Regulation of WASH-Dependent Actin Polymerization and Protein Trafficking by Ubiquitination

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SUMMARY

Endosomal protein trafficking is an essential cellular process that is deregulated in several diseases and targeted by pathogens. Here, we describe a role for ubiquitination in this process. We find that the E3 RING ubiquitin ligase, MAGE-L2-TRIM27, localizes to endosomes through interactions with the retromer complex. Knockdown of MAGE-L2-TRIM27 or the Ube2O E2 ubiquitin-conjugating enzyme significantly impaired retromer-mediated transport. We further demonstrate that MAGE-L2-TRIM27 ubiquitin ligase activity is required for nucleation of endosomal F-actin by the WASH regulatory complex, a known regulator of retromer-mediated transport. We further demonstrate that MAGE-L2-TRIM27 ubiquitin ligase activity is required for nucleation of endosomal F-actin by the WASH regulatory complex, a known regulator of retromer-mediated transport. Mechanistic studies showed that MAGE-L2-TRIM27 facilitates K63-linked ubiquitination of WASH K220. Significantly, disruption of WASH ubiquitination impaired endosomal F-actin nucleation and retromer-dependent transport. These findings provide a cellular and molecular function for MAGE-L2-TRIM27 in retrograde transport, including an unappreciated role of K63-linked ubiquitination and identification of an activating signal of the WASH regulatory complex.

INTRODUCTION

Endosomal protein recycling pathways facilitate the transfer of membrane proteins from early and late endosomes back to the trans-Golgi network (TGN) or plasma membrane (Bonifacino and Rojas, 2006). In doing so, these pathways generally function to prevent lysosomal delivery and degradation of membrane proteins. Endosome-to-Golgi transport, referred to as retrograde transport, is an important cellular process that facilitates the recycling of a variety of proteins, including sorting receptors (such as CI-M6PR), SNARE membrane fusion proteins, membrane receptors, metabolite transporters, and several proteins that undergo polarized localization/secretion (Bonifacino and Rojas, 2006; Johannes and Popoff, 2008). Importantly, retrograde transport has been implicated in a number of different human pathologies. Endosome-to-Golgi transport is essential for cellular entry of pathogenic toxins, such as Shiga, cholera, and ricin, as well as viral pathogens such as HIV (Brass et al., 2008; Sandvig and van Deurs, 2005). Furthermore, components of the retrograde transport pathway are downregulated in Alzheimer’s disease and upregulated in cancer (Scott et al., 2009; Small, 2008).

Recent studies have started to define the molecular machinery crucial for different aspects of this process, including cargo recognition, endosomal membrane budding, tubulation and scission, and vesicle transport, tethering, and fusion at the TGN (Bonifacino and Rojas, 2006; Cullen and Korswagen, 2012). One critical component is the retromer protein complex that consists of VPS26, VPS29, and VPS35 and functions to recognize retrograde cargo on endosomes (Bonifacino and Hurley, 2008). Another essential factor in retrograde transport is WASH. WASH is a member of the Wiskott-Aldrich syndrome protein (WASP) family consisting of WASP/N-WASP, WAVE, WHAMM, JMY, and WASH (Campellone and Welch, 2010). Like other WASP family members, WASH contains a carboxy-terminal VCA (verprolin homologous or WH2, central hydrophobic, and acidic) motif that binds to actin and the Arp2/3 complex to stimulate actin filament nucleation (Derivery et al., 2009; Duleh and Welch, 2010; Jia et al., 2010; Linardopoulou et al., 2007; Liu et al., 2009). Recent studies have demonstrated that WASH is endosomal-associated, exists in a macromolecular complex termed the WASH regulatory complex (SHRC) and functions downstream of the retromer complex to facilitate endosome-to-Golgi transport (Derivery et al., 2009; Duleh and Welch, 2010; Gomez and Billadeau, 2009; Linardopoulou et al.,
RESULTS

Identification of MAGE-L2 E3 RING Ubiquitin Ligase Partner and Subcellular Localization

To determine the specific E3 RING ubiquitin ligase partner of MAGE-L2 and gain insight into its cellular function, we examined MAGE-L2 binding partners by tandem affinity purification (TAP) coupled to mass spectrometry. The E3 RING ubiquitin ligase TRIM27 was identified as a major binding partner of MAGE-L2 (Figure 1A). Binding of MAGE-L2 and TRIM27 was confirmed by reciprocal communoprecipitation experiments (Figures 1B and S1C available online). Moreover, in vitro translated MAGE-L2 bound recombinant GST-TRIM27 but not GST alone, indicating the interaction between the two proteins is direct (Figure 1C). To confirm the association of MAGE-L2 and TRIM27, we stably expressed GFP-MAGE-L2 and mCherry-TRIM27 in U2OS cells and examined their colocalization by live-cell microscopy. We found that the two proteins colocalized in discrete cytoplasmic puncta (Figure 1D), as well as in a smaller nuclear pool (data not shown). These findings suggest that TRIM27 binds MAGE-L2 and that the MAGE-L2-TRIM27 ubiquitin ligase complex localizes to discrete cytoplasmic structures.

MAGE-L2-TRIM27 Binds and Localizes to Retromer-Containing Endosomes

To determine the identity of the MAGE-L2-TRIM27 structures, we reanalyzed our mass spectrometry data of MAGE-L2 interacting proteins for clues. Interestingly, MAGE-L2 was identified to interact with VPS35 and VPS26 (Figure 1A), two components of the endosomal retromer complex. mCherry-TRIM27 colocalized with GFP-tagged VPS35, VPS29, and VPS26, as well as the retromer-associated SHRC proteins, WASH and FAM21 (Figure S1A). Furthermore, costaining for endogenous VPS35 and TRIM27 clearly identified TRIM27 cytoplasmic structures as retromer-positive endosomes (Figure 1B). Unfortunately, we were unable to observe endogenous MAGE-L2 due to the lack of specific, high-quality antibodies (data not shown). More detailed analysis of TRIM27 localization revealed its localization to numerous tubular structures emanating from endosomes (Figure 1F), a property shared with the retromer complex (Argihi et al., 2004; Bonifacino and Hurley, 2008). These findings suggest that MAGE-L2-TRIM27 localizes to the retromer-positive subset of endosomes.

Next, we confirmed the interaction between MAGE-L2-TRIM27 and the retromer complex initially observed by mass spectrometry. MAGE-L2 and TRIM27 communoprecipitated with all three components of the retromer complex (Figures S1B and S1C and data not shown) and MAGE-L2 directly bound recombinant GST-VPS35 in vitro through its WH-B motif (Figures S1D, S1G, and S1H). In addition, MAGE-L2 interaction with VPS35 did not impair VPS35 binding to the SHRC component FAM21 (Figure S1I). Furthermore, MAGE-L2-VPS35 interaction is functionally important because knockdown of VPS35 dramatically inhibited MAGE-L2 and TRIM27 endosomal localization (Figures S1E and S1F and data not shown). These findings suggest that VPS35 recruits MAGE-L2-TRIM27 to retromer-positive endosomes by binding to MAGE-L2.
MAGE-L2-TRIM27 Is Required for Endosomal Protein Recycling

We next assessed whether MAGE-L2-TRIM27 participates in endosome-to-Golgi transport. Two independent siRNAs targeting MAGE-L2 or TRIM27 were identified that significantly reduced their target protein's levels but had no effects on the levels of other known essential factors required for retrograde transport (Figures S2 A–S2C). Multiple siRNAs targeting MAGE-L2 or TRIM27 resulted in impaired CI-M6PR and TGN46 trafficking to a degree similar to VPS35-RNAi (Figures 2 A–2C and S2D). Importantly, the overall organization of the TGN was unaffected in MAGE-L2- or TRIM27-RNAi cells (Figure S2 E). The steady-state defects in CI-M6PR localization were corroborated by examining transport of a small pool of surface labeled CI-M6PR in MAGE-L2- or TRIM27-RNAi cells colocalized with the endosomal marker EEA1 (Figures 2E and S2F). Furthermore, CI-M6PR protein levels were reduced in MAGE-L2- and TRIM27-RNAi cells and Cathepsin D processing and trafficking were impaired (Figure 2F). Finally, overexpression of MAGE-L2 in combination with TRIM27 increased CI-M6PR endosomal retrieval kinetics (Figure S2G). These results suggest that MAGE-L2-TRIM27 is required for endosome-to-Golgi retrograde transport.

To extend our findings, we examined the requirement of TRIM27 on the trafficking of two additional physiologically and pathologically relevant substrates of the retromer and SHRC complexes. First, knockdown of TRIM27 significantly impaired trafficking of the retromer cargo cholera toxin subunit B (CTxB) to the TGN (Figure 2G). However, the trafficking of the SHRC-independent cargo Transferrin receptor to perinuclear recycling endosomes was unaltered in TRIM27-RNAi cells (Figure 2G) (Duleh and Welch, 2010; Gomez and Billadeau, 2009; Gomez et al., 2012). In addition, the retromer and SHRC complexes have been implicated in the recycling of endosomal proteins to the plasma membrane, including integrins (Duleh and Welch, 2012; Zech...
Figure 2. MAGE-L2-TRIM27 Is Required for Endosomal Protein Recycling

(A) Cells were treated with the indicated siRNAs for 72 hr and stained for CI-M6PR (green) and DNA (blue).

(B) Quantitation of cells shown in (A). Compact juxtanuclear or dispersed CI-M6PR was scored and the percentage of cells with dispersed CI-M6PR is shown.

(C) Cells were treated with the indicated siRNAs for 72 hr and stained for TGN46 (red) and DNA (blue).

(D) Cells were treated with the indicated siRNAs. Cell surface CI-M6PR was labeled with anti-CI-M6PR antibody for one hour before imaging the pool of internalized cell surface-labeled CI-M6PR.

(E) Cells were treated with the indicated siRNAs and stained for CI-M6PR (green), EEA1 (red), and DNA (blue). XZ and YZ projection stacks and Pearson’s correlation coefficients (Rr) are shown.

(F) Cells were treated with the indicated siRNAs for 72 hr and cell lysates and media were analyzed by immunoblotting. pCatD represents unprocessed pro-CatD, iCatD indicates intermediate processed CatD, and mCatD represents fully matured CatD.

(G) Cells were treated with the indicated siRNAs for 72 hr, and transport of CTxB-488 or Tf-568 was determined at the indicated times.

(legend continued on next page)
to the wild-type protein (Figure S3B). These results suggest that the ubiquitin ligase activity of MAGE-L2-TRIM27 is important for proper retrograde transport.

Next, we examined which of the 35 different E2 ubiquitin-conjugating enzymes functions with MAGE-L2-TRIM27 to facilitate retrograde endosome-to-Golgi trafficking of CI-M6PR. Of the 35 E2 enzymes examined, knockdown of only Ube2O and Ube2L6 resulted in dramatic alteration of CI-M6PR localization (Figures 3B and S3C, and data not shown). Ube2O-RNAi showed similar penetrance as TRIM27-RNAi (Figures 3A and S3A). Importantly, the TRIM27 RING mutant localized similarly to the wild-type protein (Figure S3B). These results suggest that the ubiquitin ligase activity of MAGE-L2-TRIM27 is important for proper retrograde transport.

Next, we assessed the precise mechanism by which MAGE-L2-TRIM27, Ube2O, and K63-linked ubiquitin promotes retrograde transport. Retrograde transport requires several ordered steps to facilitate endosome-to-Golgi transport, including endosomal localization of the retromer complex and localization and activation of the SHRC to facilitate endosomal F-actin accumulation by the Arp2/3 complex (Cullen and Korswagen, 2012). We interrogated several of these specific steps to determine when ubiquitination may be important. The localization of the retromer complex to CI-M6PR substrate-containing endosomes was unaffected by depletion of TRIM27 or K63-linked ubiquitin chains (Figures S4A and S4B). In addition, the SHRC was still recruited normally to retromer-positive endosomes in TRIM27-RNAi cells (Figure S4C). However, knockdown of MAGE-L2 or TRIM27 resulted in reduced endosomal F-actin to a degree similar to WASH-RNAi (Figures 4A and 4B). Similarly, knockdown of the physiologically relevant Ube2O E2 enzyme, but not Ube2L6, resulted in a reduced endosomal F-actin (Figures 4A and 4B). Consistent with reduced endosomal F-actin, knockdown of MAGE-L2, TRIM27, or Ube2O, but not Ube2L6, significantly reduced the accumulation of the Arp2/3 complex subunit, ARPC5, on SHRC-positive endosomes (Figures 4C and 4D). Notably, there was no defect in total ARPC5 or F-actin levels (Figure 4D and data not shown). Likewise, depletion of K63-linked ubiquitin chains resulted in the specific reduction of retrograde transport.

**Retrograde Transport Requires K63-Linked Ubiquitin Chains**

We next investigated whether retrograde transport was dependent on ubiquitin, and if so, which type of polyubiquitin chain. To do so, we utilized the previously developed system (Xu et al., 2009) to inducibly knockdown ubiquitin by addition of tetracycline and replace it with a ubiquitin variant that is unable to produce a specific ubiquitin chain, namely ubiquitin in which lysine 48 or lysine 63 has been mutated to arginine (K48R or K63R, respectively; Figures S3E and S3F). We found that depletion of ubiquitin from cells dramatically affected CI-M6PR trafficking (Figures 3F and S3G). Addition of wild-type or K48R ubiquitin completely rescued CI-M6PR localization (Figures 3F and S3G), suggesting that ubiquitin is required for retrograde trafficking, but K48-linked ubiquitin chains are not. In contrast, depletion of K63-ubiquitin chains dramatically blocked CI-M6PR TGN localization and relocalized it to EEA1-positive endosomes (Figures 3F and S3G and data not shown) but had no significant effect on the overall organization of the TGN (Figure S3H). Similarly to the steady-state behavior, retrograde transport of cell surface-labeled CI-M6PR to the TGN is also dependent on K63-linked ubiquitination (Figures 3G and S3). Furthermore, depletion of K63-linked ubiquitin chains altered trafficking of the lysosomal hydrolase Cathepsin D, resulting in the reduction of mature Cathepsin D, accumulation of pro- and intermediate-Cathepsin D, and the secretion of pro-Cathepsin D into the cell culture media (Figure 3H). These results suggest that K63-linked ubiquitination is important for proper retrograde transport.

**MAGE-L2-TRIM27, Ube2O, and K63-Linked Ubiquitin Chains Are Required for Efficient Endosomal F-Actin Assembly**

Next, we assessed the precise mechanism by which MAGE-L2-TRIM27, Ube2O, and K63-linked ubiquitination promotes retrograde transport. Retrograde transport requires several ordered steps to facilitate endosome-to-Golgi transport, including endosomal localization of the retromer complex and localization and activation of the SHRC to facilitate endosomal F-actin accumulation by the Arp2/3 complex (Cullen and Korswagen, 2012). We interrogated several of these specific steps to determine when ubiquitination may be important. The localization of the retromer complex to CI-M6PR substrate-containing endosomes was unaffected by depletion of TRIM27 or K63-linked ubiquitin chains (Figures S4A and S4B). In addition, the SHRC was still recruited normally to retromer-positive endosomes in TRIM27-RNAi cells (Figure S4C). However, knockdown of MAGE-L2 or TRIM27 resulted in reduced endosomal F-actin to a degree similar to WASH-RNAi (Figures 4A and 4B). Similarly, knockdown of the physiologically relevant Ube2O E2 enzyme, but not Ube2L6, resulted in a reduced endosomal F-actin (Figures 4A and 4B). Consistent with reduced endosomal F-actin, knockdown of MAGE-L2, TRIM27, or Ube2O, but not Ube2L6, significantly reduced the accumulation of the Arp2/3 complex subunit, ARPC5, on SHRC-positive endosomes (Figures 4C and 4D). Notably, there was no defect in total ARPC5 or F-actin levels (Figure 4D and data not shown). Likewise, depletion of K63-linked ubiquitin chains resulted in the specific reduction of retrograde transport.
Figure 3. Ube2O E2 and K63-Ubiquitin Chains Are Required for Retrograde Transport

(A) Cells were treated with control or TRIM27 siRNAs for 24 hr before transfection of RNAi-resistant wild-type or RING mutant TRIM27. Forty-eight hours after transfection, cells were stained for CI-M6PR. The percentage of transfected cells with dispersed CI-M6PR is shown.

(B) Cells were treated with siRNAs targeting the indicated E2 enzymes for 72 hr, stained with anti-CI-M6PR, imaged, and quantitated. Dotted line denotes cutoff that reproducibly indicates CI-M6PR trafficking defect.

(C) Cells were treated with the indicated siRNAs for 72 hr, stained with CI-M6PR, and imaged. The percentage of cells with dispersed CI-M6PR is shown.

(D) Cells were treated with the indicated siRNAs for 72 hr and stained for CI-M6PR (green), EEA1 (red), and DNA (blue). XZ and YZ projection stacks and Pearson’s correlation coefficients (Rr) are shown.

(E) Cells were treated with the indicated siRNAs for 72 hr, stained for CI-M6PR, imaged, and CI-M6PR intensity was determined.

(F) Ubiquitin replacement cell lines were treated with or without tetracycline for 72–96 hr before steady-state CI-M6PR localization was determined by immunostaining. The percentage of cells with dispersed CI-M6PR is shown.
Data are represented as the mean ± SEM. Asterisk indicates p < 0.05. Scale bars, 20 µm. See also Figure S3.

**Endosomal F-actin and Arp2/3 complex, without affecting total F-actin, Arp2/3 complex, or SHRC levels (Figures 4E–4H, S3J, and data not shown).** Consistent with the previously described role of SHRC and endosomal F-actin in membrane tubule scission (Derivery et al., 2009; Gomez and Billadeau, 2009), TRIM27-RNAi, shUb-Ub, and shUb-Ub(K63R) cells accumulated endosomal tubules (Figures S4D and S4E). These results suggest that K63-linked ubiquitination by MAGE-L2-TRIM27 and Ube2O is required for localization of the Arp2/3 complex to the SHRC and generation of endosomal F-actin.

**Uninhibited Endosomal Actin Assembly Rescues Endosome-to-Golgi Transport Defects of TRIM27-RNAi and K63-Ubiquitin Depleted Cells**

We next examined whether the defective endosome-to-Golgi retrograde trafficking in TRIM27-RNAi or shUb-Ub(K63R) cells is due to decreased endosomal F-actin. First, we designed a fusion protein in which endosomal F-actin accumulation could be induced by an uninhibited WASH VCA motif. WASH VCA motif was fused to the C-terminal domain of FAM21 (Δ356N), which binds VPS35 for endosomal targeting (Figure 5A) (Gomez and Billadeau, 2009; Jia et al., 2012). Upon expression in cells, this fusion protein localized to retromer-positive endosomes (Figure 5A) and increased F-actin accumulation on endosomes (Figures 5A and 5B). We next determined whether this FAM21-WASH-VCA fusion could rescue retrograde transport in TRIM27-RNAi cells. Indeed, this was the case for both Cathepsin D trafficking (Figure 5C) and CI-M6PR TGN localization (Figures 5D and S4F). Furthermore, the FAM21-WASH-VCA fusion rescued CI-M6PR trafficking in cells deficient for K63-linked ubiquitin chains (Figures 5E and S4G). Therefore, the primary requirement for TRIM27 and K63-linked ubiquitin chains in retrograde trafficking appears to be for accumulation of endosomal F-actin.

**MAGE-L2-TRIM27 Ubiquitinates WASH K220 to Promote Endosomal F-Actin Assembly**

Generation of endosomal F-actin is facilitated by the SHRC, which binds the Arp2/3 complex and actin through a conserved VCA domain in WASH (Derivery et al., 2009; Gomez and Billadeau, 2009). However, the WASH VCA domain exists in an autoinhibited state that must be relieved before nucleation of F-actin by the Arp2/3 complex can be achieved (Jia et al., 2010). Therefore, we speculated that MAGE-L2-TRIM27 may facilitate WASH-VCA exposure, Arp2/3 complex and actin binding, and consequent F-actin nucleation on endosomes through targeted ubiquitination of the SHRC. Based on the highly homologous WAVE regulatory complex, regulators of SHRC activation are predicted to act on WASH itself or SWIP (Chen et al., 2010; Jia et al., 2010). Although we were unable to detect any ubiquitination of endogenous SWIP (Figure S5A), endogenous WASH was highly ubiquitinated (Figure 6A). WASH ubiquitination was TRIM27-dependent, as knockdown of TRIM27 dramatically reduced endogenous WASH ubiquitination (Figure 6B). Furthermore, WASH ubiquitination was K63 linked, as WASH was unable to be polyubiquitinated by K63R ubiquitin (Figure 6C). These findings suggest that TRIM27 is required for K63-linked ubiquitination of WASH.

Next we investigated the importance of WASH ubiquitination. To do so, we first determined the specific lysine in WASH that is conjugated to K63-linked ubiquitin chains. A single lysine, K220, in WASH had been identified in global proteomics studies to be ubiquitinated (Kim et al., 2011). Significantly, K220 is a highly conserved residue in species that express TRIM27 orthologs (Figure S5D). This residue is located in a region of WASH that is analogous to the “meander” region of WAVE, which is known to make contacts necessary for intracomplex inhibition in the wave regulatory complex (Chen et al., 2010). Therefore, we examined whether WASH ubiquitination was dependent on K220. Endogenous WASH was knocked down by RNAi and YFP-tagged wild-type or K220R WASH was re-expressed to ensure integration of the re-expressed WASH into the SHRC. Unlike YFP-WASH wild-type, YFP-WASH K220R failed to be ubiquitinated (Figure 6C), suggesting that WASH ubiquitination is dependent on K220. We next examined whether WASH ubiquitination is important for retrograde transport. Using our re-expression system, YFP-WASH K220R, unlike wild-type YFP-WASH, could not support proper retrograde transport resulting in CI-M6PR dispersion (Figures 6D and S5B) and degradation (Figures 6E and S5B) and Cathepsin D secretion (Figure 6F). These results suggest that K63-linked ubiquitination of WASH K220 by TRIM27 is required for WASH function in retrograde transport.

We next examined whether WASH K220 ubiquitination may act as a signal to relieve WASH autoinhibition and promote its activity toward the Arp2/3 complex. As previously reported (Derivery et al., 2009), knockdown of WASH resulted in reduced endosomal Arp2/3 complex localization (Figures 6G and S5C). Unlike wild-type YFP-WASH, re-expression of YFP-WASH K220R was unable to rescue proper endosomal Arp2/3 complex localization (Figures 6G and S5C). Importantly, YFP-WASH K220R localized properly to endosomes indicating that it still incorporated into the SHRC (Figure S5C). These results suggest that ubiquitination of WASH K220 is required for WASH activation and Arp2/3 complex endosomal localization.

**In Vitro Reconstitution of MAGE-L2-TRIM27 and K63-Ubiquitin-Dependent SHRC Activity**

Finally, we developed an in vitro reconstitution system to directly test whether ubiquitination of WASH is required for its actin assembling activity. To do so, we reconstituted WASH knock-out MEFs (Gomez et al., 2012) with stable expression of (G) Ubiquitin replacement cells were treated with or without tetracycline for 72–96 hr. Cell surface CI-M6PR was then labeled with anti-CI-M6PR antibody for one hour and internalized cell surface-labeled CI-M6PR was imaged. CI-M6PR localization was determined and the percentage of cells showing juxtanuclear endosomal F-actin and Arp2/3 complex, without affecting total F-actin, Arp2/3 complex, or SHRC levels (Figures 4E–4H, S3J, and data not shown). Consistent with the previously described role of SHRC and endosomal F-actin in membrane tubule scission (Derivery et al., 2009; Gomez and Billadeau, 2009), TRIM27-RNAi, shUb-Ub, and shUb-Ub(K63R) cells accumulated endosomal tubules (Figures S4D and S4E). These results suggest that K63-linked ubiquitination by MAGE-L2-TRIM27 and Ube2O is required for localization of the Arp2/3 complex to the SHRC and generation of endosomal F-actin.
Figure 4. MAGE-L2-TRIM27, Ube2O, and K63-Linked Ubiquitination Are Required for Endosomal F-Actin Assembly

(A) Cells were treated with the indicated siRNAs for 72 hr before staining VPS35 (green), F-actin (red), and DNA (blue). XZ and YZ projection stacks are shown.

(B) F-actin intensity on VPS35-positive endosomes of cells described in (A).

(C) Cells were treated with the indicated siRNAs for 72 hr before staining ARPC5 (green), FAM21 (red), and DNA (blue). XZ and YZ projection stacks are shown.

(D) Cells treated as in (C) were imaged and endosomal (solid bars) or total (open bars) ARPC5 levels were determined.

(E) shUb-Ub(K63R) ubiquitin replacement cells were treated with or without tetracycline for 96 hr before staining for WASH (green), F-actin (red), and DNA (blue). XZ and YZ projection stacks are shown.

(F) Cells described in (E) were imaged, and F-actin intensity on retromer-positive endosomes was quantitated and is shown.
HA-GFP-WASH wild-type, ΔVCA, or K220R. The intact SHRC was then purified from each of these cell lines by anti-HA chromatography to near homogeneity (Figures S6A and S6B). Under these conditions, very little free WASH is present (Figure S6B).

The activity of the reconstituted SHRCs was first assayed by examining their capacity to assemble F-actin on beads in cell lysates (Cory et al., 2003). As expected, SHRC stimulated F-actin accumulation on beads in a manner dependent on the VCA motif of WASH and was inhibited by cytochalasin D (Figure 7A). Importantly, WASH knockout MEFs reconstituted with WASH K220R mutant did not support actin assembly on beads (Figure 7A). In addition, the activity of SHRC was dependent on MAGE-L2-TRIM27, as SHRC isolated from MAGE-L2- or TRIM27-RNAi cells was significantly less active (Figure 7B). Furthermore, we examined the activity of purified SHRC in Arp2/3 complex-dependent pyrene-actin assembly assays. SHRC reconstituted

Figure 5. Uninhibited WASH-VCA Bypasses the Requirement for TRIM27 and K63-Linked Ubiquitin Chains in Retrograde Transport

(A) Schematic of uninhibited, endosomal localized WASH-VCA (top). Cells were transiently transfected for 48 hr before staining GFP-FAM21Δ356N-WASH-VCA (green), F-actin (red), VPS35 (cyan), and DNA (blue). Scale bar, 20 μm. Pearson’s correlation coefficient (Rr) is shown.

(B) Cells were transfected with GFP-alone or GFP-FAM21Δ356N-WASH-VCA and VPS35-localized F-actin intensity was determined.

(C) Cells were treated with control or TRIM27 siRNAs for 24 hr before transfection with control YFP-WASH or constitutively active GFP-FAM21Δ356N-WASH-VCA. Forty-eight hours later, the indicated proteins were examined by immunoblotting.

(D) Cells were treated with control or TRIM27 siRNAs for 24 hr before transfection with GFP alone or GFP-FAM21Δ356N-WASH-VCA. Forty-eight hours after transfection, cells were immunostained for Cl-M6PR and the percentage of transfected cells with dispersed Cl-M6PR was determined.

(E) shUb-Ub(K63R) cells were treated with or without tetracycline and transfected with either GFP alone or GFP-FAM21Δ356N-WASH-VCA. Cells were immunostained after 96 hr for Cl-M6PR and the percentage of transfected cells with dispersed Cl-M6PR was determined.

Data are represented as the mean ± SD. Asterisk indicates p < 0.05.

(G) shUb-Ub(K63R) ubiquitin replacement cells were treated with or without tetracycline for 96 hr before staining with ARPC5 (green), FAM21 (red), and DNA (blue). XZ and YZ projection stacks are shown.

(H) Cells treated as in (G) were imaged and endosomal (solid bars) or total (open bars) ARPC5 levels were determined.

Data are represented as the mean ± SD. Asterisk indicates p < 0.05. Scale bars, 20 μm. See also Figure S4.
Figure 6. WASH K63-Linked Ubiquitination by TRIM27 Is Required for Endosomal F-Actin Nucleation and Retrograde Transport

(A and B) Cells were treated with the indicated siRNAs for 24 hr before transfection of the indicated vectors. Forty-eight hours after plasmid transfection, anti-Myc IP was performed. Whole-cell lysates (WCL) or anti-Myc IP samples were immunoblotted for WASH and TRIM27.

(C) Cells were transfected with the indicated Myc-ubiquitin vectors and dual-knockdown/re-expression vectors to knockdown endogenous WASH and re-express YFP-wild-type or K220R WASH. Seventy-two hours after transfection, anti-Myc IP was performed. Whole-cell lysates (WCL) or anti-Myc IP samples were immunoblotted for WASH and Myc-ubiquitin.

(D and E) Cells were transfected with the indicated dual-knockdown/re-expression vectors to knockdown endogenous WASH and re-express YFP-WASH variants. Seventy-two hours after transfection cells were immunostained for CI-M6PR and dispersed CI-M6PR (D) and endosomal CI-M6PR abundance (E) was determined in transfected cells.

(F) Cells were transfected with the indicated dual-knockdown/re-expression vectors as indicated. Seventy-two hours after transfection, Cathepsin D secretion into the cell culture media was determined by immunoblotting. Two independent samples from each condition are shown.

(G) Cells described in (D) were immunostained for ARPC5 and the endosomal-localized pool of ARPC5 in transfected cells was quantitated.

Data are represented as the mean ± SD. Asterisk indicates p < 0.05. See also Figure S5.
with wild-type, ubiquitinated WASH displayed increased activity toward the Arp2/3 complex compared to SHRC reconstituted with nonubiquitinated WASH K220R (Figure 7C). To further examine the interplay of WASH ubiquitination and activity, we treated purified wild-type SHRC with the K63-specific deubiquitinating enzyme AMSH. This treatment deconjugated K63-ubiquitin chains from WASH (Figure 7D) and significantly inhibited SHRC activity on beads (Figure 7E) and in pyrene-actin assembly assays (Figure 7F). These results suggest that K63-ubiquitination of WASH K220 by MAGE-L2-TRIM27 facilitates the activation of SHRC in a reversible manner.

To determine if ubiquitination of WASH K220 may facilitate SHRC activation by disrupting autoinhibitory contacts in the meander region around WASH K220, we mutated WASH K220 to aspartic acid (K220D) to destabilize autoinhibitory contacts in this region. Unlike inactive WASH K220R, WASH K220D is active in vitro (Figures 7A and 7C) and facilitates endosome-to-Golgi retrograde transport (Figures 6D–6F) and endosomal Arp2/3 complex localization (Figure 6G) in cells. These results suggest that destabilizing the WASH meander region around the ubiquitination site can facilitate SHRC activity independent of ubiquitination.

**DISCUSSION**

MAGE proteins are a family of proteins that contain a conserved domain known as the MAGE homology domain. Recently, we showed that MAGE proteins function biochemically to bind to and enhance the activity of E3 RING ubiquitin ligases (Doyle et al., 2010). In this study we investigated the cellular function of one specific MAGE protein, MAGE-L2. Proteomic analysis revealed that MAGE-L2 specifically bound the TRIM27 E3 RING ubiquitin ligase. TRIM27 belongs to a large family of E3 RING ubiquitin ligases known as tripartite motif (TRIM) proteins. TRIM27 was originally identified and named Ret finger protein (RFP), due to its discovery as a gene that undergoes a translocation event with the Ret tyrosine kinase receptor in thyroid carcinomas (Saenko et al., 2003; Takahashi and Cooper, 1987). Subsequent work has implicated it in several processes, including transcriptional regulation, NF-κB signaling, CD4+ T cell homeostasis, and as an oncogene (Zha et al., 2006; Shimono et al., 2000; Zoumpoulidou et al., 2012). It will be of particular interest in the future to determine if any of these diverse functions of TRIM27 are attributed to its regulation of WASH and vesicular transport.

Cellular studies revealed that MAGE-L2 and TRIM27 colocalized on cytoplasmic structures that were determined to be retromer-positive endosomes. Of note, TRIM27 was previously shown to localize to similar structures that were unidentified at the time (Zoumpoulidou et al., 2012). Furthermore, the localization of TRIM27 was regulated by PKC, JNK, and RAS signaling pathways. To determine if any of these signaling pathways may contribute to the upstream regulation of MAGE-L2-TRIM27 and endosome-to-Golgi retrograde transport, we examined the effects of short-term inhibition of the PKC, JNK, MEK, and PI3K signaling pathways on the kinetics of surface CI-M6PR trafficking to the TGN. Specific inhibition of the JNK signaling pathway blocked CI-M6PR retrieval to the TGN (Figure S7).

Future studies into the mechanism by which JNK signaling regulates endosomal protein recycling will be of particular interest.

Our mechanistic studies uncovered that K63-linked ubiquitination of WASH K220 by MAGE-L2-TRIM27 is required for endosomal F-actin nucleation and retrograde transport. WASH K220 is predicted to exist in an analogous region on the SHRC as the meander region on the known WAVE regulatory complex structure. The meander region makes contacts with WAVE itself and SRA1 (analogous to SWIP in SHRC) and is essential for maintaining WAVE in an inactive state (Chen et al., 2010). Importantly, this region is subjected to regulation by phosphorylation (Padrick and Rosen, 2010). Thus, the proposed meander region on both the WAVE and WASH regulatory complexes may be regulated by posttranslational modifications, phosphorylation, and ubiquitination, respectively. In addition, WASH K220 is highly conserved in vertebrates where TRIM27 is found. However, it is not conserved in invertebrates where TRIM27 orthologs are not present (Boudinot et al., 2011). Thus, regulation of SHRC by ubiquitination is likely highly conserved in vertebrates, but additional forms of regulation are predicted in invertebrates.

Our results suggest that ubiquitination of WASH facilitates activation by directly destabilizing autoinhibitory contacts in the SHRC, thus allowing VCA exposure, Arp2/3 complex binding, and subsequent F-actin assembly (Figure 7G). Consistently, destabilizing these autoinhibitory contacts bypasses the requirement for WASH ubiquitination. In the context of the cell, other factors likely cooperate to further enhance activity, perhaps by clustering the active SHRC. Another likely point of regulation is in deactivating WASH after sufficient endosomal F-actin nucleation for retrograde trafficking. Indeed, we find that ubiquitin-mediated activation of SHRC is reversible. Future studies into relevant deubiquitinating enzymes will be of interest.

Our findings also provide important insights into pathological conditions associated with MAGE-L2, TRIM27, and retrograde transport. Genes required for retrograde transport are frequently amplified in melanomas, contribute to tumorigenesis, and mediate trafficking of proteins required for tumor progression (Scott et al., 2009; Zech et al., 2011). Our findings extend this work to show that the TRIM27 oncogene facilitates integrin α5 recycling, an important event in tumorigenesis. Additionally, retrograde transport has been implicated in Alzheimer’s disease, where components of the pathway are downregulated and mutated (Small, 2008). Similarly, MAGE-L2 has been reported to be downregulated in the hippocampus of patients with incipient Alzheimer’s disease (Blalock et al., 2004). Finally, retrograde transport is an essential pathway in which many microbial toxins and some viruses enter cells. Indeed, inhibition of TRIM27 blocks trafficking of cholera toxin. Our results suggest a potential strategy to combat these pathogens.

**EXPERIMENTAL PROCEDURES**

**Cell Culture, Transfections, siRNAs, and Antibodies**

Cells were cultured under standard conditions and transfected according to manufacturer’s recommendation. Detailed descriptions of cell culture conditions, transfection procedures, siRNA sequences, and antibodies are described in the Extended Experimental Procedures.
Figure 7. In Vitro Reconstitution of MAGE-L2-TRIM27 and K63-Ubiquitin-Dependent SHRC Activity

(A) Cell lysates from WASH knockout MEFs reconstituted with the indicated HA-GFP-WASH proteins were incubated with anti-HA agarose beads and F-actin stained with phalloidin-568. WT+CytoD was treated with 10 μM cytochalasin D before addition of beads.

(B) WASH knockout MEFs reconstituted with wild-type HA-GFP-WASH were transfected with the indicated siRNAs for 96 hr and actin assembly was determined as described in (A).

(C) The activity of purified SHRC from WASH knockout MEFs reconstituted with the indicated HA-GFP-WASH variants was examined by pyrene-actin assembly assays.

(legend continued on next page)
Tandem Affinity Purification and Mass Spectrometry
TAP was performed using 293/TAP-Vector or 293/TAP-MAGE-L2 stable cell lines as described previously (Doyle et al., 2010) and in the Extended Experimental Procedures.

Immunoprecipitation, Immunoblotting, and Cathepsin D Secretion Assay
Immunoprecipitation and immunoblotting were performed as described previously (Potts and Yu, 2009). Cathepsin D secretion assay details are described in the Extended Experimental Procedures.

Protein Purification and In Vitro Binding Assays
Recombinant proteins were produced using standard procedures described in the Extended Experimental Procedures. In vitro binding assays were performed as described previously (Doyle et al., 2010) and specified in the Extended Experimental Procedures.

Immunofluorescence, Microscopy, and Quantitative Measurements
Immunofluorescence was performed essentially as described previously (Potts and Yu, 2007) and in Extended Experimental Procedures. Retrograde transport of cell surface Cl-M6PR was performed as described previously (Gomez and Billadeau, 2009) and detailed in the Extended Experimental Procedures.

Actin Assembly Assays and Purification of SHRC
WASH knockout MEFs (Gomez et al., 2012) were reconstituted with HA-GFP-tagged WASH and the resulting SHRC variants were purified as described in the Extended Experimental Procedures. Bead-based and pyrene-actin assembly assays were performed as described previously (Cory et al., 2003; Jia et al., 2010) and detailed in the Extended Experimental Procedures.

SUPPLEMENTAL INFORMATION
Supplemental Information includes Extended Experimental Procedures and seven figures and can be found with this article online at http://dx.doi.org/10.1016/j.cell.2013.01.051.

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siRNAs (Thermo Dharmacon) used in this study are: FAM21 (5’-GUUCUUAACUGGAAUACAA-3’), MAGE-L2 #1 (5’-UACCUAGAGUACAGCCGAADTT-3’), MAGE-L2 #3 (5’-ACACUGAGCCCGCAGUAdTdT-3’), TRIM27 #1 (5’-CGGAGAGCUAAAGCA-GUAdTdT-3’), TRIM27 #2 (5’-GAACAGGCUGCAUUAdTdT-3’), VPS35 (5’-AUUUGGUGCGCCUCAGUAdTdT-3’), and WASH (5’-CGCCACUGUGUUCUCUAdTdT-3’). siRNAs targeting ubiquitin E2 enzymes, mouse MAGE-L2, and mouse TRIM27 were purchased as SmartPools (Thermo Dharmacon). Negative control siRNAs (siControl) against an irrelevant gene (LonRF1) were also purchased as a SmartPool (Thermo Dharmacon). Simultaneous WASH knockdown and re-expression constructs were described previously (Gomez and Billadeau, 2009).

Antibodies

Antibodies used in this study are as follows: anti-APC2 (Tang et al., 2001), anti-ARPC5 (305011; Synaptic Systems), anti-CapZ (AB23892; Abcam), anti-VPS29 (GTX104768; GeneTex), anti-VPS35 (AB10099; Abcam), and anti-WASH (SAB4200373; Sigma or Cell Signaling Technology), anti-mouse IgG-HRP (Amersham), anti-Myc (9E10; Roche), anti-rabbit IgG-HRP (Amersham), anti-Strumelin (12CA5; Roche), anti-K48-linked ubiquitin (8081; Cell Signaling Technology), anti-K63-linked ubiquitin (05-1308; Millipore; or 5621; Cell Signaling Technology), anti-goat IgG-HRP (SC-2056; Santa Cruz), anti-Golgin-97 (A-21270; Invitrogen Molecular Probes), anti-hemagglutinin (12CA5; Roche), anti-K48-linked ubiquitin (8081; Cell Signaling Technology), anti-K63-linked ubiquitin (05-1308; Millipore; or 5621; Cell Signaling Technology), anti-mouse IgG-HRP (Amersham), anti-Myc (9E10; Roche), anti-rabbit IgG-HRP (Amersham), anti-Strumelin (SC-87442; Santa Cruz), anti-SWIP (ABT70; Millipore), anti-TGN46 (T7576; Sigma), anti-TRIM27 (18791; IBL), anti-VPS26 (AB23892; Abcam), anti-VPS29 (GTX104768; GeneTex), anti-VPS35 (AB10099; Abcam), and anti-WASH (SAB4200373; Sigma or as described previously [Gomez and Billadeau, 2009]).

Tandem Affinity Purification and Mass Spectrometry

Tandem affinity purification (TAP) was performed using 293/TAP-Vector or 293/TAP-MAGE-L2 stable cell lines as described previously (Doyle et al., 2010). In brief, ten 150 mm² dishes of cells were lysed, bound to IgG Sepharose beads (GE Amersham), cleaved off the beads with TEV protease, collected on Calmodulin Sepharose beads (GE Amersham), eluted with SDS sample buffer, separated by SDS-PAGE, and stained with colloidal Coomassie blue (Pierce). Unique protein bands were excised, in-gel proteolyzed, and identified from bacterial lysates with Ni-NTA agarose (QIAGEN) and eluted with 250 mM imidazole. In vitro binding assays were performed as described previously (Doyle et al., 2010). Briefly, 15 μg of purified GST-tagged proteins were bound to glutathione Sepharose beads (Amersham) for 1 hr in binding buffer (25 mM Tris [pH 8.0], 2.7 mM KCl, 137 mM NaCl, 0.05% Tween-20, and 10 mM 2-mercaptoethanol) and then blocked for 1 hr in 5% milk powder. In vitro translated proteins (Promega SP6-TNT Quick rabbit reticulocyte lysate system) were then incubated with the bound beads for 1 hr, extensively washed, eluted with SDS sample buffer, boiled, subjected to SDS-PAGE, and immunoblotting.

Protein Purification and In Vitro Binding Assays

GST-tagged VPS35, GST-TRIM27, and His-AMSH were produced in BL21(DE3) cells by overnight induction at 16 °C with 0.5 mM isopropyl b-D-1-thiogalactopyranoside (IPTG) and 100 μM ZnCl₂ in the case of GST-TRIM27. GST-VPS35 and GST-TRIM27 were purified from bacterial lysates with glutathione Sepharose (GE Amersham) and eluted with 10 mM glutathione. His-AMSH was purified from bacterial lysates with Ni-NTA agarose (QIAGEN) and eluted with 250 mM imidazole. In vitro binding assays were performed as described previously (Doyle et al., 2010). Briefly, 15 μg of purified GST-tagged proteins were bound to glutathione Sepharose beads (Amersham) for 1 hr in binding buffer (25 mM Tris [pH 8.0], 2.7 mM KCl, 137 mM NaCl, 0.05% Tween-20, and 10 mM 2-mercaptoethanol) and then blocked for 1 hr in 5% milk powder. In vitro translated proteins (Promega SP6-TNT Quick rabbit reticulocyte lysate system) were then incubated with the bound beads for 1 hr, extensively washed, eluted with SDS sample buffer, boiled, subjected to SDS-PAGE, and immunoblotting.

Cathepsin D Secretion Assay

Cathepsin D secretion was detected as previously described (Rojas et al., 2008). In brief, 72 hr after RNAi or 96 hr after ubiquitin replacement, cells were washed and incubated for 16 hr in OPTI-MEM without serum. Adherent cells were collected in SDS sample buffer for loading controls. The cell culture media were collected and centrifuged at 15 min at 16,000 x g to pellet cell debris. The clarified supernatant was treated with 0.02% sodium deoxycholate for 30 min on ice. Proteins were precipitated in 15% trichloroacetic acid overnight at 4 °C. Precipitated proteins were pelleted at 16,000 x g for 10 min at 4 °C, washed in ice-cold acetone twice,
reconstituted with HA-GFP-tagged human WASH wild-type, D
WASH knockout MEFs were prepared and characterized previously (Gomez et al., 2012). The WASH knockout MEFs were recon-
Actin Assembly Assays and Purification of SHRC
were counted using a 10X objective.
restrictant media containing 2.5% FBS in the bottom chamber. Twenty-four hours after plating, noninvaded cells were scraped off and
cells were then plated in serum-free media on matrigel-coated 8 m HeLa cells were transfected with the indicated siRNAs for 48 hr and subsequently serum starved for 16 hr. One hundred thousand
Cell Invasion Assay
Flow Cytometry Analysis
HeLa cells were transfected with the indicated siRNAs for 72 hr before staining with 10 μg/ml anti-CD49e (Integrin α5; BD PharMingen) or IgG isotype control for 30 min on ice. Cells were washed twice and incubated with secondary anti-mouse Alexa-488 only. For live-cell microscopy, cells were grown in 4-well chambered coverglasses (Lab-Tek) and imaged on a DeltaVision inverted fluorescence microscope with environ-
mental chamber. Single plane images were acquired every 500 ms. Time-lapse images were processed and analyzed by ImageJ Software.

Immunofluorescence, Microscopy, and Quantitative Measurements
Immunofluorescence was performed essentially as described previously (Potts and Yu, 2007). Cells were washed in 1X PBS, fixed for 15 min at room temperature in 3% paraformaldehyde, washed in 1× PBS twice, and incubated in blocking solution (PBS containing 3% bovine serum albumin [BSA] and 0.1% saponin) for 20 min at 4 °C. After blocking, cells were incubated in blocking solution con-
taining approximately 2 μg/ml of each primary antibody for 60 min at room temperature. Cells were then washed three times in PBS containing 0.1% saponin and incubated for 30 min at room temperature in blocking solution containing 4 μg/ml Alexa-488, Alexa-568, or Alexa-647 secondary antibodies (Invitrogen Molecular Probes). After incubation, cells were washed three times in PBS containing 0.1% saponin, DNA stained in 1 μg/ml 4',6-diamidino-2-phenylindole (DAPI) for 2 min, washed in PBS, and mounted. For F-actin staining, 33 nM Alexa-568-conjugated Phalloidin (Invitrogen) was incubated for 30 min at room temperature following primary antibody staining.

Cells were imaged with a 63 x or 100 x objective on a DeltaVision or Nikon TiU inverted fluorescence microscope. Images were acquired with a CoolSnap HQ2 charge-coupled device camera (Photometrics) at 0.3 μm intervals, deconvolved using the nearest neighbor algorithm, and stacked to better resolve endosome structures. Intensity measurements were performed using ImageJ Software. For those experiments specifically quantitating the amount of endosomal localized F-actin or ARPC5, images were thresholded using the endosomal markers FAM21, VPS35, or WASH and intensity measurements were performed only on the F-actin or ARPC5 signal colocalizing. Quantitation of CI-M6PR trafficking was performed by blind analysis of the percentage of cells showing compact juxtanuclear (normal) or dispersed (abnormal) CI-M6PR staining. At least 50–100 cells were counted for each condition in each exper-
iment. Statistical analysis was performed using two-tailed unpaired Student’s t test.

For assaying retrograde transport of cell surface CI-M6PR, cells were incubated for 60 min or the indicated times at 37°C in serum-
free media (DMEM containing 1% BSA, and 25 mM HEPES [pH 7.4]) containing 10 μg/ml of monoclonal antibody against the luminal domain of CI-M6PR. Cells were washed in serum-free media three times to remove excess unbound anti-CI-M6PR antibody. Cells were then fixed, permeabilized, and stained with secondary antibody (anti-mouse Alexa-488) only. For live-cell microscopy, cells were grown in 4-well chambered coverglasses (Lab-Tek) and imaged on a DeltaVision inverted fluorescence microscope with envi-
ronmental chamber. Single plane images were acquired every 500 ms. Time-lapse images were processed and analyzed by ImageJ Software.

Cell Invasion Assay
HeLa cells were transfected with the indicated siRNAs for 48 hr and subsequently serum starved for 16 hr. One hundred thousand
cells were then plated in serum-free media on matrigel-coated 8 μm transwell invasion chambers (BD Biosciences) with a chemoat-
tractant media containing 2.5% FBS in the bottom chamber. Twenty-four hours after plating, noninvaded cells were scraped off and
invaded cells on the bottom of the transwell membrane were stained with DAPI and crystal violet. The numbers of cells in each field
were counted using a 10X objective.

Actin Assembly Assays and Purification of SHRC
WASH knockout MEFs were prepared and characterized previously (Gomez et al., 2012). The WASH knockout MEFs were reconsti-
WASH knockout MEFs were reconstituted with HA-GFP-tagged human WASH wild-type, ΔVCA, K220R, or K220D by retroviral infection and selection. Equal expres-
sion of mutants at levels comparable to endogenous was confirmed by immunoblotting (data not shown). In vitro actin assembly on
beads using cell lysates was performed as described previously (Cory et al., 2003). Briefly, one 15 cm² plate of WASH knockout MEFs
reconstituted with the indicated HA-GFP-tagged WASH proteins was lysed in 1 ml of NP-40 lysis buffer containing MgCl₂ (50 mM Tris-HCl [pH 7.7], 150 mM NaCl, 0.5% NP-40, 5 mM MgCl₂, and 1X protease inhibitor cocktail). Lysate protein concentrations were estimated using Bradford Assay (Bio-Rad) and normalized to 2 mg/ml. HA-GFP-WASH incorporated into SHRC was purified using anti-HA agarose beads (Sigma) by overnight incubation. Beads were extensively washed in NP-40 lysis buffer containing MgCl₂ and fixed in 3% paraformaldehyde. F-actin on SHRC-captured beads was stained with 66 nM Alexa-568-conjugated Phalloidin and beads were squashed on slides and imaged. For examining the dependence of MAGE-L2-TRIM27, WASH knockout MEFs
reconstituted with HA-GFP-WASH were transfected with mouse MAGE-L2 or mouse TRIM27 siRNAs for 72 hr before purification of

RT-QPCR
RT-QPCR was performed as described previously (Doyle et al., 2010). Briefly, total RNA was extracted (Qiagen), DNase treated
(Linvitrogen), converted to cDNA with SuperScript III (Invitrogen), and SYBR green QPCR (Bio-Rad) performed on an Abi-7900HT
instrument (Applied Biosystems).

Immunofluorescence, Microscopy, and Quantitative Measurements

Flow Cytometry Analysis
HeLa cells were transfected with the indicated siRNAs for 72 hr before staining with 10 μg/ml anti-CD49e (Integrin α5; BD PharMingen) or IgG isotype control for 30 min on ice. Cells were washed twice and incubated with secondary anti-mouse Alexa-
488 antibody for 60 min on ice. Cells were washed three times and analyzed by flow cytometry.

Cell Invasion Assay
HeLa cells were transfected with the indicated siRNAs for 48 hr and subsequently serum starved for 16 hr. One hundred thousand
cells were then plated in serum-free media on matrigel-coated 8 μm transwell invasion chambers (BD Biosciences) with a chemoat-
tractant media containing 2.5% FBS in the bottom chamber. Twenty-four hours after plating, noninvaded cells were scraped off and
invaded cells on the bottom of the transwell membrane were stained with DAPI and crystal violet. The numbers of cells in each field
were counted using a 10X objective.

Actin Assembly Assays and Purification of SHRC
WASH knockout MEFs were prepared and characterized previously (Gomez et al., 2012). The WASH knockout MEFs were reconsti-
tuted with HA-GFP-tagged human WASH wild-type, ΔVCA, K220R, or K220D by retroviral infection and selection. Equal expres-
sion of mutants at levels comparable to endogenous was confirmed by immunoblotting (data not shown). In vitro actin assembly on
beads using cell lysates was performed as described previously (Cory et al., 2003). Briefly, one 15 cm² plate of WASH knockout MEFs
reconstituted with the indicated HA-GFP-tagged WASH proteins was lysed in 1 ml of NP-40 lysis buffer containing MgCl₂ (50 mM Tris-HCl [pH 7.7], 150 mM NaCl, 0.5% NP-40, 5 mM MgCl₂, and 1X protease inhibitor cocktail). Lysate protein concentrations were estimated using Bradford Assay (Bio-Rad) and normalized to 2 mg/ml. HA-GFP-WASH incorporated into SHRC was purified using anti-HA agarose beads (Sigma) by overnight incubation. Beads were extensively washed in NP-40 lysis buffer containing MgCl₂ and fixed in 3% paraformaldehyde. F-actin on SHRC-captured beads was stained with 66 nM Alexa-568-conjugated Phalloidin and beads were squashed on slides and imaged. For examining the dependence of MAGE-L2-TRIM27, WASH knockout MEFs
reconstituted with HA-GFP-WASH were transfected with mouse MAGE-L2 or mouse TRIM27 siRNAs for 72 hr before purification of

SHRC and assaying activity as described above. In the indicated samples, clarified cell lysates were treated with 3.5 μM AMSH for 1 hr at 30°C or with 10 μM cytochalasin D before incubation with anti-HA beads.

For in vitro pyrene-actin assembly assays, the tagged WASH SHRC complex was purified to near homogeneity from ten 15 cm² plates of WASH knockout MEFs reconstituted with the indicated HA-GFP-tagged WASH proteins. Cell pellets were lysed in NP-40 lysis buffer (50 mM Tris-HCl, [pH 7.7], 150 mM NaCl, 0.5% NP-40, 1 mM DTT, and 1X protease inhibitor cocktail) and clarified by centrifugation and filtration. Clarified lysates were bound to 1 ml of anti-HA agarose beads (Sigma) and washed extensively in lysis buffer. The SHRC was eluted six times with 100 μg/ml 3X HA peptide (Sigma). The elutions were pooled, concentrated, and assayed for all SHRC components and purity by western blotting, Coomassie staining, and mass spectrometry. Note, HA-GFP-WASH was present at stoichiometric levels to other SHRC proteins, suggesting very little free HA-GFP-WASH in our preparations.

Pyrene-actin assembly assays were performed as described previously (Leung et al., 2006). Briefly, actin was purified from rabbit muscle and the Arp2/3 complex was purified from bovine thymus. Actin assembly assays contained 4 μM actin (5% pyrene labeled), 10 nM Arp2/3 complex in KMEI-15G buffer (15% (w/v) glycerol, 50 mM KCl, 1 mM MgCl₂, 1 mM EGTA, and 10 mM imidazole [pH 7.0]). In the indicated samples, purified SHRC was treated with buffer or 3.5 μM AMSH for 1 hr at 30°C before pyrene-actin assembly assays.

SUPPLEMENTAL REFERENCES


Figure S1. MAGE-L2-TRIM27 Localizes to Retromer-Positive Endosomes and MAGE-L2 WH-B Motif Binds VPS35 In Vitro, Related to Figure 1
(A) mCherry-TRIM27 and the indicated GFP-tagged proteins were expressed in U2OS cells and representative images are shown. Pearson’s correlation coefficients (Rr) are shown.
(B) Myc-VPS35 or Myc-Vector and HA-MAGE-L2 were expressed in cells. Forty-eight hours after transfection anti-Myc IP was performed and proteins were detected by anti-HA and anti-Myc western blot (WB).
(C) TRIM27 associates with the retromer complex in cells. The indicated proteins were expressed for 48 hr before anti-Myc IP and western blotting with anti-Myc and anti-HA antibodies.
(D) MAGE-L2, but not TRIM27, directly binds to VPS35 TRIM27 in vitro. Binding of the indicated Myc-tagged proteins to recombinant GST-VPS35 or GST alone was determined by GST pull-down assays. Binding of the indicated Myc-tagged proteins was determined by anti-Myc immunoblotting. Myc-VPS26 and Myc-VPS29 were used as positive controls.
(E) Localization of GFP-MAGE-L2 to endosomes requires VPS35. U2OS cells stably expressing GFP-MAGE-L2 were treated with control or VPS35 siRNAs for 72 hr before live-cell imaging of GFP-MAGE-L2.
(F) Quantitation of the number of GFP-MAGE-L2 endosomes in control- or VPS35-RNAi cells described in (E). Data are represented as the mean ± standard deviation. Asterisk indicates p < 0.05.
(G) Mapping MAGE-L2 region interacting with VPS35. Binding of the indicated Myc-MAGE-L2 fragments with recombinant GST-VPS35 or GST alone was determined by GST pull-down. Binding of the Myc-MAGE-L2 fragments was determined by anti-Myc immunoblotting. Myc-TRIM28 and Myc-TRIM27 were used as negative controls, while Myc-VPS26 and Myc-VPS29 were used as positive controls.
(H) Summary of binding experiments shown in (G).
(I) MAGE-L2 and FAM21 do not compete for binding to VPS35. Binding of Myc-MAGE-L2 and Myc-FAM21 with recombinant GST-VPS35 or GST alone was determined by GST pull-down followed by anti-Myc immunoblotting.
Scale bars, 20 μm.
Figure S2. MAGE-L2 and TRIM27 Are Required for Retrograde Transport, but Not Overall TGN Architecture, Related to Figure 2

(A) Cells were treated with the indicated siRNAs for 72 hr and the indicated proteins were detected by immunoblotting. Asterisk indicates nonspecific band. Note reduction in components of SHRC upon depletion of WASH and reduction in components of the retromer complex upon depletion of VPS35.

(B) Cells were treated with the indicated siRNAs for 72 hr and endogenous MAGE-L2 mRNA levels were determined by RT-QPCR. MAGE-L2 mRNA levels were normalized to Cyclophilin B mRNA levels and the relative abundance compared to siControl samples are shown. Data are represented as the mean ± standard deviation. Asterisk indicates p < 0.05.

(C) Cells were treated with the indicated siRNAs for 24 hr before transfection of Myc-MAGE-L2 expression vector. Forty-eight hours after plasmid transfection cell lysates were collected, subjected to SDS-PAGE, and immunoblotted for Myc-MAGE-L2 (anti-Myc) or APC2 (loading control).

(D) Cells were treated with the indicated siRNAs for 24 hr before transfection of Myc-vector control or Myc-MAGE-L2 RNAi resistant expression vectors. Forty-eight hours after plasmid transfection cells were stained with anti-Myc antibody to identify transfected cells and anti-CI-M6PR to assess retrograde transport. The percentage of cells with dispersed CI-M6PR was then determined. Data are represented as the mean ± standard deviation. Asterisk indicates p < 0.05.

(E) Cells were treated with the indicated siRNAs for 72 hr before golgin-97 (green) and DNA (blue) were stained. Representative images are shown.

(F) Cells were treated with the indicated siRNAs for 72 hr. Cell surface CI-M6PR was labeled with anti-CI-M6PR antibody for one hour at 37°C before imaging the pool of internalized cell surface-labeled CI-M6PR (green), EE1 (red), and DNA (blue). Pearson’s correlation coefficients (Rr) are shown.

(G) Cell surface CI-M6PR was labeled with mouse anti-CI-M6PR antibody for 10 or 20 min at 37°C before the pool of internalized cell surface-labeled CI-M6PR was imaged with anti-mouse Alexa-488. The percentage of cells with proper retrograde transport of CI-M6PR to perinuclear TGN region is shown. Scale bars, 20 μm.
Figure S3. MAGE-L2-TRIM27 Ubiquitin Ligase Activity, Ube2O E2, and K63-Linked Ubiquitination Required for Retrograde Transport, Related to Figure 3

(A) TRIM27 ubiquitin ligase activity is required for retrograde transport of CI-M6PR. Cells were treated with control or TRIM27 siRNAs for 24 hr before transfection of RNAi-resistant wild-type or RING mutant TRIM27. Forty-eight hours after transfection, cells were stained for CI-M6PR (green) localization and DNA (blue). Transfected cells were marked by mCherry expression (data not shown).

(B) TRIM27 RING mutant localizes similar to wild-type TRIM27. mCherry-tagged wild-type or RING mutant TRIM27 were expressed in cells for 48 hr before imaging.

(C) Cells were treated with siRNAs targeting the indicated E2 enzymes for 72 hr, stained with anti-CI-M6PR, and imaged. Representative images are shown for a subset of enzymes screened.

(D) Knockdown of Ube2L6, but not Ube2O, results in impaired TGN organization. Cells were treated with the indicated siRNAs for 72 hr before golgin-97 (green) localization and DNA (blue) were stained.

(E) Schematic of Ubiquitin replacement cell lines. (Top) Inducible shUbiquitin knockdown construct includes four hairpins targeting the Ubc and Uba52 ubiquitin encoding genes. (Bottom) Inducible ubiquitin replacement constructs. Either nothing or wild-type (WT), K48R, or K63R ubiquitin was fused to Ribosomal L40 and Ribosomal S27A encoding genes to mimic the normal ubiquitin maturation process.

(F) Validation of ubiquitin depletion cell lines. shUb-Ub(K48R), shUb-Ub(K63R), or shUb were treated with or without tetracycline for 72-96 hr and cell lysates were immunoblotted with anti-K48-linked ubiquitin, anti-K63-linked ubiquitin, or anti-polyubiquitin antibodies, respectively. Anti-CapZ was formed as a loading control.

(G) The indicated ubiquitin replacement cell lines were treated with or without tetracycline for 72-96 hr before steady-state CI-M6PR localization was determined by immunostaining: CI-M6PR (green) and DNA (blue).

(H) shUb-Ub(K63R) cells were treated with or without tetracycline for 96 hr before golgin-97 (green) and DNA (blue) were stained.

(I) The indicated ubiquitin replacement cells were treated with or without tetracycline for 72-96 hr, followed by serum starvation for 16 hr. Cell surface CI-M6PR was then labeled with anti-CI-M6PR antibody for one hour before imaging the pool of internalized cell surface-labeled CI-M6PR. CI-M6PR (green) and DNA (blue) are shown.

(J) No changes to WASH regulatory complex protein levels upon K63-linked ubiquitin depletion. shUb-Ub(K63R) cells were treated with or without tetracycline for 96 hr before cell lysates were collected and immunoblotted for the indicated proteins. Asterisk indicates nonspecific band.

Scale bars, 20 μm.
Figure S4. MAGE-L2-TRIM27 Specifically Controls Retrograde Transport through Regulation of Endosomal Actin Dynamics, Related to Figure 4

(A) Cells were treated with control or TRIM27 siRNAs for 72 hr before staining for VPS35 (green), CI-M6PR (red), and DNA (blue). Representative images are shown. Scale bars, 20 μm.

(B) shUb-Ub(WT) or shUb-Ub(K63R) cells were treated with tetracycline for 96 hr before staining for VPS35 (green), CI-M6PR (red), and DNA (blue). Representative images are shown. Scale bars indicate 20 μm.

(C) Cells were treated with control or TRIM27 siRNAs for 72 hr before staining for VPS35 (red), FAM21 (green), and DNA (blue). Representative images are shown. Scale bars, 20 μm.

(D) Cells were treated with control or TRIM27 siRNAs for 72 hr before staining with anti-VPS35 antibody. Yellow arrowheads indicate endosomal tubules. Percentage of cells with endosomal tubules is shown below. Scale bars, 1 μm.

(E) shUb-Ub(WT), shUb-Ub, and shUb-Ub(K63R) cells were treated with tetracycline for 72-96 hr before staining with anti-CI-M6PR antibody. Yellow arrowheads indicate endosomal tubules. Percentage of cells with endosomal tubules is shown below. Scale bars, 1 μm.

(F) Endosomal localized, uninhibited WASH-VCA rescues CI-M6PR retrograde transport in TRIM27-RNAi cells. Cells were treated with TRIM27 siRNA for 24 hr before transfection with GFP-FAM21Δ356N-WASH-VCA. Forty-eight hours after transfection, cells were stained for GFP-FAM21Δ356N-WASH-VCA (red), CI-M6PR (green), F-actin (cyan), and DNA (blue). Representative image is shown. Compare transfected (YFP-positive) and untransfected cells (asterisks). Scale bars, 20 μm.

(G) Endosomal localized, uninhibited WASH-VCA rescues CI-M6PR retrograde transport in K63-ubiquitin depleted cells. shUb-Ub(K63R) cells were treated with tetracycline and transfected with GFP-FAM21Δ356N-WASH-VCA. After 96 hr, cells were stained for GFP-FAM21Δ356N-WASH-VCA (green), CI-M6PR (cyan), and F-actin (red). Representative image is shown. Compare transfected (YFP-positive) and untransfected cells (asterisks). Scale bars, 20 μm.
Figure S5. Retrograde Transport and Endosomal Arp2/3 Localization Is Defective in Cells Expressing WASH K220R, Related to Figure 6

(A) No detectable ubiquitination of the WASH regulatory complex subunit, SWIP. Cells were treated with control or TRIM27 siRNAs for 24 hr before transfection of Myc-empty or Myc-Ubiquitin expression vectors. Forty-eight hours after plasmid transfection, anti-Myc IP was performed. Whole cell lysates (WCL) or anti-Myc IP samples were immunoblotted for SWIP.

(B) Retrograde transport is defective in WASH K220R cells. Cells were transfected with the indicated dual-knockdown/re-expression vectors to knockdown endogenous WASH and re-express YFP-control, YFP-WASH WT, or YFP-WASH K220R. Seventy-two hours after transfection cells were stained for CI-M6PR (red), YFP (data not shown), and DNA (blue). Note large difference in CI-M6PR localization and abundance in nontransfected cell (asterisk) in YFP-WASH K220R condition.

(C) Endosomal Arp2/3 localization is defective in WASH K220R cells. Cells were transfected with the indicated dual-knockdown/re-expression vectors to knockdown endogenous WASH and re-express YFP-control, YFP-WASH WT, or YFP-WASH K220R. Seventy-two hours after transfection cells were stained for ARPC5 (red), YFP (green), and DNA (blue). Compare transfected (YFP-positive) and untransfected cells (asterisks). Note YFP-WASH K220R localizes to endosomes normally.

(D) Ubiquitinated WASH (K220) is highly conserved in vertebrates in which TRIM27 is present, but is not conserved in invertebrates where TRIM27 is not present. Sequence alignments around human WASH K220 are shown for the indicated organisms. Dotted line separates vertebrate (top) from invertebrate (bottom). Scale bars, 20 μm.
Figure S6. Purification of Intact SHRC Complex Reconstituted with HA-GFP-WASH Variants, Related to Figure 7

(A) Whole cell lysates (WCL) from WASH knockout MEFs stably expressing HA-GFP-WASH wild-type, K220R, K220D, or ΔVCA were subjected to anti-HA chromatography and eluted with HA peptide six times (elutions 1–6). Samples from WCL, depleted WCL after anti-HA chromatography, and eluted fractions were separated by SDS-PAGE and immunoblotted with the indicated antibodies.

(B) Material described in (A) was concentrated, separated on SDS-PAGE, and stained with colloidal Coomassie blue. Purified SHRC reconstituted with HA-GFP-WASH wild-type is shown as a representative example. Note CCDC53 was below the limit of Coomassie staining, but was confirmed to be present by immunoblotting.
Figure S7. Transport of Cell Surface-Labeled CI-M6PR to TGN Is Dependent on JNK Signaling, Related to Figure 2

(A) Cells were treated with DMSO (60 min), 4 μM PKC inhibitor Bisindolylmaleimide I (BIM I) (30 min), 50 μM PI3K inhibitor LY294002 (60 min), 50 μM JNK inhibitor SP600215 (45 min), or 10 μM MEK1/2 inhibitor U0126 (60 min) prior to incubation with 10 μg/ml anti-CI-M6PR in serum-free media for 15, 30, 45, or 60 min. Cells were washed three times in serum-free media, fixed, and internalized CI-M6PR was detected by anti-mouse-Alexa 488 secondary antibody staining. Scale bar, 10 μm.

(B) Cells described in (A) were quantitated for the percentage of cells with perinuclear TGN localized surface-labeled CI-M6PR.