Carnell et al., http://www.jcb.org/cgi/content/full/jcb.201009119/DC1

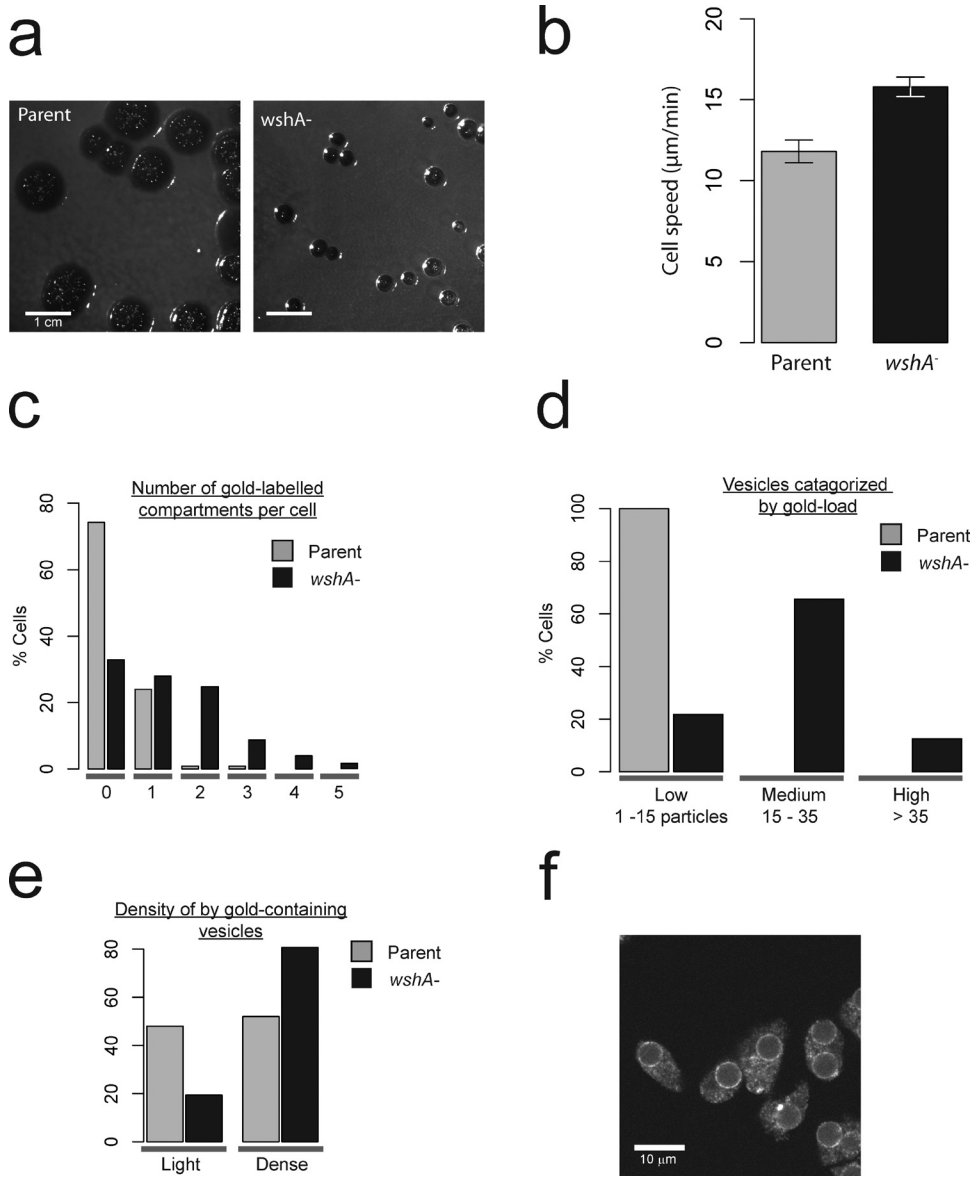


Figure S1. **Bacterial growth, migration, and EM of *D. discoideum*.** (a) Parental AX2 and *wshA*$^-$ cells were plated at low density on lawns of *Klebsiella pneumoniae* growing on SM agar to allow individual cells to grow into colonies. After 4 d, the colonies were photographed using a dissecting 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, OD_{600} = 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.

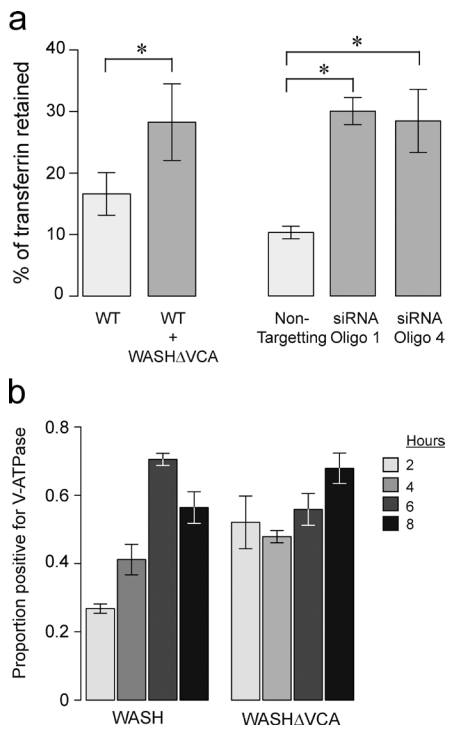


Figure S2. **V-ATPase localization in *C. neoformans*–infected macrophages.** (a) Verification of WASHΔVCA as a dominant negative. HeLa cells were loaded with 10 mg/ml Alexa Fluor 488–transferrin for 1 h and then chased with 8 mg/ml of unlabeled transferrin for 120 min before fluorimetric analysis. HeLa 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 $n = 4$.

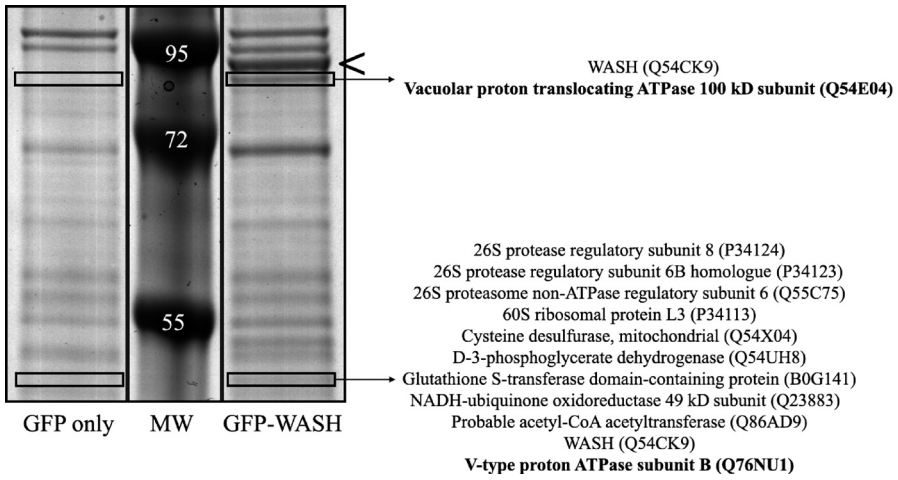
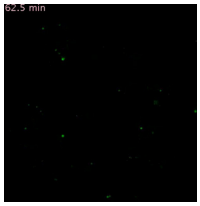
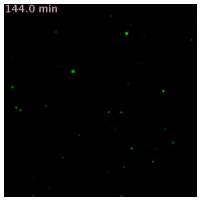


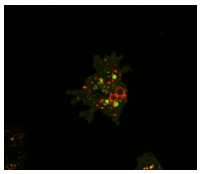
Figure S3. **Identification of proteins associated with GFP-WASH.** *wshA*$^-$ cells were transfected with GFP alone (left) or GFP-WASH (right) and lysed with Triton X-100. GFP and bound proteins were immunoprecipitated with GFP-trap (Chromatek) and separated by PAGE, and indicated gel slices were analyzed 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.



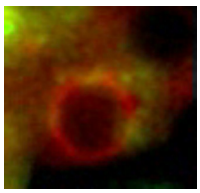
Video 1. **Neutralization of vesicles in untreated cells.** A QuickTime video showing the appearance of neutralized vesicles, as used to generate Fig. 3 b. Cells were incubated in FITC-dextran for 20 min and then washed and observed by widefield fluorescence microscopy. The appearance of fluorescent 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

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