on subdomains of enlarged early endosomes that are induced by the expression of the constitutively active Rab5 mutant Rab5-Q79L (Ceresa et al., 2001). Previous work indicates that transferrin is recycled normally through these enlarged early endosomes (Ceresa et al., 2001), although they may also contain late endocytic proteins (Wegner et al., 2010). Thus, these enlarged endosomes are an effective tool to visualize the distribution and dynamics of WASH and F-actin in live cells. The localization of WASH and dynamic F-actin to endosome subdomains suggest a function for these subdomains in sorting and/or trafficking specific cargos.

WASH and F-actin were previously shown to participate in endocytic recycling of transferrin and β2 adrenergic receptors to the plasma membrane (Derivery et al., 2009b; Puthenveedu et al., 2010; Temkin et al., 2011; Zech et al., 2011), retromer-mediated trafficking of CI-MPR to the trans-Golgi network (Gomez and Billadeau, 2009), and EGF transport to late endosomes (Duleh and Welch, 2010). We show that WASH and F-actin are also involved in the endocytic recycling of α5-integrin. In WASH-silenced cells we observed an accumulation of α5-integrin in perinuclear compartments, suggesting a role for WASH in α5-integrin trafficking. Consistent with this finding, the increase in perinuclear α5-integrin is accompanied by a concomitant decrease in α5-integrin at vinculin-positive adhesions. However, the overall α5-integrin levels were similar in WASH-depleted cells and control cells, indicating that WASH influences transport of this integrin from endocytic compartments to cellular adhesive structures. Recycling of α5-integrin occurs through several pathways depending on Arf6 and Rab GTPases (Jones et al., 2006; Pellinen and Ivaska, 2006; Ramsay et al., 2007). WASH has been localized to both Rab4- and Rab11-positive compartments (Derivery et al., 2009b; Duleh and Welch, 2010; Zech et al., 2011), suggesting a role for WASH during α5-integrin recycling to the plasma membrane via Rab4- and/or Rab11-dependent mechanisms.

In addition to reduced levels of α5-integrin at the ventral surface of the cell and in focal adhesions, the total number of focal adhesions was decreased in WASH-silenced

fibroblast cells. This observation suggests that WASH trafficking of α5-integrin, and possibly other cargos, plays an important role in establishing and maintaining focal adhesions. In WASH-depleted cells we also observed a strong defect in adherence to fibronectin, which is the primary ligand of α5β1 integrin (Humphries et al., 2006). These WASH-silenced cells also displayed a decreased affinity for both laminin and vitronectin. This may have resulted from disrupted trafficking of other α-chains paired with β1-integrin, such as α3β1, α6β1, and α7β1, which bind to laminins, and α8β1, which binds to vitronectin (Humphries et al., 2006). It is also possible that WASH is involved in the trafficking of other adhesion machinery. We propose that the lower levels of α5β1-integrin and fewer focal adhesions at the cell-substrate interface in WASH-silenced cells account for their decreased affinity for ECM proteins.

Integrin trafficking and the establishment of adhesive contacts provides the foundation for cell migration (Caswell and Norman, 2008; Huttenlocher and Horwitz, 2011; Ulrich and Heisenberg, 2009), as focal adhesions link the actomyosin network to the extracellular substratum and provide the traction that is required for cell body translocation (Mitchison and Cramer, 1996). However, too much adhesion will oppose forward translocation by anchoring the cell in place. Therefore, cells show a biphasic relationship between adhesion strength and migration velocity, with low and high adhesion strength correlated with lower velocity, and intermediate adhesion strength correlated with maximum velocity (DiMilla et al., 1991; DiMilla et al., 1993; Gupton and Waterman-Storer, 2006; Huttenlocher et al., 1996; Huttenlocher and Horwitz, 2011). Interestingly, we observed that WASH-silenced cells were less adherent and migrated significantly faster compared with control cells on 2D surfaces. A similar observation was previously made for MEF cells genetically lacking vinculin, which exhibited both decreased adhesion and increased migration rates in 2D wound healing assays (Xu et al., 1998). Based on our results, we conclude that disruption of integrin trafficking by WASH silencing leads to a decrease in cell-substrate affinity and a corresponding increase in 2D migration rates.

The increased rate of migration we observed for WASH-silenced cells is in agreement with a previous finding that *Dictyostelium discoideum* cells that are genetically deleted for WASH also exhibit faster migration (Carnell et al., 2011). However, our results differ from a recent report that WASH silencing did not affect the migration rates of A2780 ovarian cancer cells on 2D surfaces (Zech et al., 2011). The apparent discrepancy might be explained by the fact that MEF and A2780 cells have different adhesion requirements for 2D migration. Interestingly, WASH silencing in A2780 cells resulted in a strong defect in invasive motility into 3D ECM substrates (Zech et al., 2011), a process that relies heavily on integrin recycling (Caswell and Norman, 2008; Caswell et al., 2007). Thus differences exist in the requirement for WASH in 2D versus 3D migration. Nevertheless, our study is in broad agreement with previous results with regard to the role of WASH and Arp2/3 complex activity in α5β1 integrin recycling (Zech et al., 2011). Furthermore, we contribute several new findings, including identifying roles for WASH in the regulation of focal adhesion number and the strength of adhesion to specific ECM molecules in fibroblast cells.

What is the mechanistic role of WASH in the trafficking of integrins and other cargos during cell migration? One possibility is that dynamic actin assembly by WASH and Arp2/3 complex on endosomes organizes and maintains subdomains that are important for cargo sorting. A kinetic sorting model for the role of dynamic actin subdomains on endosomes was recently proposed (Puthenveedu et al., 2010). In this model, slower diffusing cargo is kinetically excluded from transient recycling tubules used by bulk cargo such as transferrin, and the slower diffusing cargo is preferentially sorted into a subset of more stable recycling tubules that are maintained by dynamic actin. In support of this hypothesis, the diffusion rate of the β2-adrenergic receptor was measured to be significantly lower than the diffusion rate of transferrin (Puthenveedu et al., 2010). Moreover, the β2-adrenergic receptor was shown to rely more heavily on WASH-dependent recycling than transferrin receptor recycling (Puthenveedu et al., 2010). It will be interesting to learn if the kinetics of integrin diffusion on early endosomes also supports this model for integrin recycling. A second possibility is that actin assembly by WASH and Arp2/3 complex is responsible for membrane remodeling, such as tubule severing, that is required for trafficking beyond early endosomes (Derivery et al., 2009b; Duleh and Welch, 2010; Morel et al., 2009), similar to the role of dynamic actin in scission events at the plasma membrane in clathrin-mediated endocytosis (Kaksonen et al., 2006). Distinguishing between these two models will require live imaging studies that precisely measure the dynamics of WASH, actin, and cargo molecules on endosomal membranes.

Although we are beginning to understand the function of WASH in endocytic trafficking, cell adhesion, and cell migration, many questions remain unanswered. One question focuses on the regulation of WASH activity in cells. WASH is incorporated into a multiprotein complex (Derivery et al., 2009b; Gomez and Billadeau, 2009; Jia et al., 2010) that requires as yet undiscovered factors for regulation (Jia et al., 2010). In *Drosophila melanogaster*, WASH functions downstream of RhoI to control actin dynamics during oogenesis (Liu et al., 2009b), indicating that WASH may be subject to regulation by GTPases, in a manner similar to other NPFs. It will be exciting to learn if mammalian WASH is also subject to regulation by Rho, Rab or other GTPases, or by regulatory mechanisms such as phosphorylation. Another question relates to the role of other cytoskeletal elements in WASH-mediated trafficking. WASH interacts with tubulin (Gomez and Billadeau, 2009; Monfregola et al., 2010) and bundles microtubules (Liu et al., 2009b), suggesting that an interaction with microtubules is important for WASH-dependent trafficking. Future work will be aimed at understanding how WASH is regulated, and how its microtubule-binding and actin nucleating activities are coordinated to enable the trafficking of α5β1 integrin and other cargos that serve important functions in cell migration.

Experimental Procedures

Plasmids, Antibodies, and Cells

Full-length human WASH cDNA (accession BC048328; Open Biosystems, Lafayette, CO) was amplified by PCR, digested with KpnI and NotI and ligated into pEGFP-C1 (Clontech, Mountain View, CA), as described previously (Duleh and Welch, 2010). We refer to the plasmid as pGFP-WASH. To construct Lifeact-3xTagBFP, pTagBFP-N (Evrogen, Moscow, Russia) was digested with AvaI, blunted with Klenow fragment, then digested with NotI to generate BFP segment one. BFP segment two was generated by digestion of pTagBFP-N with HindIII followed by blunting, then BamHI digestion. BFP segments one and two were then ligated into the NotI/BamHI sites of pBluescript-Lifeact, to generate pBluescript-Lifeact-2xTagBFP. One of two HindIII sites in pBluescript-Lifeact-2xTagBFP was removed by HincII/EcoRV digestion followed by self-ligation. Segment one was introduced into HindIII-cut, blunted, NotI digested pBluescript-Lifeact-2xTagBFP to generate pBluescript-Lifeact-3xTagBFP. To express Lifeact-3xTagBFP under the control of the CMV IE1 promoter, Lifeact-3xTagBFP was digested with SacII and NotI and ligated into pEGFP-N1 (Clontech, Mountain View, CA) that was digested with SacII and NotI. We named this plasmid pLifeact-BFP. The plasmid encoding DsRed-Rab5 (Sharma et al., 2003) was obtained from Addgene (plasmid 13050), and the Q79L mutation (glutamine 79 replaced with leucine) was generated using the QuikChange II Site-Directed Mutagenesis Kit (Stratagene, Santa Clara, CA) following the manufacturer's protocol. This plasmid is referred to as pDsRed-Rab5-Q79L in the text. Restriction endonucleases were purchased from New England Biolabs (Ipswich, MA). All plasmids were maintained in XL-1 Blue *E. coli* (Stratagene, Santa Clara, CA).

Antibodies and staining reagents

Anti-WASH antibodies were described previously (Duleh and Welch, 2010). Antibody raised against mouse α5-integrin (CD49e) was purchased from BD Biosciences (Franklin Lakes, NJ). Anti-mouse vinculin antibody (hVIN-1) was purchased from Sigma-Aldrich (St. Louis, MO). Secondary antibodies conjugated to Alexa Fluor 488 and Alexa Fluor 568 (Invitrogen, Grand Island, NY) were used for immunofluorescence. Alexa Fluor 568 phalloidin (Invitrogen) was used for F-actin staining.

Cell growth and transfections

Mouse NIH3T3 cells and mouse embryonic fibroblasts were cultured in DMEM (Invitrogen) supplemented with 10% fetal bovine serum (FBS; Atlas Biologicals, Fort Collins, CO) at 37 °C with 5% CO_2 unless otherwise indicated. For live imaging of WASH, F-actin, and active Rab5 NIH3T3 cells were transfected with the following plasmids: pGFP-WASH (200 ng), pBFP-pLifeact-BFP (200 ng), and pDsRed-Rab5-Q79L (150 ng) in a 6-well plate with lipofectamine 2000 (Invitrogen) following the manufacturer's instructions. For WASH silencing, two siRNAs targeting WASH (J-054931-09 and J 054931-12) were purchased from Dharmacon (Lafayette, CO).

NIH3T3 cells or MEFs were transfected twice with 20 nM final concentration of either siRNA, once on day 1 when cells were approximately 40% confluent, and a second time 24 h later when cells were approximately 80% confluent. Knockdown cells were analyzed 72 h following the first siRNA transfection, and both WASH-specific siRNAs resulted in approximately 80% reduction in WASH protein at this time point.

Imaging

Imaging of WASH, F-actin and Rab5 in live cells was performed on a Nikon Ti Eclipse (Melville, NY) equipped with a Yokogawa CSU-XI spinning confocal disc (Tokyo, Japan). Confocal images were captured using a 100X (1.4 NA) Plan Apo objective and a Clara Interline CCD camera (Andor, Belfast, Northern Ireland). Total Internal Reflection Fluorescence (TIRF) and epifluorescence images of α5-integrin staining were collected using a 100X (1.49 NA) CFI Apo TIRF objective and an iXon X3 EMCCD camera (Andor, Belfast, Northern Ireland). MetaMorph v7.7.40 software (Molecular Devices, Sunnyvale, CA) was used to acquire digital images. Image processing was performed with ImageJ software (NIH, Bethesda, MD).

Deconvolution images of endogenous α5-integrin, vinculin, and F-actin were acquired on a DeltaVision 4 Spectris microscope (Applied Precision, Issaquah, WA) with 100X (1.4 NA) Plan Apo objective equipped with a CH350 CCD camera (Photometrics, Tucson, AZ). SoftWoRx v3.3.6 software (Applied Precision) was used to capture digital images. Images were deconvolved using Huygens Professional v3.1.0p0 software (Scientific Volume Imaging, Hilversum, The Netherlands). ImageJ was used to process raw images to 8 bit tiff files and quantify fluorescence intensity. JACoP (Bolte and Cordelieres, 2006) was used to quantify colocalization. CellProfiler Image Analysis Software (Carpenter et al., 2006) was used to quantify focal adhesion number and morphology.

Lamellipodia dynamics were imaged using an Olympus IX71 inverted microscope (Olympus, Tokyo, Japan) with a 100X (1.35 NA) Plan Apo equipped with a Photometrics Coolsnap HQ camera (Photometrics).

Integrin trafficking assays

For integrin trafficking experiments, NIH3T3 cells were serum starved for 2 h, and 10% serum was added for 0, 5, 15, 30, or 45 min. At each time point, cells were fixed in 2.5% paraformaldehyde and immunostained for α5-integrin. Epifluorescence images (to capture total integrin intensity) and TIRF images (to capture surface-associated integrin intensity) were acquired using identical exposure conditions for at least 10 cells in each of three independent experiments. Fluorescence intensity was quantified using ImageJ. For these experiments, Arp2/3 complex inhibitor (CK-666) and inactive control compound (CK-689) were obtained from EMD Chemicals (Darmstadt, Germany). Both compounds were dissolved in DMSO and used at a final concentration of 100 μM (Nolen et al., 2009).

Cell adhesion assays

Adhesion assays were performed using the Millipore ECM cell adhesion array kit (Billerica, MA) according to the manufacturer's protocol. Briefly, 72 h following the first siRNA transfection NIH3T3 cells were lifted with PBS + 4 mM EDTA. Cells were collected by centrifugation and resuspended at 1.5 x 10^6 cells/ml in DMEM. To each well of the ECM array plate, 100 µl of cell suspension (1.5 x 10^5 cells) was added. The plate was incubated at 37 °C with 5% CO_2 for 2 h. Wells were gently washed 3 times and stained with Cell Stain Solution (Millipore). Absorbance was measured at 560 nm to determine the amount of adherent cells in each well. The resulting data represent the mean of 3 independent experiments +/- s.d.

Analysis of cell migration and lamellipodia dynamics

For cell migration assays, mouse fibroblast cells transfected with siRNAs targeting WASH or non-specific siRNAs were grown to 100% confluence (48 h after the first siRNA transfection) in a 35 mm glass-bottom dish (MatTek Corporation, Ashland, MA). Each confluent cell layer was scratched with a 27 gauge needle to create a wound. Closure of the wound was monitored by brightfield microscopy with images acquired at 1 h intervals. ImageJ was used to calculate the percent wound closure. For analysis of lamellipodia dynamics, images were acquired every 5 s and kymography analysis was performed and lamellipodial dynamic parameters were calculated as described previously (Bear et al., 2002).

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CHAPTER 4

FUTURE AIMS

I. How is WASH regulated in cells?

Work over the last five years has implicated WASH in influencing the organization, morphology, and function of early and recycling endosomes (Derivery et al., 2009b; Duleh and Welch, 2010; Gomez and Billadeau, 2009). Moreover, several receptor proteins have been identified that rely on WASH for efficient trafficking through endosomes, including the transferrin receptor (Derivery et al., 2009b; Zech et al., 2011), the β2-adernergic receptor (Puthenveedu et al., 2010; Temkin et al., 2011), and the α5β1-integrin receptor (Chapter 3) (Zech et al., 2011). Functional defects in cellular adhesion, migration, and cancer cell invasion have been observed in WASH deficient cells resulting from disrupted trafficking of these receptors (Chapter 3) (Zech et al., 2011). While we have accumulated information about events downstream of WASH activity, the upstream factors that regulate WASH in cells are not well defined.

In mammalian cells WASH is found in a multiprotein complex containing FAM21, Strumpellin, SWIP, CCDC53, and possibly CAPZ (Derivery et al., 2009b; Gomez and Billadeau, 2009; Jia et al., 2010). While purified recombinant WASH is constitutively active towards the Arp2/3 complex (Duleh and Welch, 2010; Gomez and Billadeau, 2009; Jia et al., 2010), the intact WASH complex is inhibited (Gomez and Billadeau, 2009; Jia et al., 2010). These observations raise an obvious question. What factors regulate WASH activity in cells?

Studies in *Drosophila melanogaster* suggest that WASH functions downstream of RhoI (Liu et al., 2009b). It will be interesting to learn if Rho regulates the WASH complex in mammalian cells. It is also possible that WASH is regulated by other small GTPases that colocalize with WASH in cells such as Rab4, Rab5, or Rab11 (Derivery and Gautreau, 2010; Duleh and Welch, 2010; Zech et al., 2011). Dominant negative and constitutively active mutants of these GTPases can be used in biochemical experiments that assay WASH complex activity towards the Arp2/3 complex. These experiments will reveal if WASH activity is controlled by small GTPases, similar to WASP and WAVE family NPFs, or if WASH utilizes alternative regulatory mechanisms.

WASH activity may also be influenced by post-translational modifications such as phosphorylation. Moreover, other members of the WASH complex may be subject to phosphoregulation. Mass spectrometry analysis of the WASH complex could reveal phosphorylation sites on proteins in the core WASH complex. If phosphorylation sites are identified, non-phosphorylatable and phosphomimetic mutations of these sites can be generated. These mutant proteins can then be used to determine the effect of phosphorylation on WASH complex function.

Elucidating the mechanisms that regulate WASH activity will provide valuable insight into how WASH-dependent processes are directed and coordinated in cells. In addition to understanding WASH regulation, future studies should be directed to understand the mechanics of WASH function.

II. Understanding the mechanics of WASH activity on endosomal membranes

Our studies and work from several other groups have shown that WASH and the Arp2/3 complex mediate actin assembly on the surface of endosomes (Carnell et al., 2011; Derivery et al., 2009b; Duleh and Welch, 2010; Puthenveedu et al., 2010). These actin networks do not contribute to endosome motility, but interestingly, WASH-directed actin assembly controls the shape and organization of endosomal compartments (Derivery et al., 2009b; Duleh and Welch, 2010; Puthenveedu et al., 2010; Zech et al., 2011). However, the mechanism through which WASH activity influences endosome structure and organization is not defined.

One proposed model for WASH function is that WASH and the Arp2/3 complex stimulate actin dynamics that stabilize a subset of endosomal recycling tubules (Puthenveedu et al., 2010; Temkin et al., 2011). In this model, slowly diffusing receptors are kinetically excluded from transient bulk recycling tubules. Actin dynamics confer stability to a subset of longer-lived tubules thus allowing the slowly diffusing cargo to overcome the kinetic barrier and accumulate in these tubules. Puthenveedu and colleagues demonstrate that the slowly diffusing β2-adernergic receptor accumulates in a subset of actin stabilized tubules, while the more dynamic transferrin receptor utilizes transient recycling tubules not associated with actin filaments (Puthenveedu et al., 2010). Precise measurements of protein dynamics on endosomal membranes are required to confirm if this model applies to other cargo that rely on WASH activity for efficient trafficking. How dynamic actin structures stabilize these tubules and whether actin plays a more active role in sorting these receptors remain open questions.

A second model was proposed by Derivery and colleagues based on the observation that in WASH-silenced cells there is excess tubulation of transferrin containing endosomes, suggesting that scission of these tubules is impaired in WASH deficient cells (Derivery et al., 2009b). Moreover, dynamin inhibition also increased tubulation of the these transferrin loaded compartments, indicating that both WASH and dynamin contribute to endosome scission (Derivery et al., 2009b). Consistent with this model that WASH influences endosome scission, we observed fewer and larger WASH compartments in cells where actin assembly was disrupted (Duleh and Welch, 2010). However, we have only begun to identify the factors that cooperate with WASH to reorganize endosomal membranes, and the mechanics responsible for these membrane dynamics are not yet defined.

Looking at the further characterized function of actin assembly in other dynamic membrane reorganizing processes such as clathrin-mediated endocytosis will provide insight and direction for investigating the role of WASH and actin polymerization on endocytic compartments and guide us to develop a more sophisticated model of WASH activity. In yeast, actin polymerization occurs at endocytic sites during vesicle budding from the plasma membrane (Kaksonen et al., 2005; Newpher et al., 2005). This short burst of actin assembly can be broken down into two phases. During the initial phase

motility is restricted. However, the second phase exhibits rapid motility followed by actin disassembly (Kaksonen et al., 2003). The observation that actin polymerization reaches its maximal level just before the rapid motility phase suggest that actin dynamics contribute to scission of these clathrin-coated pits (Kaksonen et al., 2006). Kaksonen and colleagues proposed a model for the mechanics of this actin-driven internalization where Las17, a yeast NPF, stimulates assembly of an actin cone surrounding a clathrin-coated invagination. This actin network is linked to the endocytic coat so that further actin assembly pulls the clathrin-coated pit away from the plasma membrane (Kaksonen et al., 2006). Type-I myosin activity also likely contributes to this process by pushing the actin network away from the membrane or stimulating additional actin assembly (Kaksonen et al., 2006). The timing of actin assembly during clathrin-mediated endocytosis events in mammalian cells is similar to that described above (Kaksonen et al., 2006; Taylor et al., 2011). However, actin is not strictly required for clathrin-mediated endocytosis in mammalian cells (Galletta et al., 2010). Instead, dynamin plays an important role in vesicle scission downstream of actin assembly (Ferguson et al., 2009). In this related model, BAR proteins and actin filaments support growth of the tubular necks of clathrin-coated pits. Subsequently, dynamin arrives and terminates actin assembly leading to vesicle scission (Ferguson et al., 2009).

These studies of clathrin-mediated endocytosis provide valuable insight into the role of actin in membrane trafficking events. Future work should be directed to see if BAR proteins or other membrane shaping machinery cooperate with actin dynamics on WASH-positive endosomes. Moreover, defining the spatial and temporal relationship of WASH, actin assembly, and other factors such as dynamin, on these endocytic compartments will be valuable for understanding the mechanics of WASH-dependent trafficking.

TIRF microscopy proved an important tool for researchers studying clathrin-mediated endocytosis. These groups generated fluorescent fusions of proteins involved in endocytosis and used TIRF microscopy to elucidate the sequence and timing that proteins arrived at endocytic sites on the plasma membrane (Kaksonen et al., 2003; Merrifield et al., 2002; Perrais and Merrifield, 2005). Additionally, TIRF microsocopy provides excellent resolution in the Z direction. Movement of proteins away from the plasma membrane can be followed by calculating the ratio of TIRF signal to epifluorescence signal during endocytosis. Unfortunately, WASH vesicles are localized on intracellular membranes well beyond the reach of TIRF microscopy. One potential solution is to artificially target WASH to the plasma membrane of cells and observe the spatial and temporal relationship of WASH and other fluorescently tagged proteins on the plasma membrane. This technique would facilitate characterization of WASH activity on membranes using powerful tools developed for characterizing plasma membrane dynamics.

How can we target WASH to the plasma membrane in a regulated manner? Toettcher and colleagues have provided a possible solution to this problem by developing the Phy-PIF system which facilitates control of plasma membrane recruitment using red and infrared light (Toettcher et al., 2011). The Phy-PIF system

takes advantage of two genetically encoded phytochrome components from *Arabidopsis thaliana*. In the presence of a small molecule phycocyanobilin, Phy-PIF association becomes light-responsive. Phy can be induced to interact with PIF upon exposure to 650 nm light. Additionally, dissociation between Phy-PIF results from exposure to 750 nm light (Levskaya et al., 2009). This system provides a non-toxic reversible switch for plasma membrane recruitment of proteins in live cells (Toettcher et al., 2011). The Phy-PIF system could be used to tightly control the recruitment of WASH to the plasma membrane. Experiments modeled after those used for characterizing clathrin-mediated endocytosis (Kaksonen et al., 2003; Merrifield et al., 2002) can then be used to define the temporal relationship between WASH, the Arp2/3 complex, actin assembly, and other membrane shaping proteins. Moreover, high resolution microscopy may reveal if WASH does indeed influence membrane tubulation and/or scission.

Of course there are several caveats to this system. The distinct membrane composition, curvature, and tension of the plasma membrane and intracellular endosomes may prevent these experiments from recapitulating WASH-mediated membrane organizing dynamics at the plasma membrane. Furthermore, adapter proteins upstream of WASH may be required to reorganize cellular membranes. However, the observation that WASH interacts directly with membranes, either alone (Carnell et al., 2011) or through FAM21, a member of the WASH complex (Carnell et al., 2011; Gomez and Billadeau, 2009; Jia et al., 2010), suggests that WASH recruitment to membranes may be sufficient to recruit additional factors including the Arp2/3 complex. Thus, these studies may provide valuable information about the temporal relationship and mechanics of WASH and other factors involved in organizing endosomal membranes.

Efforts should also be focused on biochemical experiments to characterize the activity of WASH and other factors that regulate endosome structure. In these experiments, purified endosomes or reconstituted membranes may be used in cellular extracts to recapitulate endosomal membrane dynamics in vitro. Steps have already been made to construct a functional system of membranes that can recapitulate aspects of cellular membrane dynamics (Liu and Fletcher, 2009). One of these studies demonstrated vesicle docking and membrane mixing, emulating certain aspects of exocytosis (Richmond et al., 2011). Further development of these systems will allow researchers to investigate events on endosome or endosome-like membranes in the absence of other confounding cellular structures. The ultimate goal of this work should be to identify factors involved in endosome membrane organization, thus leading to reconstitution of endosome sorting and trafficking events from purified components.

In vitro reconstitution of these cellular processes will allow precise measurements of the dynamics and forces involved in shaping endosomal membranes. These data can then be used to construct mathematical models based on experimental data and physics that describe the specific contributions of proteins, lipids, and other factors involved in cargo sorting and trafficking at endocytic compartments. These theoretical models will not only describe the mechanics driving membrane reorganization, but importantly, the models will provide predictions to direct future experimentation. Results

from these experiments will then be used to revise the theoretical models. It is this coevolution of experimentation and theoretical modeling that is required to push the field forward and truly understand the process of sorting and trafficking cargo within the cell. Efforts to understand endocytic vesicle formation by combining modeling and experimentation have proven very successful (Liu et al., 2006; Liu et al., 2009a; Liu et al., 2010). As experimental progress is made to understand the factors that cooperate with WASH during endocytic trafficking, modeling will provide mechanistic insight into the physical properties that underlie this process.

III. Future perspectives

The more complete understanding of trafficking events attained from this work will provide the foundation of knowledge required to intervene when cellular trafficking goes awry. WASH has been implicated in recycling integrin receptors that are important for cell migration, as well as cancer cell invasion (Chapter 3) (Zech et al., 2011). Therefore, elucidating the regulatory factors and mechanics that govern WASH function during integrin trafficking may reveal therapeutic targets to impede cancer metastasis.

Additionally, proper nervous system function relies on the efficiency of internalization and recycling of cargo at synapses. Intriguingly, mutations in a member of the WASH complex termed Strumpellin has been linked to hereditary spastic paraplegia (Clemen et al., 2010) suggesting that misregulation of WASH activity may contribute to the pathogenesis of this debilitating neurological disorder. Detailed characterization of WASH complex activity will guide development of therapeutics to treat disorders like spastic paraplegia and potentially other diseases influenced by misregulated intracellular trafficking.

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