Drosophila WASH is required for integrin-mediated cell adhesion, cell motility and lysosomal neutralization

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Abstract
The Wiskott-Aldrich Syndrome Protein and SCAR Homologue (WASH) is a conserved actin nucleation promoting factor controlling Arp2/3 complex activity in endosomal sorting and recycling. Previous studies have identified WASH as an essential regulator in *Drosophila* development. Here, we show that homozygous *wash* mutant flies are viable and fertile. We demonstrate that *Drosophila* WASH has conserved functions in integrin receptor recycling and lysosome neutralization. WASH generates actin patches on endosomes and lysosomes mediating both functions. Consistently, loss of WASH function results in cell spreading and cell migration defects of macrophages, and an increased lysosomal acidification that affects efficient phagocytic and autophagic clearance. WASH physically interacts with the vacuolar ATPase subunit Vha55 that is crucial to establish and maintain lysosome acidification. As a consequence, starved flies lacking WASH function show a dramatic increase in acidic autolysosomes causing a reduced lifespan. Thus, our data highlight a conserved role for WASH in the endocytic sorting and recycling of membrane proteins like integrins and the V-ATPase that increase the likelihood of survival under nutrient deprivation.
Introduction

The Wiskott-Aldrich syndrome protein (WASP) family of nucleation promoting factors (NPF) controls actin dynamics by activating the ubiquitous Arp2/3 complex, a nucleation machinery that initiates actin filament branches on sides of pre-existing filaments. One of three highly conserved WASP family members is the Wiskott-Aldrich Syndrome Protein and SCAR Homologue (WASH) protein that is found across multiple eukaryotic kingdoms (Veltman and Insall, 2010). As all class I NPFs, WASH possesses a VCA domain at its C-terminal end that activates the Arp2/3 complex \textit{in vitro} (Linardopoulou et al., 2007). It differs considerably from other members of this class at its N-terminal part, as it has a WASH homology domain (WAHD), comprised of a WASH homology domain 1 (WAHD1) and a tubulin-binding region (TBR) (Gomez and Billadeau, 2009). In cells, WASH exists in a stable pentameric complex known as the WASH Regulatory Complex (SHRC) containing four additional subunits: Strumpellin, FAM21, SWIP, and CCDC53. This complex appears to be structurally very similar to the WAVE Regulatory Complex (WRC), which controls the activity and localization of WAVE (Jia et al., 2010). Similar to the WRC, knockdown of individual WASH complex subunits results in the degradation of other WASH complex components (Derivery et al., 2009; Gomez and Billadeau, 2009; Jia et al., 2010). However, the SHRC seems to be constitutively active in contrast to the WRC, thus possibly being regulated by so far unknown mechanisms (Derivery and Gautreau, 2010; Gomez and Billadeau, 2009).

First insights into the cellular function of WASH-mediated actin polymerization emerged from the subcellular localization of WASH and from RNA interference (RNAi) studies in mammalian cell culture. These studies have demonstrated that WASH is an endosomal protein that regulates several sorting and maturation steps of endocytic vesicles. Upon WASH depletion, endosomes change shape from normally spherical to an enlarged and tubular shape (Derivery et al., 2009a; Duleh and Welch, 2010; Gomez and Billadeau, 2009), proposing that WASH plays a role in regulating endosome tubulation and scission. WASH co-localizes with Rab4, Rab7 and Rab11 suggesting an important role in both early and late endosomal recycling pathways (Derivery et al., 2009). In line with this notion, \textit{wash} deficient cells show trafficking defects in different endocytic routes, including endosome-to-Golgi transport \textit{via} the retromer complex (Gomez and Billadeau, 2009; Harbour et al., 2012; Duleh and Welch, 2012; Piotrowski et al., 2013; Zech et al., 2011) and endosome-to-plasma
membrane recycling of various cargos such as integrins, the epidermal growth factor (EGF), and transferrin receptors (Derivery et al., 2009; Zech et al., 2011). Thus, WASH activity is thought to facilitate and maintain the segregation of endosomal sorting domains.

First evidence for the in vivo role of WASH in lysosomal maturation and autophagy comes from loss-of-function studies in *Dictyostelium* (Carnell et al., 2011). Normally, endosomes and in particular lysosomes get acidified by the activity of the vacuolar (V)-ATPase, a large multimeric ATP-dependent proton pump (Maxson and Grinstein, 2014). An acidic environment provides not only an optimal pH for a wide range of acidic hydrolases, but also ensures that internalized receptors release their ligands, so that they can recycle back to the plasma membrane (Luzio et al., 2007; Pryor and Luzio, 2009). Dissociated ligands are then delivered to late endosomes while the receptors are sorted into recycling vesicles destined for the plasma membrane. The successive acidification of endosomes further leads to highly acidic lysosomes where proteins are degraded.

Before exocytosis of indigestible material can occur, lysosomes have to be neutralized by retrieving the V-ATPase from the lysosomal membrane. This task is carried out by WASH, as it interacts with the V-ATPase and generates a newly polymerized network that, in turn, pushes the V-ATPase out of the membrane (Carnell et al., 2011). Lysosomal neutralization requires a functional WASH-VCA domain suggesting that Arp2/3-mediated actin polymerization is essential for the V-ATPase retrieval (Carnell et al., 2011). Further studies in *Dictyostelium* and in mammalian cells suggest a conserved role of WASH in regulating the lysosomal network required for efficient phagocytic and autophagic digestion (Kolonko et al., 2014; King et al., 2013; Gomez et al., 2012). *Dictyostelium* cells lacking WASH are unable to digest their cytoplasm in order to survive phases of starvation (King et al., 2013). The phenotypic analysis of *wash* knockout mice confirms an important in vivo function of WASH in regulating autophagy (Xia et al., 2014). WASH deficiency results in embryonic lethality and mutant mice show extensive autophagy (Xia et al., 2014). Therefore, a model has been proposed in which WASH inhibits autophagy by suppressing ubiquitination of Beclin1/Atg 6, a core component of the conserved Vps34 complex, which promotes autophagosome formation when active (Xia et al., 2014). Remarkably, different from its activity in V-ATPase retrieval and retromer-mediated endosomal sorting, the function of WASH in autophagy does not require its
Arp2/3-activating activity. A truncated WASH protein lacking the VCA domain still rescues the accelerated autophagy induction in wash−/− mouse embryonic fibroblasts similar to full-length WASH (Xia et al., 2014). Surprisingly, rather than finding an accelerated autophagy Zavodszky and colleagues recently reported an impaired autophagy in HeLa cells depleted for WASH (Zavodszky et al., 2014a; Zavodszky et al., 2014b). Thus, the exact role of WASH as an NPF in autophagy remains unclear so far.

In Drosophila, a role of WASH in lysosomal function and autophagy has not been reported yet. The initial analysis of a deletion mutant suggests an essential function of WASH in Drosophila oogenesis and larval development (Linardopoulou et al., 2007; Liu et al., 2009). However, whether the observed lethality and the oogenesis defects are due to a loss of WASH function has not been addressed in these studies. The role of WASH in developmental dispersal of immune cells during Drosophila embryogenesis is even unclear. RNAi-mediated knockdown of WASH partially affects the stereotypic migration of embryonic macrophages (Verboon et al., 2015), but the loss-of-function mutant macrophages are indistinguishable from wild type (Evans et al., 2013). By contrast, a more recent study demonstrates a conserved function in retromer-dependent endocytic recycling of the luminal protein Serpentine in Drosophila trachea development (Dong et al., 2013). wash mutant embryos show defects in tracheal tube length phenocopying mutations in rab9 and vps35 (Dong et al., 2013).

Here, we analyzed the function of WASH in Drosophila. Different from previous studies we show that WASH is dispensable for Drosophila development. Viable mutant females show neither reduced fertility nor defects in the morphology of developing egg chambers. Instead, we found a requirement of WASH in integrin-dependent cell spreading of macrophages, and we provide further evidence for a conserved role in generating F-actin patches driving late endosomal recycling and lysosome neutralization required for efficient phagocytic and autophagic clearance.
Results

Homozygous *wash* mutants are viable and have no defects in oogenesis

The *Drosophila* genome contains a single *wash* gene located on the second chromosome (2R) at the cytological location 48E6 (Figure 1A). It consists of a single exon encoding a 75 kDa protein. In previous studies, a *wash* deletion mutant (*washΔ185*) was generated by imprecise excision of the P-element EY15549 inserted between codon 11/12 of the *wash* ORF (Linardopoulou et al., 2007). We confirmed the 1029 bp deletion in *washΔ185* mutant flies (Figure 1B; Linardopoulou et al., 2007). This deletion removes more than half of the coding region resulting in a premature stop codon. Flies homozygous for this deletion chromosome die at late third instar larvae suggesting an essential function of WASH in *Drosophila* development (Linardopoulou et al., 2007; Liu et al., 2009). However, whether the lethality is due to a loss of WASH function has not been addressed in these studies (Linardopoulou et al., 2007; Liu et al., 2009). The existence of viable flies bearing the 10 kb P-element insertion EY15549 in the *wash* ORF already suggests that the lethality of homozygous *washΔ185* mutant animals might be caused by second-hit mutations. This discrepancy prompted us to test trans-heterozygous flies with the sequence mapped deficiency Df(2R)BSC699 that removes the complete *wash* gene locus (Figure 1A, B).

Trans-heterozygous *washΔ185/Df(2R)BSC699* flies, but also the homozygous viable P-element insertion strain, completely lack WASH expression (Figure 1C). Remarkably, they are viable with no apparent developmental defects suggesting that lethality was caused by a second-site mutation. Consistently, we could establish a viable *wash* mutant fly stock (termed *washΔ185* from now onwards) by separating the lethal mutation from the *washΔ185* chromosome (see Experimental procedures). *washΔ185* mutant females produce normal eggs and are completely fertile (Figure 1E-I'). PCR and western blot analyses confirmed the complete loss of WASH expression in these flies (Figure 1D). WASH is strongly expressed in wild type ovaries and this expression is completely lost in *washΔ185* mutant animals (Figure 1D). The phenotypic analysis of homozygous *washΔ185* mutant developing eggs did not reveal any morphological defects (Figure 1F, H). Phalloidin stainings of developing mutant egg chambers further confirmed a wild typic morphology and organization of actin-rich structures including ring canals and actin baskets (Figure
wash mutant egg chambers also exhibit no significant defects in border cell migration, a well-established model for invasive collective cell migration (Figure 1J). Thus, we conclude that WASH function is not required for Drosophila oogenesis.

**wash mutant macrophages exhibit cell spreading defects**

Previous studies highlighted an important role of WASH in efficient phagocytic and autophagic digestion in Dictyostelium (King et al., 2013). In Drosophila, different types of circulating immune cells can be found. The most abundant cell types are plasmatocytes, the professional phagocytes in flies. They fulfill similar roles as mammalian monocytes and macrophages including phagocytosis of invading pathogens and clearance of apoptotic bodies controlling development, and inflammation (Stramer et al., 2005; Wood and Jacinto, 2007). Western blot analysis confirmed a specific expression of WASH in macrophage-like plasmatocytes (Figure 2A; from here on referred to as macrophages). In these cells, WASH was most prominent at large F-actin-coated subcellular structures (Figure 2B, B'), concentrated in the perinuclear region (Figure 2B'). The punctate signals were absent in wash mutant macrophages indicating antibody specificity (Figure 2C).

Interestingly, we found that wash mutant macrophages spread properly on Concanavalin A (ConA)-coated surfaces (Figure 2D-F), but displayed prominent defects in cell flattening and spreading on uncoated surfaces (Figure 2C). Detailed quantification analysis confirmed a significantly reduced spreading area of both larval and pupal washΔ185* macrophages on uncoated glass cover slips (Figure 2F). Re-expression of a WASH-EGFP transgene in washΔ185* mutant macrophages significantly restored cell spreading defects indicating that the defects resulted from a loss of WASH function (Figure 2G). Moreover, RNAi-mediated suppression of WASH and the known WASH complex components Strumpellin, SWIP, Fam21, and CCDC53 resulted in comparable spreading defects on uncoated surfaces (Supplementary figure S1).

**WASH regulates integrin-mediated cell adhesion**

ConA-induced spreading is controlled by remodeling of the actin cytoskeleton upon binding of lectins to the polysaccharide side chains of plasma membrane proteins and lipids (Rogers et al., 2003), while cell spreading on uncoated glass surfaces
depends on integrin-mediated cell adhesion (Jani and Schock, 2007). Since wash mutant cells spread properly on lectin-coated surfaces we suggest that WASH plays a role in integrin-mediated cell spreading rather than regulating lamellipodial cell protrusions. In *Drosophila*, the main β-integrin is βPS-integrin, also known as Myospheroid (Mys), which is required for cell spreading and cell migration of embryonic and pupal macrophages (Comber et al., 2013; Moreira et al., 2013). Structured illumination microscopy (SIM) revealed an overall punctate expression pattern of endogenous βPS-integrin in macrophages. The strongest signal was observed in the perinuclear region compared to a rather moderate concentration along the leading edge of the cells (Figure 2H). wash mutant cells show an increased accumulation in the perinuclear region (Figure 2I). Quantitative measurement of fluorescence intensities further confirmed an increased signal of βPS-integrin around the nuclei at the expense of peripheral staining in wash mutant cells (Figure 2J).

To further stimulate integrin-mediated cell adhesion macrophages were plated on surfaces coated with the ECM protein vitronectin. Cells spread similarly on vitronectin, but rather exhibited numerous βPS-integrin positive punctae that were often enriched along the leading edge and formed prominent foci characteristic for focal adhesion sites in mammalian cells (Figure 2K; supplementary movie M1). Under these conditions βPS-integrin clusters co-localize with the reporter fusion protein EGFP-FAT containing the Focal Targeting Sequence (FAT) domain of the Focal Adhesion Kinase (FAK; Figure 2L). Stress-fiber-like actin filament bundles were seen emerging from these bright foci, as is typical for focal adhesion sites (Figure 2L). Such dynamic focal adhesion sites are also formed in vivo in migrating pupal macrophages expressing EGFP-FAT imaged from prepupae (Supplementary movie M1). Similar to uncoated surfaces, washΔ185* mutant cells show cell spreading defects on vitronectin (Figure 2M). Moreover, loss of WASH function also resulted in a reduced length of vitronectin induced cell adhesion foci along the cell periphery marked by EGFP-FAT (Figure 2N, O; quantification in figure 2P; supplementary movie M2). Thus, our data support a conserved role of *Drosophila* WASH in integrin-mediated cell adhesion.
WASH generates dynamic actin patches on endosomes

To further analyze a possible role of WASH in regulating integrin receptor trafficking we examined the subcellular localization of WASH in Drosophila macrophages in more detail. WASH did not co-localize with EGFP-FAT at focal adhesion sites suggesting that WASH itself is not a part of them, but might rather regulate integrin receptor trafficking (Figure 3A). WASH was found in close contact to βPS-integrin and surrounded βPS-integrin punctae in the perinuclear region (Figure 3B). Live spinning disc microscopy revealed of a WASH-EGFP transgene confirmed a dynamic localization of WASH at vesicular structures of different sizes (Figure 3C; supplementary movie M3). Co-expression of WASH-EGFP with Lifeact-RFP further illustrates a striking overlap of dynamic WASH and F-actin patches on these vesicles (Figure 3D; supplementary movie M4). Such actin patches can also be observed in vivo in migrating pupal macrophages expressing Lifeact-EGFP (Supplementary movie M5). The formation of actin patches highly depends on WASH function. A wild type macrophage showed at least two distinct actin patches on large circular vesicles, whereas loss or RNAi-mediated suppression of WASH completely abolished actin patch formation (Figure 3E-G; quantification in figure 3H; supplementary movie M6).

Further co-localization analysis with different Rab-YFP/GFP transgenes as markers for different stages of endosomal maturation revealed that actin-coated WASH vesicles mainly overlap with small Rab4- and larger Rab7-marked endosomes, a compartment that also harbors the retromer complex (Figure 3I-U; quantification of co-localization in figure 3M; Seaman and Freeman, 2014; Seaman et al., 2009). This suggests that WASH might regulate either Rab4-mediated fast integrin receptor recycling from small early endosomes to the plasma membrane or it controls recycling from a Rab7 late endosomal compartment as previously proposed for α5β1-integrin recycling in mammalian cells (Zech et al., 2011). Consistently, we frequently found βPS-integrin punctae within Rab4- and Rab7-positive vesicles (Figure 4A, B). Interestingly, we observed an increased accumulation of βPS-integrin in Rab7-, but not in Rab4-marked endosomes in wash mutant macrophages (arrowheads in Figure 4C, E). Quantification of βPS-integrin fluorescence intensity confirmed a moderate, but significant increase of βPS-integrin in the lumen of late endosomes marked by endogenous Rab7 in wash mutant macrophages (Figure 4F). Thus, we propose that WASH might act predominantly at late endosomal recycling of
βPS-integrin in *Drosophila* as previously suggested for α5β1-integrin recycling in mammalian cells (Zech et al., 2011).

**WASH is required for cell migration of prepupal macrophages**

We next analyzed a possible role of WASH in integrin-mediated cell migration *in vivo*. Previous data revealed an important function of βPS-integrin-mediated cell adhesion in 2D random cell migration in white prepupae, *in vivo* (Moreira et al., 2013). Subepidermal macrophages acquire a spread morphology with broad lamellipodial protrusions and collectively initiate random single cell migration (Figure 5A, quantification in figure 5D-F; supplementary movie M7; Moreira et al., 2013; Brinkmann et al., 2016). Prepupal wash mutant cells also show a reduced cell spread area *in vivo* (Figure 5D). Macrophage-specific RNAi-mediated suppression of βPS-integrin function affects similarly cell spreading and nearly completely abolished cell migration (Figure 5B, quantification in figure 5D-F; supplementary movie M7). Loss of WASH function also resulted in significant albeit weaker cell migration defects with reduced migratory velocity and distance (Figure 5C, quantification in figure 5E, F; supplementary movie M7).

Additionally, we analyzed directed cell migration of macrophages upon laser-induced wounding in later pupal stages. At this stage, macrophages migrate in 3D rather than in 2D (Sander et al., 2013; Brinkmann et al., 2016). Wild type macrophages immediately responded to a laser-ablated single cell and migrated towards the wound (ablated cell is marked by a yellow circle in figure 5G, supplementary movie M8). βPS-integrin-depleted cells are able to respond and migrate towards wounds, however with strongly reduced directionality and velocity (Figure 5H: supplementary movie M8). By contrast, loss of WASH function has no effects on the wound response and directed cell migration at the initial phase (5-10 minutes after wounding; Figure 5I; supplementary movie M8). However, we found a reduced number of macrophages that reached the wound after 30 minutes by measuring the Histogram-based Macrophage Migration Score (HMMS) that we have previously established (Lammel et al., 2014; quantification in figure 5J). These data indicate that WASH function is important for macrophage cell motility *in vivo*. 
Conserved functional and physical interactions between WASH and Vha55 V-ATPase subunit on lysosomes

Previous studies in Dictyostelium revealed an important role of WASH in lysosome function. Given the localization of Drosophila WASH to large Rab7-positive late endosomes/pre-lysosomes we further analyzed a possible conserved role of WASH in lysosome function. Mature lysosomes in macrophage-like Drosophila S2R+ cells marked by a GFP fusion with the Lysosome-associated membrane protein 1 (Lamp1) are characterized by numerous F-actin patches visualized by a Lifeact-mCherry fusion (Figure 6A; supplementary movie M9). We also found WASH on the surface of these large lysosomes marked by either Lamp1 or Vha55, the V-ATPase regulatory B-subunit in fixed and living cells (Du et al., 2006; Figure 6B, C; supplementary movies M10, M11). Strikingly, loss of WASH function in macrophages resulted in enlarged Vha55-marked lysosomes (Figure 6D, E; quantification in figure 6F) suggesting a conserved role in lysosomal function. Supporting this notion, WASH can physically associate with the V-ATPase as found in co-immunoprecipitations with a Myc-tagged Vha55 (Figure 6G).

WASH function is required in phagolysosome neutralization

Based on the functional and physical interactions between WASH and the V-ATPase subunit we further examined possible defects in phagolysosome neutralization in wash mutant macrophages. These cells efficiently phagocytize E. coli particles conjugated with a pH-dependent fluorescent dye (pHrodo) within a few minutes. The fluorescence intensity of the pHrodo dye increases as the pH of its surrounding becomes acidic. Once a phagocytized pHrodo-conjugated E. coli enters a lysosome, the acidic environment within the phagolysosome causes a dramatic increase in fluorescent emission (Figure 7A'; supplementary movie M12). These phagolysosomes showed prominent actin patches (arrowheads in Figure 7A'; supplementary movie M12). Strikingly, WASH-EGFP is found at phagocytized E. coli pHrodo particles upon acidification and accumulates together with F-actin in distinct patches on phagolysosomes (Figure 7B, C; quantification in D; supplementary movie M13). Loss of WASH function did not affect phagocytic uptake, but rather resulted in a significant defect in lysosomal neutralization (Figure 7E, F). The neutralization defect in wash mutant macrophages becomes most obvious 60 minutes after phagocytic uptake (Figure 7E, F). From this time onwards the fluorescent emission
still increased continuously in wash mutants whereas in wild type cells the fluorescence intensity ceased after one hour (Figure 7E, F). Thus, these data strongly support a conserved role of WASH in the retrieval of the V-ATPase from lysosomes.

**Loss of WASH function promotes acidification of autophagic vesicles under starvation**

Similar to the phagocytic pathway, autophagy also utilizes lysosomes to degrade cytoplasm, ubiquitinated proteins or non-functional organelles. Autophagy can occur as a direct transport of selected proteins across the lysosomal membrane (microautophagy) or as an engulfment of cytoplasm and organelles by double-membrane vesicles that later on fuse with lysosomes (macroautophagy; Xie and Klionsky, 2007). Under starvation, autophagy is upregulated by the cells in order to supply the organism with nutrients (Russell et al., 2014; Xie and Klionsky, 2007).

The cellular function of WASH in lysosomal neutralization might not be restricted to the digestion of phagocytic cells such as macrophages, but could also be important for starvation-induced autophagy in the *Drosophila* fat body, a nutrient storage organ analogous to the vertebrate liver. This tissue has a robust, well-characterized autophagic response to starvation by which flies mobilize nutrients promoting their survival. We used Lysotracker Red (LTR) staining for the detection of lysosomes and autophagosomes. Fat bodies from well-fed wild-type third instar larvae remained essentially Lysotracker negative (Figure 8A-C). In contrast, Lysotracker staining in the fat body of fed wash mutant animals was already increased (Figure 8D-F). However, in response to amino acid starvation (sugar-only diet) we observed a more dramatic induction of enlarged punctate Lysotracker staining in mutant cells compared to wild type (compare Figure 8I, L). Induced autolysosomes in the wash mutant fat body seem to be functional since they also show an increased Magic Red staining, a marker for active Cathepsin B, suggesting that the pH within the lysosomal lumen is more acidic promoting its hydrolytic activity during starvation-induced autophagy (Figure 8M, N). Interestingly, WASH localizes to actin-enriched vesicular structures in the fat body cells, similar to what was observed in macrophages (Figure 8O). These fat body structures are also marked by an Atg8-GFP transgene (Figure 8P), a key autophagic protein that associates with autophagosome (Shpilka et al., 2011). To further test an increased induction of functional autophagic vesicles, we analyzed Atg8 lipidation by immunobloting of fat body lysates. Lipidation of Atg8
drives its association with the autophagosome and serves as an indicator of autophagy induction (Shpilka et al., 2011). The non-processed form (often referred to as Atg8-I) can easily be distinguished from its membrane-associated, faster migrating active form (Atg8-II). Consistent with our finding that lysosomal acidity was increased in the wash mutant fat body, immunoblot analysis of Atg8 showed that the amount of both the non-processed and lipidated form is increased (Figure 8Q). Thus, loss of WASH function increases starvation-induced autophagy. Finally, we examined possible changes in the life span of wash mutant flies under amino acid starvation. Under these conditions wash mutants displayed an accelerated lethality compared to control flies (Figure 8R). Increased lethality of starved wash mutant flies can be rescued by expression of the WASH-EGFP transgene in the mutant background (Figure 8R). Thus, increased acidification of autolysosomes might shorten life span rather than promote longevity as similarly found in a recent study with WASH-conditional knockout (KO) mice (Xia et al., 2013).

Taken together, our data highlight a conserved role for WASH in the endocytic sorting and recycling of membrane proteins like integrins and the V-ATPase. A loss of WASH function therefore leads to defects in cell spreading, cell motility and lysosomal neutralization. Lysosome neutralization defects in particular might contribute to a decreased life expectancy under amino acid starvation since nutrients are stuck in lysosomes and cannot nourish the starving cells or organism.

Discussion

Drosophila wash was originally identified as an essential gene required for oogenesis and larval development (Linardopoulou et al., 2007; Liu et al., 2009). Our data clearly show that both larval lethality and all morphological defects of developing eggs described so far was not due to the loss of WASH function but rather caused by a second-hit mutation. The generation of viable wash mutant flies bearing the same chromosomal deletion finally confirmed that WASH is dispensable for early Drosophila development and oogenesis. Defects in oogenesis in trans-heterozygous washΔ185/wimp mutant flies observed by Liu et al. probably are due to the wimp mutation used, as wimp is a change-of-function mutation of an RNA polymerase II subunit and reduces maternal function of a variety of genes, including, but not limited to, wash (Liu et al., 2009; Parkhurst and Ish-Horowicz, 1991). Despite the apparent
lack of any obvious phenotype during oogenesis, WASH seems to be expressed in *Drosophila* ovaries. Thus, redundant or compensatory pathways might mask WASH function in developing eggs. Previous expression data in wild type revealed an accumulation at the oocyte cortex besides an overall cytoplasmic expression in all nurse and somatic follicle cells throughout oogenesis (Rodriguez-Mesa et al., 2012). The protein A affinity-purified monoclonal antibody (mAb P3H3; Rodriguez-Mesa et al., 2012) used in this study specifically recognizes WASH in immunoblots (Figure 1B-D). However, in immunostainings of developing egg chambers we found no significant differences in the expression pattern and signal intensity between wild type and mutants.

We observed a specific and prominent expression in *Drosophila* macrophages and in the fat body that is completely lost in *wash* mutants. Our data strongly support a conserved role of *Drosophila* WASH as an endosomal specific NPF in regulating integrin trafficking and lysosome function. Important regulators of endosomal trafficking and recycling are Rab GTPases (Jordens et al., 2005; Wandinger-Ness and Zerial, 2014). Internalized integrin receptors enter early endosomes and can either be sorted by a ‘fast’ Rab4-mediated recycling or transit through a ‘slow’ Rab11-positive recycling pathway back to the plasma membrane (Bretscher, 1992; Rainero and Norman, 2013). Our data further support previous findings of a WASH function in regulating late endosomal integrin recycling in mammalian cell culture. WASH significantly co-localizes with Rab4- and Rab7-marked endosomes in macrophages. However, we only found a significant difference in ßPS-integrin signals in Rab7-marked endosomes in *wash* mutant macrophages. Thus, fast Rab4-mediated recycling of integrins might either be not dependent on WASH or might be masked by redundant functions of other WASP-protein family members. In summary, reduced focal adhesions, cell spreading and cell migration defects in *wash* mutant macrophages are most likely caused by an impaired recycling of ßPS-integrin.

In wild type, receptors that are not recycled remain within early endosomes as they undergo early-to-late endosomal maturation, a step that requires the exchange of Rab5 to Rab7. Rab7 is known to recruit the retromer on endosomal membrane and it is required for the transfer of cargo from the late endosomes to the lysosome (Priya et al., 2015; Pryor and Luzio, 2009). A conserved function of WASH in retromer-
dependent endocytic recycling of the luminal protein Serpentine has already been found in *Drosophila* trachea development (Dong et al., 2013). In macrophages, WASH also associates with large circular Rab7-positive late endosomes and mature lysosomes. We found prominent actin patches that depend on WASH function on both endosomal compartments. Such localized actin patches are thought to define discrete endosomal membrane domains facilitating protein sorting and vesicle fission (Derivery and Gautreau, 2010; Derivery et al., 2012; Derivery et al., 2009; Seaman et al., 2013). Carnell and colleagues described the first mechanistic model of how WASH-dependent actin polymerization directly controls protein sorting in *Dictyostelium* lysosomes (Carnell et al., 2011). In *Dictyostelium* WASH physically associates with the V-ATPase, a key proton pump that is crucial for the establishment and maintenance of the acidic pH in endosomes and lysosomes (Maxson and Grinstein, 2014). WASH directly drives the removal of V-ATPase from lysosomes and its recycling to small vesicles. Once the V-ATPase has been recycled, the lysosome becomes neutralized. Without WASH-driven actin polymerization, lysosomes never recycle V-ATPase into small vesicles and therefore do not neutralize to form post-lysosomes. In consequence, loss of WASH results in an insufficient retrieval of the V-ATPase and subsequently in an accumulation of undigested phagocytosed material that cannot be excreted by *Dictyostelium* (Carnell et al., 2011). In this study, we provided first evidence that this function of WASH is not unique for *Dictyostelium*, but likely represents a conserved mechanism in lysosome neutralization and V-ATPase recycling in higher eukaryotes such as *Drosophila*. *Drosophila* WASH binds Vha55, the V-ATPase regulatory B-subunit and loss of WASH function strongly affects phagolysosomal neutralization in macrophages.

Increased lysosome acidification is expected to have global effects not only on phagocytic digestion, but also on autophagy, an evolutionarily conserved process that mediates the degradation of intracellular materials in lysosomes. Supporting this notion, we observed an increased starvation-induced acidification of autolysosomes that significantly reduces the life span of mutant flies. A similar, but more severe phenotype has recently been reported in *wash* knockout mice (Xia et al., 2014). WASH deficiency results in extensive autophagy that most likely causes embryonic lethality (Xia et al., 2014). Thus, WASH function inhibits rather than stimulates autophagy as previously proposed based on RNAi cell culture experiments.
The mechanisms promoting autophagy in mice and flies, however, seem to be different. Increased autolysosomal acidification seems to promote autophagy in wash mutant flies whereas in mice WASH might directly inhibit the formation of autophagosomes before they fuse with lysosomes to form functional autolysosomes. This direct effect of WASH on autophagosome formation is independent of its function as an NPF, but seems to be mediated by inhibiting ubiquitination of Beclin1/Atg6 (Xia et al., 2014). This tumor suppressor is part of a lipid kinase complex that mediates the initial stages of autophagosome biogenesis by recruiting other Atg proteins (Levine et al., 2015; Wirawan et al., 2012). However, Beclin1/Atg6 proteins are known to exert numerous non-autophagic functions including protein sorting, endocytic, and phagosomal maturation (Levine et al., 2015; Wirawan et al., 2012). In flies, Atg6 is also required for multiple vesicle trafficking pathways and hematopoiesis (Shravage et al., 2013). Like in mammals, Drosophila Atg6 also directly interacts with the lipid kinase Vps34 and co-expression of Atg6 and Vsp34 is sufficient to induce autophagy. Consistent with its role as a tumor suppressor, loss of Atg6 function causes defects in blood cell differentiation resulting in an overproduction of macrophages (Shravage et al., 2013). Given the inhibitory function of WASH on Beclin1/Atg6 we overexpressed WASH in flies. However, neither macrophage-specific nor ubiquitous overexpression of WASH results in an increased number of macrophages or the formation of melanotic blood cell masses (data not shown). Thus, gain of WASH function cannot phenocopy atg6 mutants suggesting distinct or more complex regulatory mechanisms in flies compared to mammals.

**Experimental procedures**

**Fly genetics**

All crosses were performed at 25 °C unless indicated otherwise. washΔ185 flies were obtained from S. Parkhurst (Linardopoulou et al., 2007). Lethal mutations on the washΔ185 chromosome were removed by multiple outcrossing to wild type (w1118). The following strains were used: pUASt-Vha55-EGFP (Davies et al., 1996); hmlΔ-Gal4 (Sinenson and Mathey-Prevot, 2004); pUASt-EGFP-FAT (Sander et al., 2013), and UASt-Lifeact-EGFP, UAS-Lifeact-RFP, Df(2R), P{UASp-YFP.Rab4}, P{UAS-Rab7.GFP} the Bloomington Stock Center (IDs 26500, 58362, 9767, 24616, 42706); RNAi lines from the Vienna Drosophila RNAi Center (VDRC, IDs: 24642, 39769,
Transgenic pUAS-t-WASH-EGFP flies were generated using \( \Phi C31 \)-mediated transgenesis (\( M\{3xP3-RFP.attP\}ZH-86F \); (Bischof et al., 2007).

**Cell culture, cell transfection**

*Drosophila* S2R\(^+\) cells were propagated in 1\( \times \) Schneider’s *Drosophila* medium as described previously (Stephan et al., 2008). S2R\(^+\) cells were transfected as described previously (Bogdan et al., 2004).

**Structured illumination microscopy (SIM) imaging**

SIM images were taken with an ELYRA S.1 Microscope (CellObserver SD, 63\( \times \)/1.4 oil-immersion objective; Zeiss) using software ZEN 2010 D (Zeiss). Immunostainings and image acquisition were done as previously described (Brinkmann et al., 2016; (Zobel and Bogdan, 2013). Primary antibodies were used as follows: α-WASH (P3H3 from DSHB purified by protein-A affinity chromatography, 1:100), α-βPS-integrin (CF.6G11 from DSHB, 1:10); α-Rab7 (1:3000; Tanaka and Nakamura, 2008).

**Spinning disk microscopy imaging**

Live imaging of macrophages cultures was performed using a CellObserver SD spinning disk microscope (Zeiss) as reported (Sander et al., 2013).

**Co-immunoprecipitation experiments**

Co-immunoprecipitation with WASH-EGFP and Vha55-myc were performed as previously described (Fricke et al., 2009). Western blots were stained with α-myc (9E10 from DSHB, 1:10) and α-WASH (P3H3 from DSHB, 1:100) as primary antibodies.

**Sterility assay**

Single female (1 d old) wild type and wash\( \Delta 185^* \) mutant flies were crossed with 4 male (1 d old) wild type flies and left on *Drosophila* standard food for 7 days. 14 days after the crosses, obtained offspring were counted for each cross. The Mann-Whitney test was used for statistics; P value < 0.2121 (two-tailed).
Immunohistochemistry, pHrodo uptake assay and fat body staining

For immunohistochemistry, female wild type and washΔ185* mutant flies were kept on *Drosophila* standard food supplemented with fresh yeast for 1.5 days before the ovaries were dissected. Isolated ovarioles were fixed for 15 min with 4% paraformaldehyde (Sigma Aldrich) in PBS, permeabilized for 1 h in 0.1% Triton X-100 (Invitrogen) in PBS, blocked for 30 min in 3% BSA in PBS, and subsequently stained with phalloidin 488 and DAPI for 2 h. The *E. coli* particle pHrodo uptake assay, Lysotracker and Magic Red staining were performed according to manufacturer instructions (Molecular Probes, USA).

Quantifications of Vha55 vesicles, actin spots, and focal adhesion length

A total number of 50 wild type and 47 washΔ185* mutant macrophages were imaged and all Vha55-positive vesicles in a 0.5 µm stack were measured using the AxioVision SE64 Rel 4.9 software. The Mann-Whitney test was used for statistics; *P* value < 0.0001 (two-tailed). To quantify the number of actin spots at vesicular structures per cell, a total number of 80 wild type, 95 washΔ185* and 88 hmlΔ-Gal4 > wash RNAi macrophages expressing UASt-Lifeact-EGFP were imaged. The Mann-Whitney test was used for statistics; both times *P* value < 0.0001 (two-tailed).

βPS-integrin fluorescence intensity in Rab7-positive vesicles and βPS-integrin distribution

To quantify βPS-integrin immunostaining signal intensity in Rab7-positive vesicles, a total number of 6 wild type and 6 washΔ185* mutant macrophages were imaged using SIM. 12 vesicles in wild type and 19 in washΔ185* were analyzed using FIJI. The *t*-test was used for statistics; *P* value < 0.047. To quantify βPS-integrin immunostaining signal intensity and distribution, a total number of 23 wild type and 23 washΔ185* mutant macrophages were imaged using SIM. A diametrical line was drawn across each cell thereby determining the quantification section, which was used. To overcome differences in length, each line was rescaled by setting the beginning point to 0 and the endpoint to 1. In this rescaled line the position of each pixel is also rescaled between 0 and 1. To have relevant data for statistical purposes the rescaled line was divided into 100 equidistant slices. The immunostaining signals within each slice were pooled and the mean of intensity was calculated. Ribbons
represent SEM. For focal adhesion quantification, a total number of 75 wild type and 53 \textit{wash}\textDelta 185* mutant macrophages expressing UAS\textit{t}-EGFP-FAT were imaged using spinning disk microscopy, and quantified (1727 in wild type and 1346 in \textit{wash}\textDelta 185*) using the ZEN lite 2012 software. The Mann-Whitney test was used for statistics; \(P\) value < 0.0001 (two-tailed).

\textit{In vivo migration of pupae}
Live imaging of macrophages in prepupae and in the pupal wing was performed as reported recently (Sander et al. 2013; Brinkmann et al., 2016). Number of flies used for 2D migration: WT (\(n = 68\)), \textit{wash}\textDelta 185* (\(n = 143\)), \textit{hml}\textDelta -Gal4 > \textit{mys} RNAi (\(n = 64\)). The Mann-Whitney test was used for statistics; both times: \(p\) value < 0.0001 (two-tailed). Number of flies used for 3D wound ablation: WT (\(n = 18\)), \textit{wash}\textDelta 185* (\(n = 22\)), \textit{hml}\textDelta -Gal4 > \textit{mys} RNAi (\(n = 10\)).

\textit{Quantification of border cell migration}
100 wild type and 100 \textit{wash}\textDelta 185* mutant stage 10 egg chambers were stained for F-actin (phalloidin) and nuclei (DAPI). Egg were divided into four regions: 75-100 \%, 50-75 \%, 25-50 \%, and 0-25 \% motility and border cells were scored. The Wilcoxon matched-pairs signed rank test was used for statistics; \(P\) value > 0.9999 (two-tailed).

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Figure 1 Homozygous wash mutant flies are viable and fertile

(A) Schematic overview of the wash gene locus. The wash gene consists only of one exon. The dashed lines mark the chromosomal deletion in the washΔ185 mutant stock. The deficiency Df(2R)BSC699 spans the complete wash gene region. The insertion of the P-element used for imprecise excision is indicated by the red triangle.

(B-D) PCR confirmed a deletion of about 1 kb of wash in washΔ185 mutant flies (B). The originally published mutant fly stock crossed over the deficiency, the stock carrying the P-element as well as the viable washΔ185* stock we established show a complete loss of WASH expression in ovaries in western blot analysis (C, D).

(E) The sterility of washΔ185* female flies was tested by single females crossed with wild type males. There is now significant difference between the number of wild type and washΔ185* offspring (n = 111; wild type and n = 112; washΔ185*; Error bars represent SEM).

(F-I’) Egg chamber morphology was analyzed in more depth using a laser scanning confocal microscopy. Neither in earlier stages of germ line cyst development, nor in the formation of actin baskets surrounding the nucleus in washΔ185* nurse cells showed any defects (H-I’) compared to the wild type (F-G’). Furthermore, the laid eggs also were wild typical.

(J) Quantification of border cell migration. To quantify possible defects in border cell migration in washΔ185* mutants, each stage 10 egg chamber stained for F-actin (phalloidin) and nuclei (DAPI) was divided into four regions: 75-100 % motility (50-75 % motility, 25-50 % motility and 0-25 % motility. Border cells were scored and the percentages presented in histogram form. BC, border cell; ns, not significant.
Figure 2 Loss of WASH function results in integrin-dependent cell spreading defects

(A) Western blot analysis of lysates from isolated wild type and washΔ185* macrophages confirmed a complete loss of WASH expression in mutant cells. (B-C) Structured illumination microscopy (SIM) images of isolated (B) wild type and (C)
washΔ185* macrophages. Scale bars represent 10 µm. (B') In wild type macrophages, WASH localizes to F-actin coated subcellular structures in the perinuclear region. (D, E) SIM images of (D) wild type and (E) washΔ185* macrophages plated on ConA and stained for F-actin (phalloidin) and nuclei (DAPI). Scale bars represent 10 µm. (F, G) Quantification of cell spreading. (F) When spread on the lectin Concavalin-A (ConA), macrophages of both wild type and washΔ185* spread properly. (G) When settled on glass, washΔ185* mutant macrophages exhibit spreading defects, reducing the cell area to about the half of the wild type cell area (n = 250 per genotype and treatment). Macrophages re-expressing a WASH-EGFP transgene significantly restored their ability to spread. Overexpression of WASH-EGFP leads to a reduction in the overall spread cell area when compared to wild type (n = 150 per genotype). (H, I) SIM images of (H) wild type and (I) washΔ185* mutant macrophages stained for endogenous βPS-integrin (red), F-actin (green, phalloidin) and nuclei (blue, DAPI). Scale bars represent 10 µm. (J) Quantitative measurement of fluorescence intensities revealed an increased signal of βPS-integrin around the nuclei at the expense of peripheral staining in washΔ185* mutant cells. A total number of 23 wild type and 23 washΔ185* mutant macrophages were quantified. βPS-integrin distribution is altered in washΔ185* macrophages compared to wild type. Ribbons represent SEM. (K, M). SIM images of (K) wild type and (M) washΔ185* mutant macrophages plated on vitronectin-coated surfaces stained for endogenous βPS-integrin (red), F-actin (green, phalloidin) and nuclei (blue, DAPI). washΔ185* mutant macrophages exhibit spreading defects. Note: βPS-integrin localizes in distinct foci enriched along the leading edge of wild type cells. Scale bars represent 10 µm. (L) βPS-integrin marked foci co-localize with EGFP-FAT. (N, O) Frames from spinning disk movies of (N) wild type and (O) washΔ185* mutant macrophages expressing the focal adhesion marker EGFP-FAT. Scale bars represent 10 µm. (P) Quantification of focal adhesion lengths in washΔ185* macrophages compared to wild type (*** = p < 0.0001, error bars represent SD).
Figure 3 WASH induces dynamic actin patches on early and late endosomal vesicles

(A, A”) SIM images of a macrophage expressing the focal adhesion marker EGFP-FAT (green) stained for endogenous WASH (red) and F-actin (white, phalloidin). WASH did not co-localize with EGFP-FAT at focal adhesion sites. White box
highlights the area of the magnified inset shown in A’. Scale bars represent 10 µm. (B, B’’) SIM images of a wild type macrophage expressing WASH-EGFP (green) stained for βPS-integrin (red) and F-actin (white, phalloidin). WASH did not co-localize with EGFP-FAT at focal adhesion sites. White boxes highlight the areas of the magnified inset shown in B’ and B’’. Scale bars represent (B) 10 µm, (B’, B’’) 1 µm. WASH was found in close contact to βPS-integrin and surrounded βPS-integrin punctae in the perinuclear region. (C) Still images from a spinning disk movie of wild type macrophages expressing WASH-EGFP at indicated time points. WASH-EGFP localizes at dynamic vesicles of different sizes (arrowheads). Scale bars represent 10 µm. (D) Still images from a spinning disk movie of a wild type macrophage co-expressing WASH-EGFP with Lifeact-RFP. WASH-EGFP co-localizes with F-actin at dynamic vesicles. Scale bars represent 10 µm. (E-G) Still images from spinning disk movies of (E) wild type (F) wash knockdown and (G) washΔ185* mutant macrophages expressing a Lifeact-EGFP transgene. Loss of WASH function results in a complete loss of actin patches (arrowheads in E). White boxes highlight the areas of the magnified insets. Scale bars represent 10 µm. (H) Quantification of actin patches in macrophages of indicated genotype (wild type, n = 80; hmlΔ-Gal4 > wash RNAi = 88; washΔ185* = 95). *** = p < 0.0001, box = 25th and 75th percentiles, bars = min and max values. (I-U) SIM images of macrophages of the indicated genotypes stained for F-actin (phaloidin, white). Actin-coated WASH vesicles co-localizes with small Rab4-YFP vesicles (J-L) and larger Rab7 late endosomes marked by either (O, P, Q) a Rab7-GFP transgene or (S-U) an anti-Rab7 antibody. White boxes highlight the areas of the magnified insets. Scale bars represent 10 µm. (M) Analysis of co-localization between WASH and F-actin (n = 50), Rab4-YFP (n = 58), Rab7-GFP (n = 54) and endogenous Rab7 (n = 40) based on Pearson’s colocalization coefficient.
Figure 4 Loss of WASH function results in an increased accumulation βPS-integrin in Rab7-marked late endosomes

(A-E) SIM images of macrophages of the indicated genotypes stained for F-actin (phalloidin, white). Scale bars represent 10 µm. Macrophages expressing (A) a Rab4-YFP transgene or (B) wild type macrophages co-stained for βPS-integrin (red)
and F-actin (white). βPS-integrin punctae are frequently found in the lumen of Rab4- and larger Rab7-marked vesicles. (C) Increased βPS-integrin signals are found within Rab7-GFP positive late endosomes (arrowhead). (D, E) Endogenous Rab7 marks late endosomes in (D) wild type and (E) washΔ185* mutant macrophages with increased luminal βPS-integrin punctae (arrowheads). (F) Quantification of βPS-integrin fluorescence intensity confirmed a moderate but significant increase of βPS-integrin in the lumen of late endosomes marked by endogenous Rab7 in washΔ185* mutant macrophages. A total number of 6 wild type and 6 washΔ185* mutant macrophages were imaged using SIM. 12 vesicles in wild type and 19 in washΔ185* were analyzed using FIJI. * = p < 0.047. Error bars represent SEM.
Figure 5 WASH function is required for cell migration in vivo

(A–C’) Frames of spinning disk microscopy videos of migrating (A) wild type (B) βPS-integrin knockdown and (C) washΔ185* mutant macrophages expressing an EGFP transgene imaged from a living prepupae (2 h APF). Migratory tracks of individual cells are indicated (colored, jagged lines). Scale bars represent 10 µm. RNAi-mediated suppression of βPS-integrin function strongly affects random cell migration. washΔ185* mutant macrophages show reduced migration velocity and distance. (D) Quantification of cell spread area in living wild type (n= 55), washΔ185* mutant (n =
60) and βPS-integrin knockdown (n = 48) prepupae (2 h APF). The t-test was used for statistics; P value *** < 0.0001. (E, F) Quantification of the mean cell speed and distance of macrophages of indicated genotypes; wild type (n = 68), washΔ185* (n = 143), mys knockdown (n = 64). p-values were calculated using Mann-Whitney test; *** indicates p < 0.0001. (G-I’) Scale bars represent 10 μm. (G-G’) Frames of a spinning disk microscopy video of wild type macrophages that migrate towards a laser-ablated cell (indicated by the yellow circle). (H-H’) Frames of a spinning disk microscopy video of βPS-integrin knockdown macrophages that show a strongly impaired migratory behavior towards the wound (indicated by the yellow circle). (I-I’) washΔ185* mutant macrophages respond properly towards the wound without changes in directionality and velocity. Scale bars represent 10 μm. (J) Mean HMMS values for wild type (blue, n = 18), βPS-integrin knockdown (red = 10) and washΔ185* mutant (green, n = 22), macrophage (17h APF) movies plotted for 1800 seconds after wounding. An overall higher mean HMMS value is observed for the wild type cells compared to βPS-integrin knockdown and washΔ185* mutant cells.
Figure 6 WASH localizes to lysosomal vesicles in *Drosophila* macrophages as well as in S2R\(^+\) cells

(A) Still images of spinning disk movie of a S2R\(^+\) cell co-expressing the lysosome-associated membrane protein Lamp1-EGFP and Lifeact-mCherry. (B, C) SIM images of S2R\(^+\) cells expressing (B, B\(^{'}\)) Lamp1-EGFP (green) and (C, C\(^{'}\)) stained for endogenous WASH (red) and F-actin (white, phalloidin). (D, E) SIM images of isolated (D, D\(^{'}\)) wild type and (E, E\(^{'}\)) wash\(^{Δ185}\) mutant macrophages expressing the
V-ATPase subunit Vha55-EGFP (green) stained for F-actin (red). White boxes highlight the areas of the magnified insets. F-actin patches are present at Vha55-EGFP positive vesicles in wild type macrophages (arrowheads in D'). *washΔ185* mutant macrophages show enlarged Vha-55-marked lysosomes. Scale bars represent 10 µm. (F) Quantification of Vha55-EGFP vesicle size in wild type and *washΔ185* macrophages. Wild type cells have an average size of 0.36 µm² (arrowheads, n = 628), whereas in mutant cells the vesicle size is increased to an average size of 0.59 µm² (n = 549). *** = p < 0.0001, error bars represent SEM. (G) Co-immunoprecipitation of WASH and Vha55 reveals that both proteins physically associate.
Figure 7 WASH is required in phagocytic neutralization of macrophages

(A) Still image of a spinning disk movie of a macrophage expressing Lifeact-EGFP (green) with phagocytosed *E. coli* particle conjugated with pH-sensitive fluorescent dye (pHrodo, magenta). Arrowheads mark F-actin patches on phagolysosomes. The white box highlights the area of the magnified insets shown in (A') at different, indicated time points. Arrows mark phagocytosed *E. coli* particle that enters a...
lysosome with an increase in fluorescent intensity. (B) SIM image of a macrophage expressing WASH-EGFP (green) and phagocytosing pHrodo (magenta) show an increase of WASH-EGFP patches at vesicles that are positive for pHrodo (magenta) and F-actin (white). Scale bar represents 10 µm. (C) Still images of spinning disc movie of a macrophage expressing WASH-EGFP that is recruited to a phagocytized E. coli pHrodo particle upon acidification. (D) Quantification of increased WASH-EGFP and E. coli pHrodo fluorescence intensities over time. Error bars represent SEM. (E) Time course of fluorescence intensity of phagocytized pHrodo labeled E. coli particles demonstrate lysosomal neutralization defects in washΔ185* macrophages. Images are taken at indicated time points. (F) Quantification of E. coli pHrodo particle fluorescence intensities in wild type and washΔ185* mutant macrophages over time. The mean of three independent experiments is depicted (n between 7 and 30 cells per genotype and time point, error bars represent SEM).
Figure 8 Increased acidification of autophagic vesicles in wash mutant flies

(A-L) Confocal microscopy images of Drosophila fat bodies stained with Lysotracker Red of (A-C, G-I) wild type and (D-F, J-L) washΔ185* mutant larvae under fed (A-F, scale bar represents 50 µm) and starved (G-L, scale bar represents 20 µm) conditions. In the washΔ185* mutant fat body, an increase of acidified vesicles under starved conditions can be observed. (M, N) Lysosomes in wash mutants seem to be functional, as they are able to process Cathepsin B (visualized by Magic red staining), scale bars represent 10 µm. (O) WASH localizes to F-actin enriched vesicular structures in the fat body cells. (P) WASH localizes at distinct domains of Atg8-EGFP positive vesicles (scale bars in O, P represent 20 µm). (Q) The lipidation of Atg8 is also increased in the washΔ185* mutant larval fat body. (R) washΔ185* mutant female flies have a decreased life span compared to wild type, which can be rescued by re-expressing WASH-EGFP (the mean of three independent experiments).
is depicted; \( n = 150 \), wild type and \( \text{wash}^{\Delta 185^*} \), and \( n = 100 \), rescue, error bars represent SE).
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Supplementary material

Supplementary figure S1
WASH is associated in a pentameric complex whose stability depends on each member. When single complex members are knocked down by RNAi, macrophages are unable to spread properly, comparable to the wash mutant situation (n = 200 per genotype). *** = p < 0.0001, error bars represent SEM.
Supplementary Movie M1

Spinning disc microscopy time-lapse movie of macrophages expressing EGFP- FAT using the hmlΔGal4 driver ex vivo plated on coated and uncoated surfaces, as well as in vivo in a prepupa.
Supplementary Movie M2

Spinning disc microscopy time-lapse movie of ex vivo cultured wild type and washΔ185* mutant macrophages expressing EGFP-FAT using the hmlΔGal4 driver.
Supplementary Movie M3

Spinning disc microscopy time-lapse movie of ex vivo cultured macrophages expressing WASH-EGFP using the hmlΔGal4 driver.
**Supplementary Movie M4**

Spinning disc microscopy time-lapse movie of *ex vivo* cultured macrophages expressing WASH-EGFP and Lifeact-RFP using the *hmlΔGal4* driver.
Supplementary Movie M5

Spinning disc microscopy time-lapse movie of *ex vivo* cultured macrophages expressing Lifeact-EGFP using the *hmlΔGal4* driver.
Supplementary Movie M6

Spinning disc microscopy time-lapse movie of *ex vivo* cultured (A) wild type (B) *wash* RNAi depleted and (C) *washΔ185* mutant macrophages expressing Lifeact-EGFP using the *hmlΔ* Gal4 driver.
**Supplementary Movie M7**

Spinning disc microscopy video of migrating (A) wild type, (B) βPS-integrin knockdown and (C) *washΔ185* mutant macrophages expressing a EGFP transgene imaged from a living prepupa (2 h APF). Migratory tracks of individual cells are indicated (colored, jagged lines).
Supplementary Movie M8
Spinning disc microscopy video of migrating (A) wild type, (B) βPS-integrin knockdown and (C) washΔ185* mutant macrophages expressing a EGFP transgene imaged from a pupal wing (17 h APF) upon laser-induced cell ablation.
Supplementary Movie M9
Spinning disc microscopy time-lapse movie of cultured S2R+ cells transfected with Lifeact-mCherry and Lamp1-EGFP.
Supplementary Movie M10

Spinning disc microscopy time-lapse movie of cultured S2R⁺ cells transfected with WASH-mCherry and Lamp1-EGFP.
Supplementary Movie M11

Spinning disc microscopy time-lapse movie of cultured S2R* cells transfected with WASH-mCherry and Vha55-EGFP.
Supplementary Movie M12
Spinning disc microscopy time-lapse movie of an ex vivo cultured macrophage expressing Lifeact-EGFP using the hmlΔGal4 driver phagocytosing pHrodo-conjugated E. coli
Supplementary Movie M13

Spinning disc microscopy time-lapse movie of an ex vivo cultured macrophage expressing WASH-EGFP (green) and phagocytosing pHrodo (magenta). WASH-EGFP is recruited to phagolysosomes with internalized *E. coli* pHrodo particles upon acidification.