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

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Abstract

WASH is a nucleation-promoting factor for the Arp2/3 complex that is implicated in multiple endocytic trafficking pathways including receptor recycling, cargo degradation, and retromer-mediated receptor retrieval. We sought to examine whether WASH plays an important role in trafficking of specialized cargo molecules such as integrins, for which trafficking is highly regulated during cell migration. We observed that subdomains of early/sorting endosomes associated with dynamic WASH and filamentous actin, and $\alpha 5$ -integrins trafficked through this population of endosomes. Depletion of WASH caused accumulation of $\alpha 5$ -integrins in intracellular compartments, reduction of $\alpha 5$ -integrin localization at focal adhesions, and reduction in focal adhesion number. Transport of $\alpha 5$ -integrins from internal endocytic structures to focal adhesions was disrupted upon WASH depletion or Arp2/3 complex inhibition. Furthermore, WASH-depleted cells displayed greatly reduced affinity for specific ECM proteins including fibronectin, and impaired cell spreading ability. Interestingly, the reduced adhesion capacity of WASH-depleted cells resulted in their migrating more rapidly than control cells in wound healing assays. Our results define a requirement for WASH, Arp2/3 complex, and actin in specialized trafficking of integrins. These findings highlight a role for actin dynamics in 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.

Keywords

actin; endocytic trafficking; integrins; WASH; 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 [Pellegrin and Mellor, 2007]. 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.

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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., 2009; 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 β 2 adrenergic receptors, retromer-mediated trafficking of CI-MPR to the trans-Golgi network, and EGF transport to late endosomes [Derivery et al., 2009; 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 via 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 α 5 β 1 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 α 5 β 1 integrin is internalized by clathrin-dependent [Pellinen et al., 2008] and caveolar-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 α 5 β 1 integrin.

Here we show that actin nucleation by WASH and the Arp2/3 complex plays an important role in transport of α 5-integrin from intracellular compartments to ventral adhesive structures in fibroblast cells. Furthermore, WASH is crucial for maintaining focal adhesion number, promoting adherence to specific ECM proteins, and enabling efficient cell spreading. 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.

Materials and Methods

Plasmids

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 $\alpha 5$ -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% CO₂ 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), Lifeact-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 (Figure S1).

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 $\alpha 5$ -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 $\alpha 5$ -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 Cordelières, 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).

FRAP experiments were performed on a Nikon Ti Eclipse microscope equipped with a Yokogawa CSU-XI spinning confocal disc, as detailed above. NIH3T3 cells transfected with $\alpha 5$ -integrin-GFP or paxillin-GFP were plated onto 35 mm MatTek dishes coated with 20 μ g/ml fibronectin and were imaged 24 h later. Five prebleach events were acquired before a single 200 ms pulse with a 405 nm laser of a single focal adhesion or an equivalent area of non-focal adhesion associated $\alpha 5$ -integrin-GFP. Fluorescence recovery was followed at 3 s intervals until recovery reached a steady plateau. ImageJ FRAP Profiler was used to calculate the half-time for fluorescence recovery and to quantify the mobile fraction for each fluorescent protein. Each experiment was carried out on at least five cells for a minimum of three independent trials.

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 $\alpha 5$ -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 μ M [Nolen et al., 2009].

Cell adhesion and spreading 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 1.5×10^6 cells/ml in DMEM. To each well of the ECM array plate, 100 μ l of cell suspension (1.5×10^5 cells) was added. The plate was incubated at 37 °C with 5% CO₂ for 1 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 $\pm$ s.d.

Cell spreading assays were performed as previously described [Berrier and LaFlamme, 2005]. Briefly, suspended NIH3T3 cells were plated on glass coverslips coated with 0, 2, 20, 200 μ g/ml fibronectin for 45 min. Adherent cells were fixed, processed, and imaged as described above for immunofluorescence microscopy. Relative areas of individual cells were quantified using CellProfiler image analysis software [Carpenter et al., 2006].

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].

Results

WASH and F-actin colocalize on subdomains of endosomes that contain $\alpha 5$ -integrin

Previous observations indicate that WASH and F-actin localize to subdomains of early and recycling endosomes [Derivery et al., 2009; 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 (Fig. 1A,B). Moreover, live cell imaging revealed dynamic F-actin polymerization at endosomal WASH subdomains (Movie 1 and Movie 2, Supporting Information). Thus, WASH frequently colocalizes with dynamic F-actin on subdomains of early endosomes.

To determine if $\alpha 5$ -integrin is trafficked through these same enlarged endosomes, cells transfected with pDsRed-Rab5-Q79L and pLifeact-BFP were stained for $\alpha 5$ -integrin by immunofluorescence (Fig. 1C,D). Although $\alpha 5$ -integrin was present in many enlarged endosomes, its localization only partially overlapped with domains containing F-actin (Fig. 1C,D). Moreover, much of the $\alpha 5$ -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 $\alpha 5$ -integrin traffics through compartments that contain WASH and dynamic F-actin.

WASH is important for $\alpha 5$ -integrin localization to focal adhesions and for maintaining focal adhesion number

The observation that $\alpha 5$ -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 $\alpha 5$ -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 (Fig. S1, Supporting Information). In cells treated with the control siRNA, $\alpha 5$ -integrin was primarily localized to focal adhesions (Fig. 2A–C). In contrast, WASH-silenced cells exhibited aberrant $\alpha 5$ -integrin localization in large internal punctae (Fig. 2A) and significantly less $\alpha 5$ -integrin colocalized with vinculin-positive focal

adhesions (Fig. 2B,C). Interestingly, WASH deficient cells also displayed significantly fewer focal adhesions than control cells (Fig. 2D). These data implicate WASH in regulating $\alpha 5$ -integrin localization to adhesive structures, and in controlling the overall number of focal adhesions in fibroblast cells.

We also measured the dynamic behavior of $\alpha 5$ -integrin-GFP in focal adhesions and internal structures, as well as the dynamics of the focal adhesion protein α -actinin-GFP, using fluorescence recovery after photobleaching (FRAP). However, we did not detect significant differences in the mobility of these proteins in WASH-silenced cells compared to cells treated with non-targeting siRNA (Fig. S2, Supporting Information). This suggests that WASH depletion affects focal adhesion number, but not necessarily the dynamic behavior of focal adhesion proteins.

WASH and the Arp2/3 complex play a role in $\alpha 5$ -integrin transport to adhesion sites

Next, we investigated whether WASH and its downstream effector the Arp2/3 complex play a role in $\alpha 5$ -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 $\alpha 5$ -integrin at adhesion sites, and epifluorescence microscopy to measure the total cellular $\alpha 5$ -integrin. The ratio of TIRF intensity to epifluorescence intensity (TIRF:EPI ratio), which is a measure of the fraction of cellular $\alpha 5$ -integrin at adhesion sites, was followed over time to assay integrin trafficking to ventral adhesive structures (Fig. 3). Cells silenced for WASH (with either of two siRNAs) exhibited an approximately 25% reduction in the TIRF:EPI ratio compared with cells treated with the non-targeting siRNA in the presence or absence of serum and at all time points (Fig. 3A–C). To investigate the role of the Arp2/3 complex in $\alpha 5$ -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 also exhibited a significantly reduced proportion of $\alpha 5$ -integrin at adhesive sites compared with cells treated with the inactive control drug CK689 (Fig. 3D). The effect was apparent at all time points and in the presence or absence of serum. The fact that inhibiting WASH or the Arp2/3 complex caused a reduction in the steady state ratio of $\alpha 5$ -integrin at adhesive sites versus internal stores, but did not affect the rate of accumulation at adhesive sites following serum stimulation, suggests that they are important for the efficiency but not the rate of transport of $\alpha 5$ -integrin to these sites.

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 $\alpha 5$ -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 (Fig. 4). Interestingly, the affinity for fibronectin was reduced by 40% in WASH-silenced cells compared to control cells (Fig. 4). Moreover, adherence to laminin and vitronectin was decreased by 15–20% in cells treated with WASH siRNAs (Fig. 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 $\alpha 5$ -integrin part of the $\alpha 5\beta 1$ integrin heterodimer that is the primary fibronectin-binding integrin in these cells.

WASH influences cell spreading on intermediate concentrations of fibronectin

To gain further insight into the role of WASH in cell adhesion, we observed the ability of cells to spread on various concentrations of fibronectin, a process that relies on the formation of multiple adhesive contacts as cells flatten onto the substrate. On glass alone or glass coated with low concentrations of fibronectin (2 $\mu\text{g/ml}$), no defect in cell spreading was detected (Fig. 5). In contrast, at an intermediate concentration of fibronectin (20 $\mu\text{g/ml}$), WASH-silenced cells spread significantly less than cells treated with non-targeting siRNAs (Fig. 5A). The effect of WASH depletion was rescued by plating cells on high concentrations of fibronectin (200 $\mu\text{g/ml}$). Additionally, WASH-deficient cells accumulated $\alpha 5$ -integrin in the perinuclear region as compared with control cells, which displayed more peripherally-distributed $\alpha 5$ -integrin (Fig. 5B,C). The cell spreading phenotype establishes a link between the roles of WASH in integrin localization/trafficking and cell adhesion.

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 (Fig. 6). 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 (Fig. S3, Supporting Information). Therefore, we propose that WASH influences cell migration by regulating cell adhesion, and not the protrusion functions of lamellipodia.

Discussion

The molecular pathways that connect actin function in cell migration and membrane trafficking remain poorly understood. Here, we implicate the Arp2/3 NPF WASH in cell adhesion and migration via its role in the endocytic trafficking of $\alpha 5$ -integrin. We find that WASH and F-actin form dynamic subdomains on endosomes that contain $\alpha 5$ -integrin, and that WASH is important for trafficking $\alpha 5$ -integrin to ventral adhesive structures. WASH is also important for maintaining focal adhesion number, enabling adhesion to specific ECM proteins including fibronectin, and cell spreading. 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., 2009; Duleh and Welch, 2010; Gomez and Billadeau, 2009; Puthenveedu et al., 2010]. Here we show that WASH 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 $\beta 2$ adrenergic receptors to the plasma membrane [Derivery et al., 2009; 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 $\alpha 5$ -integrin. In WASH-silenced cells we observed an accumulation of $\alpha 5$ -integrin in perinuclear compartments, suggesting a role for WASH in $\alpha 5$ -integrin trafficking. Consistent with this finding, the increase in perinuclear $\alpha 5$ -integrin is accompanied by a concomitant decrease in $\alpha 5$ -integrin at vinculin-positive adhesions. However, the overall $\alpha 5$ -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 $\alpha 5$ -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., 2009; Duleh and Welch, 2010; Zech et al., 2011], suggesting a role for WASH during $\alpha 5$ -integrin recycling to the plasma membrane via Rab4- and/or Rab11-dependent mechanisms.

In addition to reduced levels of $\alpha 5$ -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 $\alpha 5$ -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 $\alpha 5\beta 1$ 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 $\beta 1$ -integrin, such as $\alpha 3\beta 1$, $\alpha 6\beta 1$, and $\alpha 7\beta 1$, which bind to laminins, and $\alpha 8\beta 1$, 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 $\alpha 5\beta 1$ -integrin and fewer focal adhesions at the cell-substrate interface in WASH-silenced cells account for their decreased affinity for ECM proteins. In support of this hypothesis, we found that cell spreading on an intermediate concentration of fibronectin was impaired in WASH-silenced cells. Importantly, this spreading phenotype was rescued at high concentrations of fibronectin. These data suggest that WASH influences cell spreading when high levels of $\alpha 5\beta 1$ -integrin are required to efficiently adhere to the substratum. However, at very low or high concentrations of fibronectin cells are not as reliant on $\alpha 5\beta 1$ -integrin for adherence, and therefore, WASH is not required for efficient spreading under these conditions.

Integrin trafficking and the establishment of adhesive contacts provides a 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, there is 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 agreement with previous results with regard to the role of WASH and Arp2/3 complex activity in $\alpha 5 \beta 1$ integrin recycling [Zech et al., 2011]. Furthermore, we contribute several new findings, including identifying roles for WASH in the regulation of focal adhesion number, the strength of adhesion to specific ECM molecules, and the process of cell spreading 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 $\beta 2$ -adrenergic receptor was measured to be significantly lower than the diffusion rate of transferrin [Puthenveedu et al., 2010]. Moreover, the $\beta 2$ -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., 2009; 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., 2009; 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., 2009], 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., 2009], 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 $\alpha 5\beta 1$ integrin and other cargos that serve important functions in cell migration.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. WASH and F-actin colocalize on subdomains of enlarged endosomes containing $\alpha 5$ -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 $\alpha 5$ -integrin (green) by immunofluorescence. (D) Enlarged insets from (C). Scale bars, 10 μ m. See also supplementary Movies 1 and 2.

Figure 2. WASH depletion disrupts $\alpha 5$ -integrin localization to focal adhesions and decreases focal adhesion number

(A) $\alpha 5$ -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) $\alpha 5$ -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 $\alpha 5$ -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 μ m.

Figure 3. $\alpha 5$ -integrin transport to adhesion sites is disrupted in WASH-silenced cells
 (A) Epifluorescence images of $\alpha 5$ -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 $\alpha 5$ -integrin transport to the ventral surface of the cells following serum treatment in siRNA- or drug-treated cells. Data represent the mean TIRF:Epi fluorescence 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. Asterisks (*) indicate a p-value < 0.05 as determined by a Student's t-test. Scale bar, 10 μ m.

Figure 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. Single asterisk (*) indicates a p-value < 0.05 and two asterisks (**) indicate a p-value < 0.001 as determined by a Student's t-test.

Figure 5. Cell spreading on intermediate concentrations of fibronectin is disrupted in WASH-depleted fibroblast cells

(A) Spread cell area (μm^2) at the indicated concentrations of fibronectin. At least 30 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. **(B)** Cells treated with siRNA targeting WASH (siWASH 1; right) exhibited perinuclear accumulation of $\alpha 5$ -integrin compared with peripherally-located $\alpha 5$ -integrin in cells treated with control siRNA (siNS; left). Scale bar, 10 μm .

Figure 6. 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. Scale bar, 10 μ m. Asterisk (*) indicates a p-value < 0.05 by a Student's t-test.