RESEARCH ARTICLE

HPS6 interacts with dynactin p150\textsuperscript{Glued} to mediate retrograde trafficking and maturation of lysosomes

Ke Li\textsuperscript{1,2}, Lin Yang\textsuperscript{1}, Cheng Zhang\textsuperscript{1}, Yang Niu\textsuperscript{1,2}, Wei Li\textsuperscript{1} and Jia-Jia Liu\textsuperscript{1,*}

ABSTRACT

Hermansky-Pudlak syndrome 6 protein (HPS6) has originally been identified as a subunit of the BLOC-2 protein complex that is involved in the biogenesis of lysosome-related organelles. Here, we demonstrate that HPS6 directly interacts with the dynactin p150\textsuperscript{Glued} subunit of the dynein–dynactin motor complex and acts as cargo adaptor for the retrograde motor to mediate the transport of lysosomes from the cell periphery to the perinuclear region. Small interfering RNA (siRNA)-mediated knockdown of HPS6 in HeLa cells not only partially blocks centripetal movement of lysosomes but also causes delay in lysosome-mediated protein degradation. Moreover, lysosomal acidification and degradative capacity, as well as fusion between late endosomes and/or multivesicular bodies and lysosomes are also impaired when HPS6 is depleted, suggesting that perinuclear positioning mediated by the dynein–dynactin motor complex is required for lysosome maturation and activity. Our results have uncovered a so-far-unknown specific role for HPS6 in the spatial distribution of the lysosomal compartment.

KEY WORDS: HPS6, p150\textsuperscript{Glued}, DCTN1, lysosome, dynein–dynactin motor complex

INTRODUCTION

The intracellular transport driven by motor proteins plays crucial roles in membrane trafficking, which is essential for organelle biogenesis, homeostasis and function. Cytoplasmic dynein, a microtubule-based motor, drives the movement of a wide range of cargoes towards the minus ends of microtubules and is primarily responsible for retrograde transport of materials from the cell periphery to the center (Allan, 2011). Functions of dynein require the activity of dynactin, a multisubunit protein complex that not only enhances its processivity along microtubule tracks (Culver-Hanlon et al., 2006; King and Schroer, 2000; Moore et al., 2009) but also links it to cargoes. Although several proteins that tether the dynein–dynactin complex to its membranous cargoes have been identified and their functions in intracellular trafficking have been characterized (Engelender et al., 1997; Hong et al., 2009; Hoogenraad et al., 2001; Johansson et al., 2007; Tai et al., 1999; Traer et al., 2007; Wassmer et al., 2009; Watson et al., 2005; Yano and Chao, 2005), adaptor proteins for many other organelles and vesicular cargoes that are retrogradely transported from the cell periphery to the center remain to be discovered.

Lysosomes are catabolic organelles of eukaryotic cells that contain hydrolytic enzymes required for degradation of macromolecules, including proteins, lipids, nucleic acids and polysaccharides. They play a central role in the degradation of macromolecules that are received from endocytic, phagocytic and autophagic pathways, and are essential for many cellular and physiological processes, such as receptor-mediated signaling, antigen presentation, pathogen clearance and cholesterol homeostasis (Saftig and Klumperman, 2009; Settembre et al., 2013). The intracellular trafficking and steady-state distribution of lysosomes rely upon molecular motors, and both the actin and microtubule cytoskeletons (Burkhardt et al., 1997; Cantalupo et al., 2000; Cordonnier et al., 2001; Harada et al., 1998; Jordens et al., 2001; Matsushita et al., 2004; Rosa-Ferreira and Munro, 2011; Santama et al., 1998). Although it has been reported that lysosomes change their intracellular positioning in order to coordinate cellular responses to nutrients through mTOR signaling and autophagic flux (Korolchuk et al., 2011), the physiological relevance of perinuclear steady-state clustering of lysosomes in cells remains largely unexplored.

Lysosome-related organelles are a class of tissue-specific compartments that share many features with lysosomes, but store and secret proteins for cell-type-specific functions (Marks et al., 2013; Raposo et al., 2007). They include melanosomes, platelet-dense granules, lytic granules, lamellar bodies and many others (Raposo et al., 2007). Defects in the biogenesis of these organelles cause Hermansky–Pudlak syndrome (HPS), a human autosomal recessive disorder that is characterized by partial loss of pigmentation and prolonged bleeding, and that occurs in nine forms (Wei and Li, 2013). Except for the HPS2 gene (also known as \textit{AP3B1}) – which encodes the β-subunit of the adaptor protein (AP) complex 3 (AP-3), genes mutated in the other eight forms of HPS encode subunits of three protein complexes that are known as biogenesis of lysosome-related organelles complex (BLOC)-1, BLOC-2 and BLOC-3. Of these, HPS7, HPS8 and HPS9 constitute BLOC-1; HPS3, HPS5 and HPS6 constitute BLOC-2; and HPS1 and HPS4 constitute BLOC-3 (Di Pietro et al., 2004; Gautam et al., 2004; Li et al., 2003; Wei and Li, 2013; Zhang et al., 2002; Zhang et al., 2003). Although it has been established that the BLOC complexes are important for the biogenesis of lysosome-related organelles (Chiang et al., 2003; Di Pietro et al., 2006; Martina et al., 2003; Setty et al., 2007) – biological functions of BLOC-1 and BLOC-3 have been identified most recently (Gerondopoulos et al., 2012; John Peter et al., 2013; Kloer et al., 2010) – the exact molecular functions of BLOC-2 subunits remain elusive.

In this study, we found that HPS6 is a cargo adaptor for the dynein–dynactin motor complex. We present evidence that HPS6 binds to dynactin p150\textsuperscript{Glued} (also known as DCTN1), and that this...
interaction is required for the perinuclear positioning of lysosomes. Depletion of HPS6 in HeLa cells not only caused mislocalization of LAMP1- and LAMP2-positive lysosomes to the cell periphery but also impaired lysosomal degradation of endocytic cargoes. This – however – could be rescued by overexpressing full-length HPS6 but not by HPS6 truncation mutants that are unable to bind p150<sub>Glued</sub>. Furthermore, lysosomal acidification as well as fusion between late endosomes and/or multivesicular bodies (hereafter referred to as LEs/MVBs) and lysosomes were also impaired in HPS6-depleted cells. We, therefore, propose that HSP6 facilitates retrograde lysosomal trafficking by linking the retrograde motor to lysosomes, and that perinuclear positioning of lysosomes is crucial for the delivery of endocytic cargos to lysosomes, for lysosome maturation and function.

**RESULTS**

**HPS6 directly interacts with the dynactin p150<sub>Glued</sub> subunit of the dynein–dynactin complex**

In search of cargo adaptors for the dynein–dynactin motor, we performed a yeast two-hybrid screen of a human fetal brain cDNA library by using the C-terminus of human dynactin p150<sub>Glued</sub>, amino acid residues (aa) 718–1278, as bait (Hong et al., 2009). Of 20 positive clones, one was found to encode an N-terminal fragment (aa 66–197) of HPS6. To verify the interaction between HPS6 and p150<sub>Glued</sub> in mammalian cells, we transiently co-transfected HEK293 cells with plasmids encoding epitope-tagged full-length p150<sub>Glued</sub> and HPS6, respectively, and performed co-immunoprecipitation (co-IP) with immobilized antibodies against the Flag tag. HPS6 and p150<sub>Glued</sub> reciprocally co-immunoprecipitated with each other (Fig. 1A), indicating that the interaction between HPS6 and p150<sub>Glued</sub> also occurs in mammalian cells. To further verify the interaction, we performed co-IP with HEla cell lysates and detected not only HPS3 and HPS5 – the other two subunits of the BLOC-2 complex – but also p150<sub>Glued</sub> and the dynein intermediate chain (hereafter referred to as DIC) in the immunoprecipitates of HPS6 (Fig. 1B).

To map the HPS6 interaction site on p150<sub>Glued</sub>, we again performed yeast two-hybrid assays (Fig. 1D) and co-IP experiments in cultured mammalian cells with a series of p150<sub>Glued</sub> fragments. In mammalian cells, although the extreme C-terminus of p150<sub>Glued</sub> was not expressed, co-IP of cell extracts from cells that co-expressed HPS6 and p150<sub>Glued</sub> fragments showed that the C-terminal p150<sub>Glued</sub> fragment of aa 911–1281 interacts strongly with HPS6 (Fig. 1E,F). Together, these data indicate that the HPS6-binding site on p150<sub>Glued</sub> resides in its extreme C-terminus. We also attempted to map the p150<sub>Glued</sub> interaction site(s) on HPS6 by yeast two-hybrid assays and co-IP of cell extracts from cells that co-expressed p150<sub>Glued</sub> and a variety of HPS6 fragments. Interaction between the N-terminal region of HPS6 (aa 66–200) and the extreme C-terminus of p150<sub>Glued</sub> (aa 1138–1281) was verified in the yeast two-hybrid assay (Fig. 1G). Although the extreme N-terminal fragments of HPS6 were not expressed in mammalian cells, surprisingly, all of the N-terminal fragments (aa 1–400, 1–500 and 1–700) that were expressed in cultured cells failed to co-immunoprecipitate with p150<sub>Glued</sub> (Fig. 1H), suggesting that the region adjacent to the extreme N-terminus inhibits its interaction with p150<sub>Glued</sub> through aberrant protein folding. Intriguingly, among the truncation mutants expressed, an N-terminally truncated fragment (aa 201–805) was able to bind p150<sub>Glued</sub>, and another N-terminal truncation fragment (aa 301–805) showed weak p150<sub>Glued</sub> binding (Fig. 1H). Taken together, the finding that HPS6 interacts with p150<sub>Glued</sub> prompted us to hypothesize that HPS6 serves as cargo adaptor to mediate the retrograde transport driven by the dynein–dynactin motor complex.

**The perinuclear distribution of HPS6 requires activity of the dynein–dynactin complex**

Immunofluorescence staining of HeLa cells with antibodies against HPS6 revealed that it primarily localized to perinuclear vesicular structures (supplementary material Fig. S1). To test whether the perinuclear distribution of HPS6-associated membranous structures requires retrograde transport mediated by the dynein–dynactin motor complex, we treated cells with nocodazole to disrupt the microtubule cytoskeleton, which serves as track for the retrograde motor. Indeed, nocodazole treatment resulted in disassembly of the microtubule network and dispersal of HPS6 signals to the cell periphery (Fig. 2A). Moreover, overexpression of a Flag-tagged p50 dynamitin (DCTN2) subunit of dynactin, which disassembles the dynein–dynactin protein complex (Echeverri et al., 1996; Melkonian et al., 2007), also caused dispersal of HPS6 signals to the cell periphery (Fig. 2B), indicating that the perinuclear distribution of HPS6-associated vesicles requires both an intact microtubule cytoskeleton and activity of the dynein–dynactin motor complex.

Because p150<sub>Glued</sub> binds to HPS6 through its C-terminus, and because its N-terminal and middle regions mediate its interaction with DIC and the Arp1 subunit of dynactin, respectively (Fig. 1C) (Schoer, 2004), we reasoned that in the absence of the cargo-recognition site, the N-terminal fragment of p150<sub>Glued</sub> alone could serve as a dominant-negative mutant to compete with the full-length protein in the motor complex. In our previous study, we found that SNX6-mediated, dynein–dynactin-complex-driven retrograde transport of CI-MPR from endosomes to the TGN is disrupted by overexpression of a p150<sub>Glued</sub> truncation mutant (aa 1–910, Flag-p150<sub>Glued-N</sub>) that lacks the SNX6-binding C-terminus (Hong et al., 2009). In agreement with this, we found in our current study that overexpression of the same protein fragment perturbed HPS6 distribution, thereby causing cytoplasmic dispersal of HPS6. However, overexpression of its HPS6-binding C-terminus (aa 911–1281, Flag-p150<sub>Glued-C</sub>) had no obvious effect (Fig. 2B), probably because it cannot assemble into the dynactin complex by itself and, thus, cannot compete with the endogenous, wild-type protein for vesicular cargoes (Johansson et al., 2007). Therefore, our data establish that the perinuclear distribution of HPS6 requires retrograde transport driven by the dynein–dynactin motor complex.

**HPS6 associates with the lysosomal compartment**

To investigate the cellular function(s) of HPS6, we first analyzed its subcellular compartmental distribution in HeLa cells by immunofluorescence staining and confocal microscopy. HPS6 partially colocalized with the LE/MVB marker CD63 (also known as LAMP-3) (Fig. 3A,B). Intriguingly, HPS6 colocalized to a higher extent with lysosome-associated membrane proteins 1 and 2 (LAMP1 and LAMP2) (Fig. 3A,B), two of the most abundant lysosomal proteins (Eskenilien, 2006). In contrast, little colocalization between HPS6 and the early endosome marker EEA1 or the trans-Golgi network (TGN)-resident protein p230 (GOLGA4) was observed (Fig. 3A,B). However, not only p150<sub>Glued</sub>, but also LAMP1 and LAMP2 were detected on
HPS6-positive vesicles that had been immunoisolated from membrane fractions of HeLa cells (Fig. 3C). Moreover, immunoisolation of LAMP1-positive vesicles from membrane fractions showed that not only LAMP2 but also HPS6 and p150Glued associate with LAMP1-positive lysosomes (Fig. 3D). To further determine whether HPS6 is, indeed, associated with...
lysosomes, we performed ultrastructural analysis of immunoisolated HPS6-associated vesicles on magnetic beads by transmission electron microscopy. Electron-dense lamellar lysosomal structures were found to be associated with HPS6-conjugated beads, whereas no vesicular structures were detected in association with IgG-conjugated beads (Fig. 3E). In contrast, when immunoinosolation was performed with antibodies against the cis-Golgi marker GM130, Golgi-cisternae-like structures were found associated with GM130-conjugated beads (Fig. 3E). Together these data indicate that HPS6 associates with lysosomes.

**HPS6 mediates minus-end-directed microtubule motility and perinuclear positioning of lysosomes**

Having established that HPS6 requires the dynein–dynactin motor complex for perinuclear distribution and that HPS6 associates with lysosomes, we reasoned that HPS6 plays a role in the retrograde transport and subcellular distribution of lysosomes. To investigate the role(s) of HPS6 in lysosomal transport and positioning, we depleted HPS6 by small interfering RNA (siRNA)-mediated RNA interference (RNAi) (supplementary material Fig. S1E,F). Immunofluorescence microscopy showed that lysosomes labeled with LAMP1 or LAMP2 were evenly distributed in cells that had been depleted of HPS6 (Fig. 4A,B).

The HPS6-deficiency-induced subcellular distribution phenotype of LAMP1 was rescued by Flag-tagged, siRNA-resistant HPS6 (Fig. 4D,E), indicating that HPS6 is specifically required for the perinuclear positioning of lysosomes. Furthermore, overexpression of the HPS6 N-terminal fragment (aa 1–700), which cannot bind to p150Glued but retains the ability to bind to the other BLOC-2 subunit HPS5 (Fig. 4C), did not rescue the lysosome dispersal phenotype (Fig. 4D,E), indicating that the p150Glued-binding ability of HPS6 is required for the perinuclear positioning of lysosomes. Overexpression of Flag-HPS6, however, did not affect the subcellular distribution of LAMP1 lysosomes in HeLa cells (Fig. 4F,H).

Having established that HPS6 is required for lysosome perinuclear positioning and that HPS6 interacts with the dynein–dynactin motor complex, we next asked whether HPS6 mediates retrograde transport of lysosomes by linking the motor complex to lysosomal cargoes. Since we have demonstrated that overexpression of p150Glued-N caused cytoplasmic dispersal of HPS6, possibly by competing with endogenous p150Glued for binding of the dynein–dynactin motor complex, we reasoned that this p150Glued fragment can also block retrograde transport mediated by HPS6. Indeed, immunofluorescence microscopy showed that overexpression of Flag-p150Glued-N but not Flag-p150Glued-C caused dispersal of LAMP1-labeled lysosomes to the cell periphery (Fig. 4G,H), indicating that the interaction of HPS6 with p150Glued is required for lysosomal positioning in the perinuclear region. Similarly, consistent with previous studies (Burkhardt et al., 1997), overexpression of Flag-p50, which disrupts the dynein–dynactin protein complex, caused peripheral distribution of lysosomes (Fig. 4G,H).

To test whether HPS6 is required for the association of the dynein–dynactin motor complex with lysosomes, we performed immunoisolation of lysosomes from HeLa cell lysates with antibodies to LAMP1. Immunoblot analysis indicated that there is a significant decrease in the levels of p150Glued as well as DIC co-immunoisolated with LAMP1 in cells depleted of HPS6 (Fig. 4I). It has been reported that, in the fibroblasts from Hps1 mutant mice (pale ear, BLOC-3 deficient), LAMP1-positive compartments are less concentrated than wild-type, whereas their perinuclear clustering and surface levels were normal in fibroblasts from Hps3 mutant mice (cocoa, BLOC-2 deficient) (Falcón-Pérez et al., 2005; Salazar et al., 2006). To determine whether loss of HPS6 function in mouse causes a phenotype of LAMP1 mislocalization similar to that caused by HPS6 gene knockdown, we performed immunofluorescence staining of mouse embryonic fibroblasts (MEFs) from ruby-eye (ru) mice, which harbor an internal deletion (aa 187–189) mutant of HPS6 (Fig. 5A). Confocal microscopy analysis showed that, compared to wild-type (WT) MEFs, both LAMP1 and LAMP2 were more peripherally distributed in ru MEFs (Fig. 5B), indicating that loss of HPS6 function, indeed, leads to the cytoplasmic dispersal of lysosomes. Consistently, immunoinosolation from brain lysates of ru mice by using antibodies against LAMP1 detected less p150Glued and DIC on LAMP1 vesicles compared with those from wild-type mice (Fig. 5C). Moreover, the cytoplasmic dispersal phenotype of LAMP1 in ru MEFs was also rescued by the full-length HPS6 fused to enhanced green fluorescent protein (EGFP) (Fig. 5D).

To further verify that HPS6, indeed, mediates retrograde transport of lysosomes driven by the dynein–dynactin complex, we performed live imaging experiments. Image analyses of trajectories of mCherry-LAMP1-labeled vesicles indicate that,
compared with control cells, there was, indeed, a significant decrease in movement of LAMP1 vesicles towards the cell center in HPS6-depleted cells (supplementary material Fig. S2, Movies 1 and 2). Taken together, these results indicate that the perinuclear positioning of lysosomes requires retrograde transport that is driven by the dynein–dynactin complex and that HPS6-p150 Glued interaction mediates the retrograde transport of lysosomes to the cell center. That HPS6 is required for perinuclear distribution of lysosomes prompted us to ask whether it is also required for the subcellular distribution of late endosomes and multivesicular bodies. Immunofluorescence microscopy of HeLa cells by using antibodies against Rab7 and CD63 – markers for late endosomes and multivesicular bodies, respectively – did not detect any changes in the distribution pattern of Rab7 and CD63-positive structures in HPS6-depleted cells (supplementary material Fig. S3A-D). Taken together, these results indicate that the perinuclear positioning of lysosomes requires retrograde transport that is driven by the dynein–dynactin complex and that HPS6-p150 Glued interaction mediates the retrograde transport of lysosomes to the cell center. Previously it has been reported that RILP binds to p150 Glued and mediates retrograde transport of lysosomes towards the cell center (Johansson et al., 2007). To test whether there is functional redundancy between HPS6 and RILP in lysosomal movement and positioning, we performed double knockdown of HPS6 and RILP and examined lysosome distribution by immunostaining followed by quantitative analysis. Radial profile plots showed that lysosomes were more peripherally distributed in RILP- or HPS6-depleted cells, and double knockdown caused a further shift of LAMP1 fluorescence towards the cell periphery (supplementary material Fig. S4A-C), suggesting that partially redundant or compensatory mechanisms exist to mediate centripetal movement and perinuclear positioning of lysosomes. Moreover, we examined colocalization of HPS6 with RILP-labeled lysosomes and found that, consistent with
Fig. 4. See next page for legend.
Fig. 4. The perinuclear distribution of lysosomes requires HPS6-mediated dynein-dynactin activity. (A) HeLa cells were transfected with a control non-targeting siRNA, or siRNAs targeting HPS6 and stained with phallolidin and antibodies against LAMP1 or LAMP2, respectively, KD, knockdown. (B) Radial profile plots of signal distribution of LAMP1 and LAMP2 relative to the nucleus in A. Shown is the distribution of fluorescent signals starting from the centroid and extending toward the cell periphery. Values are averages corresponding to the fraction of total fluorescence present in each concentric circle drawn from the centroid (Mean ± s.e.m., n=3 experiments, 20–22 cells/experiment). (C) HEK293 cells overexpressing Flag-tagged HP5, Myc-tagged HPS6, HP6-N (aa1–700), or the internal deletion mutant of HPS6 (Δ1187–189) were lysed for co-IP with immobilized Flag antibody. Input and bound proteins were analyzed by SDS-PAGE and immunoblotting with antibodies against Myc and Flag. (D) HPS6-depleted cells were transfected with Flag vector, Flag-tagged siRNA-resistant murine HPS6 or HPS6-N and stained with antibodies against LAMP1. (E) Radial profile plots of signal distribution of LAMP1 relative to the nucleus in D (mean ± s.e.m., n=3 experiments, 20–22 cells/experiment). (F) HeLa cells were transiently transfected with Flag-tagged HPS6 for 24 h and immunostained with antibodies against Flag and LAMP1. (G) HeLa cells were transiently transfected with Flag vector, Flag-tagged p50, p150Glued, or p150Glued-C for 24 h and immunostained with antibodies against Flag and LAMP1. (H) Radial profile plots of signal distribution of LAMP1 relative to the nucleus in F and G (mean ± s.e.m., n=3 experiments, 20–22 cells/experiment). (I) Immunoprecipitation of LAMP1-associated membranous organelles from HeLa cells. Input and bound proteins were analyzed by SDS-PAGE and immunoblotting with antibodies to p150Glued, EEA1, LAMP1, LAMP2, HPS6, DIC and β-actin. Relative amount of p150Glued and DIC co-immunolysolated with LAMP1 was determined with NIH ImageJ. Data represent mean ± s.e.m. (n=3 experiments, **P<0.01). Scale bars: 10 μm.

Previous reports (Cantalupo et al., 2001; Jordens et al., 2001), RILP colocalized with LAMP1 and Rab7 (supplementary material Fig. S4D). In contrast, RILP did not colocalize with HPS6 (supplementary material Fig. S4D), suggesting that they mediate different retrograde lysosomal transport pathways.

HPS6 and its interaction with p150Glued are required for lysosomal degradation of endocytosed cargoes and lysosomal enzyme activity

Previous studies have found that lysosomal positioning coordinates nutrient responses through the regulation of mTORC1 signaling and autophagic flux (Korolchuk et al., 2011). To assess the physiological relevance of HPS6-mediated perinuclear positioning of lysosomes, first we examined lysosomal activity in HPS6-depleted cells. To this end, we incubated HeLa cells with the fluorogenic substrate DQ-BSA and measured its fluorescence intensity, which is an indication of lysosomal degradation capacity as endocytosed DQ-BSA became fluorescent upon proteolytic cleavage in lysosomes (Vázquez and Colombo, 2009). Compared with control cells, weaker DQ-BSA fluorescence was detected in HPS6-depleted cells up until 3 hours of incubation (Fig. 6A,B), indicating that HPS6 depletion delays the degradation of endocytosed cargo in lysosomes. Similarly, when we incubated cells with Magic Red cathepsin B (MR catB), a membrane permeable probe that fluoresces upon cleavage by the lysosomal protease cathepsin B (Creasy et al., 2007a), a decrease in the mean intensity of fluorescent puncta was detected in HPS6-depleted cells (Fig. 6C,D), indicating that lysosomal enzyme activity was impaired. Overexpression of siRNA-resistant full-length HPS6 restored lysosomal degradation of DQ-BSA and MR catB, whereas the truncation mutant that failed to interact with p150Glued but retained HPS5-binding ability had no effect (Fig. 6E-H). Moreover, overexpression of p50 or

Fig. 5. Overexpression of HPS6 rescues lysosome dispersal phenotype in MEF cells from ru mouse. (A) HEK293 cells overexpressing Flag-tagged p150Glued or Flag-tagged HP55 and Myc-tagged HPS6 or Myc-tagged HPS6 deletion mutant (Δ1187–189) were lysed for co-IP with immobilized Flag antibody. Input and bound proteins were analyzed by SDS-PAGE and immunoblotting with antibodies to Flag and Myc. (B) Primary cultured MEF from WT or ru mouse were immunostained with antibodies against LAMP1 and LAMP2. (C) HeLa cells overexpressing Flag-tagged HPS6 for 24 h and immunostained with antibodies against Flag and LAMP1. (D) MEF cells from ru mouse were transfected with constructs expressing EGFP or EGFP-HPS6 for 24 h and immunostained with antibodies against LAMP1. Scale bar: 10 μm.
p150\textsuperscript{Glued-N} fused to EGFP caused a decrease in the fluorescence intensity of MR catB substrate puncta, whereas the EGFP-p150\textsuperscript{Glued-C} fusion protein did not (Fig. 6I,J). Together these data indicate that HPS6 and its interaction with p150\textsuperscript{Glued} are required for both degradation of endocytic cargo and enzyme activity of lysosomes.

Fig. 6. See next page for legend.
Lysosomes fuse with LEs/MVBs and mature in the perinuclear region

The fact that knockdown of HPS6 caused both cytoplasmic dispersal of lysosomes and impaired delivery of endocytic cargos to lysosomes prompted us to speculate that fusion between LEs/MVBs and lysosomes takes place in the perinuclear region. When we then plotted the extent of colocalization of Dextran–488 and Dextran–647 as a function of radial distance starting from the center of the nucleus determined by its measured centroid (Niu et al., 2013), the radial profile plot revealed that the extent of colocalization of Dextran–488 and Dextran–647 peaked near the nucleus in both control and HPS6-depleted cells (Fig. 8A), as well as in cells that overexpressed p50 or p150Glued-N (Fig. 8B).

Similarly, we investigated the relationship between lysosomal enzyme activity and subcellular distribution with radial profile plots of the fluorescent signals of MR catB substrate. The mean intensity of MR catB fluorescence peaked near the nucleus not only in control knockdown and HPS6-depleted cells (Fig. 8C), but also in cells that overexpressed p50 or p150Glued-N (Fig. 8D), which indicates that lysosomal enzyme activity is highest in the perinuclear region. Because enzymatic activity of lysosomal proteases is dependent on the pH of lysosomes, and because there is evidence that lysosomal acidification is dynamic and tightly regulated (Majumdar et al., 2011; Majumdar et al., 2007; Mindell, 2012), we speculated that lysosomal acidification also takes place in the perinuclear region. To this end, we first incubated cells overexpressing mCherry–LAMP1 with LysoSensor (LysoSensor™ green DND-189), a membrane-permeable pH probe (pKᵢ = 5.2) that accumulates and fluoresces green in acidic compartments within a pH range of 4–5 (Lin et al., 2001; Siemasko et al., 1998). Intriguingly, compared with control cells, there was a decrease in both the intensity of LysoSensor fluorescent puncta and in the colocalization of LysoSensor with mCherry–LAMP1 in HPS6-depleted cells (Fig. 8E,F). This indicates that HPS6 knockdown also impaired the acidification of lysosomes, thereby contributing to the lower lysosomal enzyme activity that was detected by using the MR catB substrate (Fig. 6C,D). Furthermore, the radial profile plots of LysoSensor showed that its mean fluorescence intensity peaked near the nucleus in both control and HPS6-depleted cells (Fig. 8G), indicating also a positive correlation between lysosomal acidification and perinuclear positioning. Together, these data suggest that heterotypic fusion between LEs/MVBs and lysosomes as well as lysosome acidification and maturation most frequently take place in the perinuclear region.

The fact that lysosomes fuse with endocytic cargos and mature in the perinuclear region prompted us to speculate that fusion of lysosomes with LEs/MVBs plays a role in lysosomal maturation and/or acidification. It has been reported previously that Rab7 is required for the transfer of endocytic cargo from the LEs/MVBs to the lysosomes (Vanilihan and Ceresa, 2009), probably by regulating the fusion competence of the LEs/MVBs. To determine whether fusion between LE/MVB and lysosome is required for lysosomal maturation and acquisition of enzyme activity, we depleted Rab7 in HeLa cells by knockdown with siRNA (Fig. 8H). We detected lysosomal degradative capacity with MR catB fluorescence. Compared to control cells, there was a significant decrease in MR catB fluorescence in Rab7-depleted cells (Fig. 8I,J), indicating that Rab7-mediated fusion between LEs/MVBs and lysosomes, indeed, plays an important role in lysosomal maturation.

HPS6 depletion and disruption of dynein–dynactin complex activity impair fusion between LEs/MVBs and lysosomes

Direct fusion between LEs/MVBs and lysosomes was observed, and is required for delivery of endocytic cargos to lysosomes for degradation (Bright et al., 2005; Bright et al., 1997; Futter et al., 1996; Mullock et al., 1998). To determine whether the decreased degradation of endocytic cargo in HPS6-depleted cells is caused by a failure in the fusion between LEs/MVBs and lysosomes, we labeled lysosomes by incubating cells in medium containing dextran conjugated to Alexa-Fluor-647 (Dextran–647) for 12 h followed by an 8 h chase in conjugate-free medium. Cells were then incubated in medium containing dextran conjugated to Alexa-Fluor-488 (Dextran–488) for 3 h followed by confocal imaging (Bright et al., 2005). Confocal microscopy analysis showed no difference in the mean intensity and number of fluorescent puncta (Fig. 7A,B), indicating that HPS6 knockdown did not affect the uptake of dextran. However, there was a decrease in the colocalization of Dextran–488 and Dextran–647 in HPS6-depleted cells (Fig. 7A,B), suggesting that fusion between LEs/MVBs and lysosomes requires perinuclear distribution of lysosomes. Similarly, less Dextran–488 colocalized with mCherry–LAMP1 in HPS6-depleted cells (Fig. 7C,D), indicating that there was less fusion between LEs/MVBs and lysosomes. Furthermore, ultrastructural analysis of HPS6-depleted cells revealed that, although no significant difference in the size of lysosomes was detected (supplementary material Fig. S3I), compared with control cells, there was an increase in the number of MVBs and a decrease in the number of lamellar-structured and electron-dense lysosomes (Fig. 7E,F). Consistently, overexpression of mCherry-p50 or mCherry-p150Glued-N caused a decrease in the colocalization between Dextran–488 and Dextran–647 (Fig. 7G,H), whereas with mCherry fused to p150Glued-N, no change in colocalization was seen. Together, these data indicate that the fusion between endocytic cargos and lysosomes requires HPS6 and an active dynein–dynactin complex.
Fig. 7. Knockdown of HPS6 results in a decrease in fusion between LE/MVB and lysosome. (A) Snapshots of control or HPS6 knockdown cells preloaded with Dextran-647 to label lysosomes and then incubated for 3 h with Dextran-488 to allow labeling of endocytic endosomes. (B) Quantification of the puncta number and mean intensity of endo-lysosomal compartments labeled with Dextran-488 or −647 and their degree of overlap in A. Data represent mean ± s.e.m. (ctrl KD, n=38 cells; HPS6 KD, n=39 cells. ***P<0.001). (C) Snapshots of control or HPS6 knockdown cells transfected with mCherry-LAMP1 for 24 h and then incubated with Dextran-488 for 3 h. (D) Quantification of colocalization between Dextran-488 and mCherry-LAMP1 in C. Data represent mean ± s.e.m. (n=35 cells, *P <0.05). (E) Representative electron micrographs of control (left) or HPS6 knockdown (middle) cells. Arrows indicate MVBs, solid arrowheads indicate lysosomes. Right panels are representative higher magnification images of structures with a typical morphology of MVB or lysosomes. (F) Quantification of indicated organelles in E. Data represent mean ± s.e.m. (ctrl KD, n=101 cells; HPS6 KD, n=103 cells. *P=0.0221, ***P<0.001). (G) Cells transfected with mCherry, mCherry-p50, mCherry-p150Glued-N or mCherry-p150Glued-C for 16 h were loaded with Dextran-647 and Dextran-488 sequentially as in A, stained live with Hoechst 33342 for 30 min and imaged by confocal microscope. (H) Quantification of colocalization of Dextran-488 and Dextran-647-labeled compartments in G. Data represent mean ± s.e.m. (n=40–60 cells, ***P <0.001). Scale bars: 10 μm (A,C,G).
DISCUSSION

In this study, we set out to find the interaction partner(s) of p150\textsuperscript{Glued}, a subunit of the dynein–dynactin complex. We identified HPS6, a subunit of the BLOC-2 that is involved in LRO biogenesis and Hermansky–Pudlak syndrome. In HeLa cells, HPS6 is mostly absent from early endosomes and the TGN; knockdown of HPS6 has no effect on the distribution of these organelles, neither does it affect the distribution of late endosomes and multivesicular bodies. Moreover, depletion of HPS6 has no effect on endocytic trafficking or trafficking between endosomes and the TGN. In contrast, although disruption of the activity of the dynein–dynactin complex results in a block of retrograde transport and peripheral distribution of lysosomes, HPS6 depletion causes cytoplasmic dispersal of lysosomes. Disruption of the interaction between HPS6-p150\textsuperscript{Glued} and the dominant negative p150\textsuperscript{Glued} N-terminus.
Fig. 8. Lysosomes fuse with LEs/MVBs and mature in the perinuclear region. (A) Radial profile plots of colocalized signals of Dextran-488 and Dextran-647 in Fig. 7A. Shown is the distribution of colocalized fluorescent signals starting from the centroid and extending toward the cell periphery. Values are averages corresponding to the fraction of total fluorescence present in each concentric circle drawn from the centroid (mean ± s.e.m., n=3 experiments, >11 cells/experiment). (B) Radial profile plots of colocalized signals of Dextran-488 and Dextran-647 in Fig. 7G (mean ± s.e.m., n=3 experiments, 15 cells/experiment). (C) Radial profile plots of signal distribution of MR catB substrate relative to the nucleus in Fig. 6C (mean ± s.e.m., n=3 experiments, >11 cells/experiment). (D) Radial profile plots of signal distribution of MR catB substrate relative to the nucleus in Fig. 6I (mean ± s.e.m., n=3 experiments, 15 cells/experiment). (E) Snapshots of control or HPS6 knockdown cells expressing mCherry-LAMP1 and incubated with LysoSensor DND-189 for 30 min. (F) Quantification of LysoSensor signal distribution relative to the nucleus in E (mean substrate per cell. Data represent mean ± s.e.m., n=3 experiments, 15 cells/experiment). (G) Radial profile plots of LysoSensor signal distribution relative to the nucleus in E (mean ± s.e.m., n=3 experiments, >11 cells/experiment). (H) Immunostaining analysis of HeLa cell showing Rab7 silencing with a mixture of Rab7-targeting siRNA in cells; HPS6 KD, mCherry-LAMP1 (right) in E. Data represent mean ± s.e.m., (ctrl KD, n=31 cells; HPS6 KD, n=40 cells. ***P<0.001). (I) Snapshots of control or Rab7 knockdown cells incubated with MR catB substrate for 2 h. (J) Quantification of mean intensity of MR catB substrate per cell. Data represent mean ± s.e.m. (n=3 experiments, ***P<0.001). (K) Model for lysosomal degradation defects in the absence of HPS6. Lysosomes move along microtubules in a bidirectional manner. HPS6 serves as dynein–dynactin cargo adaptor by interacting with p150Glued and tethering the dynein–dynactin complex to lysosomes. Depletion of HPS6 impairs retrograde transport of lysosomes, thus resulting in cytoplasmic dispersal of lysosomes. Moreover, it delays lysosomal degradation of endocytosed proteins, possibly resulted from reduced fusion of LEs/MVBs with perinuclear lysosomes. Scale bars: 10 μm.

has a similar effect, which indicates that HPS6 is specifically involved in retrograde transport of lysosomes towards the cell center that is driven by the dynein–dynactin complex (Fig. 8K).

Lysosomes are dispersed in Lmp1+/−/Lmp2−/− cells (Huynh et al., 2007) and the dynein–dynactin complex is required for the centripetal movement of lysosomes (Burkhardt et al., 1997; Cantalupo et al., 2001; Harada et al., 1998; Jordens et al., 2001). However, mechanisms by which the retrograde motor is recruited to lysosomes are only partially understood. Previous studies have identified multiple dynein-recruitment and -regulatory factors that include RILP (Cantalupo et al., 2001; Johansson et al., 2007; Jordens et al., 2001) and LIC1/2 (Tan et al., 2011) – dynein cargo adaptors for late endosomes and lysosomes in HeLa cells. Moreover, in HPS1-deficient (pale ear) murine fibroblasts, not only the perinuclear clustering of LAMP1-positive LEs and lysosomes but also the frequency of the movement of LAMP1-GFP-labeled organelles along microtubules was reduced, suggesting that BLOC-3 regulates the attachment of late endosomes and lysosomes to microtubule motors (Falcón-Pérez et al., 2005; Nazarian et al., 2003). In this study, we identified HPS6 as yet another dynein cargo adaptor for lysosomes that is required for lysosome perinuclear positioning. Conceivably, these results indicate that multiple dynein-dynactin recruitment mechanisms exist for lysosome trafficking, which explains the mild phenotypes caused by HPS6 depletion. Alternatively, different dynein adaptors for late endosomes and/or lysosomes or lysosome-related organelles might provide cargo specificity/ selectivity that is tissue or cell type-specific and/or organelle specific.

Most recently, BLOC-3 was identified as a guanine nucleotide exchange factor for Rab32 and Rab38, two small GTPases that are required for melanosome biogenesis (Gerondopoulos et al., 2012). BLOC-1 was shown to promote endosomal maturation by recruiting the Rab5 GTPase-activating protein Msb3 to the endosomal membrane in yeast (John Peter et al., 2013). However, molecular function(s) of BLOC-2 subunits remain elusive. Our study on HPS6 reveals that it has a role in mediating lysosomal trafficking and functioning in the HeLa cells, which is illustrated in the model in Fig. 8K. It remains to be elucidated whether HPS6, as the subunit of the BLOC-2 complex, also serves as a dynein adaptor in the biogenesis and trafficking of lysosomes, and of lysosome-related organelles in specialized cell types, such as fibroblasts and melanocytes.

Fusion between LEs/MVBs and lysosomes is a crucial step in lysosomal biogenesis and function. Previous studies have demonstrated that Rab7 is required for efficient fusion between LEs/MVBs and lysosomes in HeLa cells, probably through its interaction with the HOPS (homotypic fusion and vacuole protein sorting) complex (Luzio et al., 2007; Vanlantingham and Ceresa, 2009). In our study, fusion between LEs/MVBs and lysosomes is impaired in cells depleted of HPS6, accompanying the more peripheral distribution of LAMP1- and/or LAMP2-positive compartments, suggesting that the perinuclear clustering of lysosomes is required for the correct fusion between LEs/MVBs and lysosomes (Fig. 8). Further, blocking LE/MVB-lysosome fusion by knocking down Rab7 also causes a reduction in lysosomal degradative capacity, supporting the notion that fusion between LEs/MVBs and lysosomes to form endo-lysosomes is required for full maturation and activation of the lysosomal compartment. Previous studies on lysosomal degradation of amyloid β in microglial cells have provided strong evidence that lysosomal acidification is tightly regulated (Majumdar et al., 2011; Majumdar et al., 2008; Majumdar et al., 2007). It will, thus, be important to investigate mechanism(s) by which heterotypic fusion between LEs/MVBs and lysosomes triggers further acidification and enzyme activation in the lysosomal compartment.
purposes. The p150
gleted fragments were PCR-amplified from the Flag-
mp150
gleted plasmid and cloned into PCMv-Tag2b (Stratagene, La Jolla) and pEGFP-C2 (Clontech, CA). The p50 full-length cDNA was PCR-
amplified from the Flag-p50 construct and cloned into pmCherry-C1 and pEGFP-C2. The mCherry-LAMP1 and Myc–RILP constructs were

Wang (Xiamen University, China), respectively.

Cell culture, siRNA and transfection

HeLa, HEK293 or MEF cells were grown in DMEM (HyClone, Logan, UT) supplemented with 10% fetal bovine serum (HyClone, Logan, UT), 2 mM L-glutamine at 37°C with 5% CO2. For DNA transfection, cells were grown to 50% confluency and transfected with plasmid DNA using LipofectamineTM 2000 (Invitrogen, Carlsbad, CA) following the manufacturer’s instructions. For transfection of small interfering RNA (siRNA), HeLa cells seeded on day 0 were transfected with 50 nM siRNA duplex by using Lipofectamine TM 2000 following the manufacturer’s instructions on day 1, and seeded and transfected again on day 2; experiments were performed 72 h post-transfection. For Rab7 knockdown, a mixture of oligonucleotide duplexes (Vanlandingham and Ceresa, 2009) was transfected as previously described. For rescue experiments, cells were transfected with plasmid DNA by using LipofectamineTM 2000 on day 3 after transfection with siRNA, and analyzed for the experiment on day 4. siRNA sequences used in this study were: 5’-GAGGCUUAGAACAGCUGAATT-3’ and 5’-GUACAAAGCCACAAUAGGAUU-3’, and 5’-AAACGGA-

Microscope Sciences, Hatfield, PA), 2.5% sucrose in 0.1 M PB

Electron microscopy

The ultrastructural analysis of cells was performed as described (Vanlandingham and Ceresa, 2009). HeLa cells were transfected with control or HPS6 siRNA and 72 h post-transfection, cells were washed once with ice-cold PBS and fixed in 2.5% glutaraldehyde (Electronic Microscope Sciences, Hatfield, PA), 0.2% tannic acid (Electronic Microscope Sciences, Hatfield, PA), and 2.5% sucrose in 0.1 M PB buffer (pH 7.4) at 4°C for 2 h, washed three times with 0.1 M PB buffer (pH 7.4), scraped, and pelleted. Cells were then post-fixed with 1% OsO4 in 0.1M PB for 1.5 h at 4°C. Samples were then dehydrated in ascending acetone series (30%, 50%, 70%, 90%, 100% three times for 3 min, followed by dehydration in pure ethanol: pure acetone (1:1) for 3 min, and dehydration in pure acetone for 3 min. The samples were then embedded in Embed 812 (Electronic Microscope Sciences, Hatfield, PA).

Immunoprecipitation

Immunoprecipitation experiments were performed as previously described (Hong et al., 2009). For immunoprecipitation assay, HeLa or HEK293 cells were washed once with PBS and lysed either with lysis buffer A (0.05% [vol/ vol] NP-40, 20 mMTris·HCl pH 7.4, 50 mM NaCl, protease inhibitors) for endogenous IP or with lysis buffer B (1% [vol/ vol] Triton X-100, 50 mMTris·HCl pH 7.4, 150 mM NaCl, 1 mM EDTA and protease inhibitors) for all other IPs.

Immunofluorescence staining and confocal microscopy

Cells were grown on coverslips, fixed in 4% paraformaldehyde/PBS (pH 7.4), and permeabilized with either PBS containing 0.1% saponin (Sigma-Aldrich, St Louis, MO), 1% goat serum, 1% BSA for staining of LAMP1, LAMP2 and CD63 or PBS containing 0.3% Triton X-100, 1% goat serum, 1% BSA for staining of other proteins. Cells were incubated with primary antibodies for 1 h at room temperature; secondary antibodies conjugated to Alexa-Fluor-488, Alexa-Fluor-555 or Alexa-Fluor-647 were used for detection. Images were taken with the Confocal Microscope DIGITAL ECLIPSE CiSI (Nikon, Japan).

Yeast two-hybrid assay

Matchmaker Gal4 two-hybrid system 3 (Clontech, Mountain View, CA) was used as previously described (Hong et al., 2009). Yeast strain AH109 was transformed with pGBK7T and pGAD7T vectors, and plated on tryptophan- and leucine-deficient SD medium (SD-Leu-Trp). The transformed colonies were further plated on tryptophan-, leucine-, histidine- and adenine-deficient plates (SD-Leu-Trp-Ade-His) for interaction assay. The interaction was assessed by comparing the growth and blue color of colonies on SD-Leu-Trp-Ade-His containing 2 mg/ml X-a-Gal.

Immunoisolation

HeLa cells were homogenized in buffer (10 mM HEPES pH 7.4, 1 mM EDTA, 0.25 M sucrose, and protease inhibitors) and centrifuged at 800 g for 10 min to collect the supernatant. The supernatant was pooled with the supernatant from previous centrifugation and subjected to high-speed centrifugation at 100,000 g at 4°C for 1 h (TLS-55 rotor, OptimaTM MAX Ultracentrifuge; Beckman Coulter, Germany). The pellet was resuspended in homogenization buffer (membrane fraction) and subjected to immunoisolation with Dynabeads Protein G (Invitrogen, Carlsbad, CA) coupled with specific antibodies or control IgG as previously described (Niu et al., 2013). Beads were washed four times with 5% BSA in PBS, and once with 0.1% BSA in PBS before elution. For detection of organelle markers by immunoblotting, the beads were eluted by boiling in 2× SDS gel loading buffer and samples were analyzed by SDS-PAGE and immunoblotting. For electron microscopy, beads were processed as follows.

Electron microscopy

Immunoprecipitated bead-bound vesicles were fixed with 2.5% glutaraldehyde (Electronic Microscope Sciences, Hatfield, PA), washed three times with 0.1M PB buffer (pH 7.4), post-fixed with 1% OsO4 (SPI Supplies, West Chester, OH), dehydrated in ethanol of ascending purity (30%, 50%, 70%, 80% and 90%) at 3 min per dilution, and in 100% ethanol three times for 3 min, followed by dehydration in pure ethanol: pure acetone (1:1) for 3 min, and dehydration in pure acetone for 3 min. The samples were then embedded in Embed 812 (Electronic Microscope Sciences, Hatfield, PA).

Live cell imaging

Dextran internalization assay was performed as previously described (Bright et al., 2005). Cells were loaded with 0.2 mg/ml Alexa-Fluor-647-conjugated dextran (10,000 MW, Invitrogen, Carlsbad, CA) at 37°C for 8 h and incubated in conjugate-free medium for 12 h. The cells were then incubated with 0.5 mg/ml Alexa-Fluor-488-conjugated dextran for 3 h, rinsed with dextran-free medium and processed for imaging using confocal microscope DIGITAL ECLIPSE CiSI (Nikon, Japan).

To test lysosomal activity, cells transfected with control or siRNA targeting HPS6 for 48 h were seeded in 4-well coverglass chambers (Thermo Fisher Scientific, Waltham, MA). After 16 h, cells were incubated for 1–6 h with 0.05 mg/ml DQ-BSA Green (Invitrogen) (Vázquez and Colombo, 2009), or 2 h with Magic Red cathepsin B substrate (MR caB, ImmunoChemistry Technology, Bloomington, MN) (Creasy et al., 2007b) following manufacturer’s instructions. In brief, 26X MR caB solution was added directly to culture medium at a ratio of 1:26, and then the overlay cell medium was gently mixed to ensure even exposure to MR caB. After incubating for 2 h at 37°C, culture medium containing MR caB was removed, and cells were rinsed twice with PBS. To inhibit the lysosomal V-ATPase activity, 1 μM bafilomycin A1
Statistical analyses were performed using GraphPad Prism 5 (GraphPad Software, La Jolla, CA). Pairwise significance was calculated using two-tailed unpaired Student’s t-test or nonparametric Student’s t-test (Mann-Whitney test). For evaluation of statistical significance of three or more groups of samples, one-way analysis of variance with a Tukey post test was used. Results are described as mean ± s.e.m. Data were considered statistically significant at P<0.05, *P<0.05; **P<0.01; ***P<0.001.

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Competing interests
The authors declare no competing interests.

Author contributions
Experiments were designed by K.L., W.L. and J-J.L. Experiments were conducted by K.L., L.Y. and C.Z. Data were analyzed by K.L. and Y.N. The manuscript was written by J-J.L. with help from K.L., L.Y., C.Z., Y.N. and W.L.

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Supplementary material
Supplementary material available online at http://jcs.biologists.orglookup/suppl/dis1.41978/DC1

References


Fig. S1. HPS6 is efficiently knocked down in HeLa cells. (A) IPTG-induced expression of histidine (His)-tagged HPS6 (aa 401-805) fusion protein in *E. coli*. C, control. T, induced. P, pellet. S, supernatant. (B) Recombinant His-HPS6 (aa 401-805) expressed from *E. coli*. (C) Immunoblotting analysis of His-HPS6 (aa 401-805, immunogen), mouse brain lysates, protein extracts of HEK293 cells expressing myc-tagged HPS6 or Flag-tagged HPS6 using affinity-purified rabbit polyclonal antibodies raised against the recombinant protein in B. A His-tagged HPS5 fragment serves as control. (D) Immunoblotting analysis of wild-type and *ruby-eye* (*ru*) C57BL/6J mouse brain lysates using the rabbit polyclonal antibodies against HPS6. β-actin serves as loading control. (E) Immunoblotting analysis of HeLa cell extracts showing HPS6 silencing with siRNA against HPS6. Relative amount of HPS6 was determined with NIH ImageJ (n = 3 experiments). (F) Immunostaining analysis of HeLa cells showing HPS6 silencing with siRNA against HPS6. A non-targeting siRNA was used as negative control. Scale bar, 10 μm.
Fig. S2. Retrograde transport of lysosomes is impaired in HPS6-depleted cells. (A) Snap shots of control or HPS6-depleted, mCherry-LAMP1-expressing HeLa cells imaged live. Three-dimensional (3D) data sets of live cells were acquired and isosurfaces of cell, nucleus and mCherry-LAMP1 vesicles reconstructed. The mCherry-LAMP1 vesicles were tracked in three dimensions. Only displacement length that were longer than 0.35 μm were evaluated to reliably determine the overall directionality of vesicle movements. (B) The distance of representative vesicles towards the cell center is plotted over time. Directionality of vesicle movements is color coded (red: towards the cell surface, green: towards the center). (C) The mCherry-LAMP1 vesicles were tracked and it was determined whether they moved to the surface (anterograde) or to the center of the cell (retrograde). Data represent means ± s.e.m., 120-303 vesicles/cell, n = 5 cells. ***P <0.001.
Fig. S3. Neither the subcellular distribution patterns of Rab7, CD63, CD-MPR and CI-MPR, nor the size of lysosomes is altered in HPS6-depleted cells. HeLa cells were transfected with a control non-targeting siRNA or siRNA against HPS6 and immunostained with phalloidin and antibodies against Rab7 (A), CD63 (C), CD-MPR (E) or CI-MPR (G), respectively. Scale bar, 10 μm. (B, D, F and H) Radial profile plots of signal distribution of markers relative to the nucleus in A, C, E and G, respectively (mean ± s.e.m., n = 3 experiments, 10 cells/experiment). (I) Scatter plot depicts the size distribution (nm) of the maximum diameter of lysosomes in electron micrographs of control and HPS6-depleted cells (ctrl KD, n=111; HPS6 KD, n=155). The maximum diameter of lysosomes was calculated using the ‘Measure’ function of NIH imageJ. The red line represents the median of all samples.
Fig. S4. HPS6 and RILP do not colocalize on lysosomes. (A) RILP knockdown efficiency was evaluated by immunoblotting of lysates of HeLa cells co-transfected with myc-RILP expressing construct and siRNA duplexes targeting RILP. (B–C) HeLa cells growing in 24-well plate were transfected with siRNA duplexes targeting HPS6, RILP or both for 48 h. Cells were fixed and immunostained with antibodies to LAMP1. Shown are representative confocal images in B and radial profile plots of distribution of LAMP1 signals relative to the nucleus (mean ± s.e.m., n = 3 experiments, 10 cells/experiment) in C. siRNA sequences used in this study were as previously described (Progida et al., 2007): siRNA-RILP1, 5′-GCAGCGGAAGAAGAUCAAGTT-3′; and siRNA-RILP2, 5′-GAUCAAG-GCCAAGAUGUATT-3′. (D) HeLa cells were transiently transfected with constructs expressing myc-RILP, or myc-RILP and RFP-Rab7 or mCherry-LAMP1. Cells single-transfected with myc-RILP were immunostained with antibodies against HPS6. Myc-RFP was immunostained with antibodies against Myc. Shown are representative confocal images. Right most panels are blow-ups of the boxed regions in left panels. Scale bars, 10 μm.
**Movie S1.** Control knockdown cells transfected with mCherry-LAMP1 for 24 h were imaged by time-lapse fluorescence microscopy. Shown is a representative movie of mCherry-LAMP1 dynamics in live cell. Three-dimensional data sets of mCherry-LAMP1 vesicles were detected and tracked by Imaris.

**Movie S2.** HPS6 knockdown cells transfected with mCherry-LAMP1 for 24 h were imaged by time-lapse fluorescence microscopy. Shown is a representative movie of mCherry-LAMP1 dynamics in live cell. Three-dimensional data sets of mCherry-LAMP1 vesicles were detected and tracked by Imaris.