Actin polymerization driven by WASH causes V-ATPase retrieval and vesicle neutralization before exocytosis

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Introduction

Arp2/3 and nucleators

The Arp2/3 complex is normally inactive, but when it binds to nucleation-promoting factors (NPFs; Pollard, 2007), it becomes able to start new, growing actin filaments from the side of existing ones. Different NPFs initiate actin filaments in different locations. A related process, the expulsion of the lethal endosomal pathogen Cryptococcus neoformans from mammalian macrophages, also uses WASH-coated vesicles, and cells expressing dominant negative WASH mutants inefficiently expel C. neoformans. D. discoideum WASH causes filamentous actin (F-actin) patches to form on lysosomes, leading to the removal of vacuolar adenosine triphosphatase (V-ATPase) and the neutralization of lysosomes to form postlysosomes. Without WASH, no patches or coats are formed, neutral postlysosomes are not seen, and indigestible material such as dextran is not exocytosed. Similar results occur when actin polymerization is blocked with latrunculin. V-ATPases are known to bind avidly to F-actin. Our data imply a new mechanism, actin-mediated sorting, in which WASH and the Arp2/3 complex polymerize actin on vesicles to drive the separation and recycling of proteins such as the V-ATPase.

WASH and SCAR homologue (WASH) is a recently identified and evolutionarily conserved regulator of actin polymerization. In this paper, we show that WASH coats mature Dicyostelium discoideum lysosomes and is essential for exocytosis of indigestible material. A related process, the expulsion of the lethal endosomal pathogen Cryptococcus neoformans from mammalian macrophages, also uses WASH-coated vesicles, and cells expressing dominant negative WASH mutants inefficiently expel C. neoformans. D. discoideum WASH causes filamentous actin (F-actin) patches to form on lysosomes, leading to the removal of vacuolar adenosine triphosphatase (V-ATPase) and the neutralization of lysosomes to form postlysosomes. Without WASH, no patches or coats are formed, neutral postlysosomes are not seen, and indigestible material such as dextran is not exocytosed. Similar results occur when actin polymerization is blocked with latrunculin. V-ATPases are known to bind avidly to F-actin. Our data imply a new mechanism, actin-mediated sorting, in which WASH and the Arp2/3 complex polymerize actin on vesicles to drive the separation and recycling of proteins such as the V-ATPase.

Supplemental Material can be found at:
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It binds directly to the retromer complex, associating it with retrograde flow from endosomes to the trans-Golgi network. RNAi of WASH in mammalian cells causes a hard to interpret phenotype involving the dispersal of endosomes containing the mannose 6-phosphate receptor and the tubulation of endosomal structures. The FAM21 complex subunit also associates with capping protein in mammals, suggesting a complex role in actin dynamics (Hernandez-Valladares et al., 2010).

**Actin in Dictyostelium discoideum exocytosis**

In this paper, we discuss the role of WASH in generating F-actin coats in *D. discoideum* lysosome maturation. *D. discoideum* growing in axenic medium have a well-defined endocytic cycle using most of the components seen in mammalian cells but with a far more rapid transit time. One difference is in lysosome maturation. Wild-type *D. discoideum* grows by phagocytosing bacteria; indigestible or inedible material is constitutively exocytosed between 90 and 120 min after endocytosis (Clarke et al., 2010). This process involves a small proportion of total vesicular traffic; most liquid is extracted from vesicles early in the cycle, and most membrane is recycled between the endosomal and lysosomal stages. Constitutive exocytosis is rarely seen in mammalian cells, presumably because of a combination of the lower rate of flux through the endolysosomal system and a greater range of retrograde pathways.

F-actin is polymerized on vesicles at two stages of the pathway. First, a fairly long-lived coat is seen between the lysosomal and postlysosomal stages (Drengk et al., 2003). A second phase of actin polymerization is seen immediately before neutralized vesicles are exocytosed (Clarke et al., 2010). By comparison, this is short lived, involving multiple dynamic flashes of F-actin, and only occurs in the 2–4 min before expulsion. This process is thought to position postlysosomes for exocytosis. In this manuscript, we show that WASH is essential for the first phase of actin polymerization during lysosome maturation and that this actin is essential for the neutralization and recycling of the vacuolar ATPase (V-ATPase).

**Results and discussion**

The *D. discoideum* WASH is well conserved, containing all the domains seen in human and *D. melanogaster* (Fig. 1 a), and, like WASP and SCAR, is encoded by a single gene. We generated several disruptants of the gene (*wshA*) in an AX2 background, giving identical phenotypes. *wshA* mutants grew at near-normal rates in liquid medium, though we observed slower growth on bacteria (Fig. S1 a). They also showed no defects in cell migration, moving, if anything, faster than parental cells (Fig. S1 b). When *wshA* cells were fixed and stained with phallolidin, there were also no visible changes in pseudopods or filopods at the leading edge. However, the large F-actin–coated intracellular vesicles seen in most normal cells were entirely absent from *wshA* cells (Fig. 1 b). These do not colocalize with WASP or SCAR and are distinct from early endocytic actin structures such as phagosomes and macropinosomes, which are seen at normal levels. They have been previously observed using GFP fused to the actin-binding proteins ABP120 (Lee and Knecht, 2002) and coronin (Drengk et al., 2003) some time before indigestible material is exocytosed (Rauchenberger et al., 1997). These vesicles are present in >95% of normal cells but completely lost from *wshA* cells (Fig. 1 c).

Expression of a GFP-WASH fusion in *wshA* cells fully rescues the loss of these actin-coated vesicles (Fig. 1 d). The GFP-WASH and actin coats localize to the same vesicles (Fig. 1 d, green and red, respectively), suggesting that WASH directly induces the actin. We confirmed this using GFP-WASH in which the Arp2/3- and the actin-binding VCA domains have been deleted (Fig. 1 e, GFP-WASHΔVCA). This localizes normally to the large vesicles but does not rescue the actin coats, implying that the actin coats are directly generated by WASH acting as an NPF for the Arp2/3 complex.

**Exocytosis is blocked in WASH mutants**

We measured the rate of fluid phase uptake in *wshA* cells using fluorescent dextran (Fig. 2 a). The initial rate of endocytosis was unaltered, although after incubations of >1 h, the *wshA* cells accumulated significantly more dextran than the parent (Fig. 2 a, inset). Dextran is indigestible, and like the remains of food phagosomes (Clarke et al., 2002), it is normally exocytosed ~1–2 h after passing through the endocytic and lysosomal pathways (Maniak, 2001; Neuhaus et al., 2002), leading to a plateau in accumulation. Therefore, this implies a defect in exocytosis rather than during earlier traffic. Thus, we measured the rate at which fluorescent dextran was expelled after loading. As shown in Fig. 2 b, whereas wild-type cells expelled nearly everything within 2 h, *wshA* cells were completely unable to exocytose dextran (Fig. 2 b). Again, GFP-WASH rescued exocytosis near perfectly but had no effect when missing its VCA domain (Fig. 2 c). Thus, WASH-stimulated actin polymerization is essential for exocytosis. This has serious consequences to cells that encounter indigestible material (including, for example, bacterial cell walls; Clarke et al., 2002), presumably explaining the growth defect on bacteria. When cells were grown in 20% of 60-kD dextran, which barely affects normal cells, *wshA* cells became extremely distended with large fluid-filled vesicles (Fig. 2 d) and divided far more slowly (Fig. 2 e).

We loaded cells with BSA 15-nm colloidal gold (Neuhaus et al., 2002), washed them for 2 h, and examined them by transmission EM (Fig. 2 f). Few wild-type cells contained BSA-containing vacuoles; those few were usually single, contained few gold particles, and were often empty or filled with low-density material (Fig. S1 c). However, 70% of the *wshA* cells contained BSA-gold (Fig. 2 g), frequently at a high density and in densely stained vesicles. Single lysosomes often contained compacted deposits from several different endosomes, and fusion events were occasionally seen (Fig. 2 g), suggesting that the *wshA* lysosomes are a dynamic “trash bin” containing the indigestible remains of multiple endosomes.

**WASH in expulsion of Cryptococcus neoformans from macrophages**

Constitutive exocytosis of endocytosed material is rare in mammals. To test the generality of this pathway, therefore,
in expulsion (Fig. 2 i). Thus, WASH activity is important for
*C. neoformans* expulsion in macrophages as well as *D. discoi-
decum* postlysosomal exocytosis.

WASH- and actin-driven lysosome neutralization

When cells are loaded with a mixture of FITC- and TRITC-labeled
dextran, neutral vesicles show yellow, but acidic vesicles are red
due to FITC losing fluorescence (Jenne et al., 1998). Most intra-
cellular vesicles are acidic, but they neutralize as they mature to
postlysosomes (Fig. 3 a, arrows). In *wshA* cells, neutral post-
lysosomes were never seen. Again, this phenotype was fully
rescued by GFP-WASH but not GFP-WASH\*VCA (Fig. 3 d).
This suggests that *wshA* cells cannot exocytose dextran be-
cause lysosomes are never able to neutralize and thus do not

Figure 1. WASH localizes to postlysosomes
and is required for postlysosomal actin coats.
(a) Domain architecture of WASP subfamilies.
(b) Deconvolved widefield images of parent
(AX2) and WASH-null (*wshA*-) cell lines fixed
and stained with phalloidin. WASH-null cells
lack large F-actin-coated vesicles (arrows in
AX2). (c) Loss of actin-coated vesicles. Parental
(*n*= 82) and *wshA* (*n*= 79) cells were fixed
and stained with phalloidin. Vesicles were
counted in all planes of focus; macropino-
somes were excluded. Error bars represent
SEM. (d) Expression of GFP-WASH (middle
panel and green) in fixed, phalloidin-stained
(left panel and red) *wshA* cells. (e) Expres-
sion of GFP-WASH\*VCA (green) in fixed,
phalloidin-stained (left panel and red) *wshA*
cells. Bars, 10 µm.
Figure 2. **WASH is required for exocytosis.** (a) Parental AX2 (○) and wshA− (●) cells were incubated with FITC-dextran, and uptake was measured fluorimetrically. The inset shows a longer time course of the same data. (b and c) Parental AX2 (○), wshA− cells (●), and wshA− cells expressing GFP-WASH (∆) and GFP-WASHΔVCA (▲) were loaded with FITC-dextran for 2 h and then washed. Expulsion of dextran was measured fluorimetrically. (d) Cells were grown for five generations in medium with 20% dextran. wshA− cells, but not the parental strain, grew large and accumulated multiple dense vesicles. Bars, 5 µm. (e) Cells were grown for five generations in medium with and without 20% dextran. wshA− growth is greatly slowed by the presence of indigestible dextran. (f and g) Transmission electron micrographs of parental AX2 (f) and wshA− (g) cells incubated overnight with BSA–colloidal gold and chased for 2 h. Cells were examined as described in Hagedorn et al. (2009). Arrowheads in g indicate vesicular structures containing gold particles. Bars, 2 µm. (h) *C. neoformans*–containing vesicles in macrophages are also coated in WASH. Cultured J774 cells (left) were allowed to phagocytose *C. neoformans* and were then fixed with 4% formaldehyde and stained with anti-WASH (middle) and phalloidin (right). Bar, 10 µm. (i) Impairment of *C. neoformans* exocytosis caused by WASHΔVCA expression. Cells were transfected with full-length WASH or WASHΔVCA, incubated with *C. neoformans* for 2 h, and then observed for 24 h. The difference is significant to P = 0.05 (Fisher’s exact test). Error bars in each case show the SD. n = 3 in each case in a–c and e.
loading, but this was still sufficient to block vesicle neutralization completely (Fig. 3 b and Video 2). The effect was rapidly reversible; washing away latrunculin allowed neutralization to restart within a few minutes. Thus, actin polymerization is essential for vesicle neutralization.

WASH and V-ATPase recycling

The V-ATPase, which consists of a membrane channel (V0) and a cytoplasmic ATP-hydrolyzing proton pump (V1; Jefferies et al., 2008), mediates vesicle acidification. When it is removed, vesicles neutralize through proton leaks. It has been known for several years that subunits of V1 have a surprisingly high affinity for actin. Each V1 contains three B subunits, which have a sub-micromolar affinity for F-actin (Holliday et al., 2000), and the single C subunit also binds actin (Vitavska et al., 2003). Together, these imply an extremely high avidity for F-actin. We hypothesized that the WASH-derived F-actin coat on lysosomes was neutralizing them by binding and removing the V-ATPase. This was supported by coimmunoprecipitation from detergent lysates (Fig. S3). VatM and VatB subunits (from V0 and V1, respectively) were identified with 100% confidence from GFP-WASH pull-downs but not from GFP alone (Fig. S3). Thus, WASH can physically associate with V-ATPase either directly or as part of a larger protein assembly. However, our data suggest that this interaction results in V-ATPase recycling and is thus short lived.

We simultaneously imaged VatB-RFP and GFP-WASH in cells that had been fed 2-µm indigestible agarose beads, which made lysosomes large enough that they could be imaged continuously. As seen in Fig. 4 a and Video 3, the V1 subunit began disappearing from the lysosome as soon as puncta of GFP-WASH appeared. Quantitation shows V-ATPase levels start to drop within 1 or 2 min of the arrival of WASH (Fig. 4 b). Unexpectedly, however, WASH was only present in puncta, whereas the V-ATPase was removed; all detectable V-ATPase had gone well before formation of the contiguous coat of WASH seen on postlysosomes.

In yeast, V-ATPase is inactivated by dissociation of V1 from the V0 subunit (Parra and Kane, 1998), though it is unclear how universal this mechanism is for metazoans. Therefore, we examined GFP-tagged VatM, a V0 subunit (Fig. 4 c), but both halves are recycled at a similar stage. The neutralization mediated by WASH, therefore, is not caused by a yeast-like mechanism in which V1 is removed and V0 is left on the vesicle. The implication is that the D. discoideum V-ATPase is recycled as an intact complex, but we have been unable to test this directly, as GFP-V1 and RFP-V0 appear to be impossible to coexpress. GFP-coronin, a marker for actin and the presence of the dynamic Arp2/3 complex (Humphries et al., 2002), was recruited around the same time as WASH. GFP fused to vacuolin A, the D. discoideum flotillin homologue (Wienke et al., 2006), accumulated earlier than WASH (Fig. 4 b). Unexpectedly, however, WASH was only present in puncta, whereas the V-ATPase was removed; all detectable V-ATPase had gone well before formation of the contiguous coat of WASH seen on postlysosomes.

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If this were true, lysosomal neutralization would be stopped by blocking actin polymerization. To test this hypothesis, we preloaded cells with FITC-dextran and counted the rate at which vesicles neutralized. As shown in Fig. 3 b and Video 1, vesicle neutralization started ∼45 min after loading. We then used latrunculin A to block actin polymerization. To minimize secondary effects, latrunculin was only added from 40 min after Figure 3. WASH and actin polymerization are essential for vesicle neutralization. (a) Vesicle neutralization assay. Cells were loaded with FITC- and TRITC-dextran to distinguish between acidic and neutral vesicles. Neutral postlysosomes (arrows) are absent from WASH-null cells and are rescued by expression of GFP-WASH but not GFP-WASHΔVCA (GFP is not seen under these conditions). (b) Latrunculin A completely but reversibly blocks the neutralization of vesicles. AX2 cells were loaded with a pulse of FITC-dextran, and the appearance of neutral vesicles was monitored in the presence or absence of 10 µg/ml latrunculin A. The figure shows measurements of Videos 1 and 2. The representative curves are from an experiment that was performed at least five times.
Figure 4. WASH causes recycling of the V-ATPase. (a) Confocal imaging of wshA<sup>−</sup> cells expressing GFP-WASH and VatB-mRFP after endocytosis of 0.5-µm agarose beads. Frames are taken from Video 3. Insets show a magnified view of the vesicle on which WASH is acting. (b) Quantification of various vesicular proteins during postlysosome formation. Graphs show that the loss of V-ATPase immediately follows the arrival of WASH and coronin, whereas the previously described (Maniak, 2001) postlysosome marker vacuolin is present much earlier than neutralization and rises steadily. The representative curves are from an experiment that was performed at least five times. (c) Rapid widefield oblique illumination imaging of small vesicles containing both GFP-WASH and VatB-mRFP budding a single lysosome. Frames are taken from Video 4. Bar, 1 µm.
Figure 5. The role of WASH and actin in lysosome neutralization. A schematic diagram of the model proposed in this paper: (a) WASH recruited to acidic lysosomes; (b) WASH puncta cause local actin polymerization, and F-actin binds to V-ATPase; (c) small recycling vesicles bud off, and V-ATPase is recruited by WASH/F-actin; (d) loss of V-ATPase causes the vesicle to neutralize and mature to postlysosome; (e) WASH is removed some time after neutralization.

The vesicle traffic and recycling pathways of metazoa are more complex than those of D. discoideum. However, it seems likely that the underlying physiological role is consistent. The functions of other NPFs are conserved across species. RNAi of WASH from mammalian cells causes a much less emphatic phenotype involving two or more recycling pathways at different stages of endocytic transport (Derivery et al., 2009; Gomez and Billadeau, 2009), but each step involves retrograde pathways, thus requiring neutralization. Although constitutive exocytosis of the D. discoideum kind is unusual in mammals, the example we have tested, the expulsion of C. neoformans, requires WASH, and expression of WASH-ΔVCA also altered the pattern of V-ATPase recruitment and recycling in C. neoformans−fed macrophages (Fig. S2 b), again implying that these pathways are conserved.

Actin-mediated sorting

Overall, our data imply that the role of WASH and the Arp2/3 complex is to perform actin-mediated sorting, generating F-actin to sequester and remove specific proteins from vesicles. The V-ATPase has a particularly strong avidity for F-actin, but many other recycled proteins—in particular, growth factor receptors—have unexplained actin-binding activities that may in fact be tags for actin-mediated sorting. Furthermore, the unusually clear, experimentally tractable phenotype we see in D. discoideum will enable genetic dissection of WASH function that would be impossible given the subtle phenotypes seen in mammalian cells.

Materials and methods

Cell culture and growth assessment

D. discoideum cells were grown at RT in HL5 medium plus glucose (Formedium) either in Petri dishes or shaken in flasks. All subsequent experiments and imaging were also performed at RT. Measurement of generation time was performed in shaken culture with and without 20% dextran (wt/vol, molecular mass = 60–90 kD), with samples taken periodically and analyzed for population density and cell size distribution using a cell counter (CASY; Roche). Cultures exceeding 2 × 10^5 cells/ml were diluted to 10^5 cells/ml. After 4 d of growth, differential interference contrast images of those cells that adhered to glass-bottomed dishes were taken using a microscope (TE2000-E; Nikon) with a 60x 1.4 NA objective fitted with a
camera (Retiga EXi; QImaging) and captured using µManager 1.3 software (MicroManager).

Gene disruption

D. discoideum WASH-null cell lines were generated by targeted gene disruption using homologous recombination. A plasmid was constructed containing a flanked Blastocidin resistance cassette flanked with regions homologous to genes upstream (dictyBase database accession no. DDB_G0292876) and downstream (dictyBase database accession no. DDB_G029272) of WASH (dictyBase database accession no. DDB_G0292878). This construct was linearized and electroporated into AX2 cells. Blastocidin-resistant cells were screened by Western blotting using a peptide anti-WASH antibody (BioGenes GmbH) for loss of WASH.

Fluorescent fusion proteins

Exogenously expressed proteins were cloned from cDNA by PCR. WASH and related genes were cloned into the extrachromosomal GFP fusion expression plasmid pDM351 (Veltman et al., 2009). vATM and vATB (without a stop codon) were cloned into mRFPmars shuttle vectors pDM411 and pDM413, respectively (Veltman et al., 2009). These were then excised and inserted into a GFP-WASH extrachromosomal vector to create dual expression constructs.

Cell fixation

Cells were seeded onto coverslips and allowed to adhere for 30 min. They were then fixed with 2% (wt/vol) formaldehyde, 15% (vol/vol) saturated picric acid, and 10 mM Pipes, pH 6.5, for 15 min (Hagedorn et al., 2009). Coverslips were washed in PBS before postfixation for 2 min with 70% ethanol followed by sequential washes in PBS glycine. Cells were incubated with 33 nm Texas red phalloidin (Invitrogen) in PBS for 1 h. Coverslips were washed in PBS and mounted on glass slides using antifade reagent (ProLong Gold; Invitrogen). Cells were imaged using an inverted widefield microscope (IX81; Olympus) moved by a piezo-electric stage using a 100× 1.4 NA objective. Images were captured with a camera (CoolSnap HQ2; Photometrics) using Velocity software (PerkinElmer). Iterative deconvolution was performed with a calculated point spread function using Volocity 3D software (PerkinElmer).

Endocytosis/exocytosis assays

Rates of endocytosis and exocytosis were assessed by measuring the intracellular level of FITC-dextran after its addition or removal from HL6 growth medium, respectively. For measuring rates of endocytosis, cells were suspended in 10 ml H/S medium at 10⁶ cells/ml and shaken at 120 rpm. Samples were taken to measure protein content before the start of the experiment to normalize results between replicates. 20 mg FITC-dextran was added to flask, and 500-pl samples were taken at 0-, 15-, 30-, 45-, 60-, 90-, 120-, 180-, 240-, and 300-min time points. To measure exocytosis, 10-ml cultures at 5 x 10⁶ cells/ml were incubated overnight with 20 mg FITC-dextran. Cells were pelleted and suspended in fresh H/S medium, and samples were taken at the aforementioned time points. Samples were washed in ice-cold KK2 buffer and pelleted. Pellets were lysed in 50 mM Na2HPO4, pH 9.3, and 0.2% Triton X-100 and measured on a fluorimeter (470-nm excitation and 515-nm emission; Photon Technology International).

Postlysosome visualization

Visualization of C. neoformans postlysosomes was performed using mRFP and TRITC-conjugated dextran as previously described by Rivero and Maniak (2006) and Jenne et al. (1998). Cells were seeded onto glass-bottomed dishes and incubated in LoFlo medium (Formedium) at 5% CO2 and 37°C. After fixation, cells were labeled with anti-WASH antibody and TRITC phalloloid. RAW macrophages transiently expressing WASH or WASH-null cell lines were generated by targeted gene disruption using homologous recombination. A plasmid was constructed containing a flanked Blastocidin resistance cassette flanked with regions homologous to genes upstream (dictyBase database accession no. DDB_G0292876) and downstream (dictyBase database accession no. DDB_G0292878). This construct was linearized and electroporated into AX2 cells. Blastocidin-resistant cells were screened by Western blotting using a peptide anti-WASH antibody (BioGenes GmbH) for loss of WASH.

Lactrinulin A (EMD) was added to the dish at a final concentration of 2 μM at various times during imaging. For washout experiments, the dish was removed from the microscope, rinsed twice with fresh LoFlo medium, and immediately returned to the microscope. Images were acquired using Velocity software, and postlysosomes were quantified with ImageJ using its built-in Analyze Particles routine.

Live cell imaging

For imaging of cells expressing fluorescent fusion proteins, cells were placed in LoFlo medium at least 2 h before imaging. Cells were incubated for 20 min with agaroase beads and then compressed under a layer of 1% (wt/vol) agarose made using LoFlo medium. For quantitative microscopy, cells were imaged on an A1 confocal microscope (GFP, 488-nm excitation and 500–550-nm emission; mRFP, 561.4-nm excitation and 570–620-nm emission) with a 60x 1.4 NA objective. For qualitative high speed acquisition, cells were imaged on a TE2000-U widefield microscope with a 100x 1.49 NA objective, with illumination provided by 473- and 561-nm solid-state lasers (Deepstar; Omicron Laserprodukte GmbH). Images were captured with a camera (Cascade II; Photometrics) using Metamorph version 7 software (Universal Imaging). ImageJ was used for the quantification of fluorescence around phagocytosed particles using a doughnut-shaped region of interest to obtain mean intensity.

C. neoformans expulsion

RAW macrophages transiently expressing WASH or WASHΔVCA were incubated with opsonised (1.98 μg/10⁶ cells/100 μl, 1 h rotating at RT) C. neoformans strain H99-GFP (Noelz et al., 2010) for 2 h. After phagocytosis of Cryptococcus, J774 macrophage cells were washed three times in DMEM without phenol red. Cells were imaged on a microscope (TE2000) enclosed in a temperature-controlled and humidified environmental chamber (okolab) with 5% CO2 at 37°C. Time-lapse images were captured with a camera (Digital Sight DS-Qi1MC; Nikon) with a 20x objective (Ph1 PLAN APO 0.45 NA) using NIS-Elements AR software. Fluorescence and phase-contrast images were captured every 2 min for 24 h. The occurrence of expulsion was analyzed as described previously (Mo et al., 2006).

WASH and V-ATPase localization on C. neoformans-containing phagosomes

J774 macrophages were fixed with formaldehyde 3 h after phagocytosis of C. neoformans and incubation at 5% CO2 and 37°C. After fixation, cells were labeled with anti-WASH antibody and TRITC phalloloid. RAW macrophages transiently expressing WASH or WASHΔVCA were fixed with formaldehyde at the time points indicated after phagocytosis of C. neoformans and were labeled with anti-AFP6V1B2. Images were captured using a Digital Sight DS-Qi1MC camera with a 60x objective (PLANO APO 1.4 NA) using NIS-Elements AR software. The A1R confocal used in this research was obtained through Birmingham Science City Translational Medicine Clinical Research and Infrastructure Trials Platform with support from Advantage West Midlands.

Online supplemental material

Fig. S1 shows bacterial growth, migration, and EM of D. discoideum. Fig. S2 shows V-ATPase localization in C. neoformans-infected macrophages, and Fig. S3 shows the identification of proteins associated with GFP-WASH. Videos 1 and 2 show the neutralization of vesicles in untreated cells and after latrunculin, respectively. Video 3 shows WASH association and V-ATPase removal from vesicles, and Video 4 shows the detail of a single lysosome during neutralization. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.201009119/DC1.

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Figure S1. **Bacterial growth, migration, and EM of *D. discoideum***. (a) Parental AX2 and wshA⁻ cells were plated at low density on lawns of Klebsiella pneumoniae growing on SM agar to allow individual cells to grow into colonies. After 4 d, the colonies were photographed using a dissecting microscope. The wshA⁻ colonies are clearly smaller at all times. (b) Vegetative cells were allowed to chemotax under agar toward a source of folate (Blagg et al., 2003) and imaged using Nomarski differential interference contrast. The speeds of cells were calculated using ImageJ as described in Materials and methods. Error bars represent one SEM, and n = 3. (c–e) Parental AX2 and wshA⁻ cells were incubated overnight with BSA–colloidal gold (15 nm, OD₆₀₀ = 5) and then washed and incubated for 2 h. Cells were fixed, stained, and examined by transmission EM (Hagedorn et al., 2009) as shown in Fig. 2 (f and g). Quantitative analyses were conducted on multiple cells from a single set of samples. The number of gold-loaded vesicles in each cell was counted, and the proportion of cells with each number is shown in c. Over 70% of parental cells contained no gold-labeled vesicles, whereas wshA⁻ cells contained many more. For each vesicle, the number of gold particles was counted and shown in d. wshA⁻ cells contained vesicles with many more gold particles than parental. The density of other material in the vesicles was assessed; approximately half of the parental vesicles were mostly filled with low-density material (Fig. 2 f), whereas nearly all wshA⁻ vesicles were densely stained (e). (f) wshA⁻–null cells were incubated for 6 h with 4.5-mm beads and fixed with picric acid and formaldehyde. Cells were stained with an antibody against vacuolin, a protein previously described (Blagg et al., 2003) as a marker for postlysosomes in *D. discoideum*. This, along with quantitative analysis in wild-type cells shown in Fig. 4, reveals that vacuolin is present on vesicles before lysosome neutralization.
Figure S2. V-ATPase localization in C. neoformans–infected macrophages. (a) Verification of WASHΔVCA as a dominant negative. HeLa cells were loaded with 10 mg/ml Alexa Fluor 488–transferrin for 1 h and then chased with 8 mg/ml of unlabeled transferrin for 120 min before fluorimetric analysis. HeLa cells expressing WASHΔVCA show a comparable defect in transferrin recycling to cells in which WASH has been depleted by siRNA. *, P < 0.05 relative to control. Dominant-negative experiments are the mean of three experiments and siRNA the mean of six experiments. WT, wild type. (b) Proportion of phagosomes positive for V-ATPase in samples fixed at regular time intervals after incubation with C. neoformans. Error bars show the SD, and n = 4.

Figure S3. Identification of proteins associated with GFP-WASH. wshA− cells were transfected with GFP alone (left) or GFP-WASH (right) and lysed with Triton X-100. GFP and bound proteins were immunoprecipitated with GFP-trap (Chromatek) and separated by PAGE, and indicated gel slices were analyzed by mass spectrometry. The arrowhead marks the position of GFP-WASH. Proteins identified in the GFP-WASH, but not the GFP-only lanes, are listed on the side. MM, molecular mass indicated in kilodaltons.
Video 1. **Neutralization of vesicles in untreated cells.** A QuickTime video showing the appearance of neutralized vesicles, as used to generate Fig. 3 b. Cells were incubated in FITC-dextran for 20 min and then washed and observed by widefield fluorescence microscopy. The appearance of fluorescent vesicles is caused by neutralization, causing FITC to regain fluorescence. Frame rate, 1/30 s.

Video 2. **Neutralization of vesicles after latrunculin.** A QuickTime video showing the appearance of neutralized vesicles during and after latrunculin A treatment, as used to generate Fig. 3 b. Cells were incubated in FITC-dextran for 20 min and then washed and observed by widefield fluorescence microscopy. Latrunculin was included for 30 min from t = 40 min to t = 70 min and was then washed out and replaced with latrunculin-free medium. Frame rate, 1/30 s.

Video 3. **WASH association and V-ATPase removal from vesicles.** A QuickTime video showing correlation of the recruitment of GFP-WASH puncta (green) with the disappearance of V-ATPase (red, labeled with VatB-mRFPmars). Double-labeled cells were fed with small (~0.5 µm) agarose beads, compressed with an agar overlay, and then imaged on a confocal microscope. Frame rate, 1/min.

Video 4. **Detail of a single lysosome during neutralization.** An enlarged and rapid view of a single vesicle during V-ATPase recycling under the same conditions as Video 3. The video shows small vesicles budding off a maturing lysosome containing both GFP-WASH puncta (green) and V-ATPase (red, labeled with VatB-mRFP). Cells were labeled and treated as in Video 3 but were imaged with oblique illumination in a widefield microscope. Frame rate, 1/5 s.

**References**
