Lysosomal fusion and SNARE function are impaired by cholesterol accumulation in lysosomal storage disorders

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The function of lysosomes relies on the ability of the lysosomal membrane to fuse with several target membranes in the cell. It is known that in lysosomal storage disorders (LSDs), lysosomal accumulation of several types of substrates is associated with lysosomal dysfunction and impairment of endocytic membrane traffic. By analysing cells from two severe neurodegenerative LSDs, we observed that cholesterol abnormally accumulates in the endolysosomal membrane of LSD cells, thereby reducing the ability of lysosomes to efficiently fuse with endocytic and autophagic vesicles. Furthermore, we discovered that soluble N-ethylmaleimide-sensitive factor attachment protein (SNAP) receptors (SNAREs), which are key components of the cellular membrane fusion machinery are aberrantly sequestered in cholesterol-enriched regions of LSD endolysosomal membranes. This abnormal spatial organization locks SNAREs in complexes and impairs their sorting and recycling. Importantly, reducing membrane cholesterol levels in LSD cells restores normal SNARE function and efficient lysosomal fusion. Our results support a model by which cholesterol abnormalities determine lysosomal dysfunction and endocytic traffic jam in LSDs by impairing the membrane fusion machinery, thus suggesting new therapeutic targets for the treatment of these disorders.

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Introduction

Lysosomes are cellular organelles that have a pivotal role in the cell homeostasis being involved in degradation and recycling processes. The function of lysosomes is mediated by their membrane and its ability to efficiently fuse with several target membranes (Luzio *et al*, 2007).

Lysosomal storage disorders (LSDs) are a group of inherited diseases caused by the deficiency of lysosomal and nonlysosomal proteins with consequent accumulation of several types of substrates in the lysosomes (Suzuki, 2002). Increasing evidence supports the idea that LSDs are associated with a global impairment of the endolysosomal trafficking pathways and in particular with a defect of autophagy, the major lysosome-mediated degradation system (Tanaka *et al*, 2000; Cao *et al*, 2006; Fukuda *et al*, 2006; Liao *et al*, 2007; Settembre *et al*, 2008a, b).

Although the involvement of a global lysosomal dysfunction in the progression of LSDs is well established, the molecular mechanisms underlying such dysfunction are largely unknown. Cholesterol together with other lipids accumulate as primary or secondary storage in several LSDs and have been proposed to jam the endolysosomal system (Simons and Gruenberg, 2000; Sobo *et al*, 2007; Walkley and Vanier, 2009). Accordingly, the perturbation of cellular endocytic membrane traffic has been directly linked to cholesterol homeostatic defects in different LSDs (Puri *et al*, 1999; Choudhury *et al*, 2004; Miedel *et al*, 2008). However, the mechanisms underlying cholesterol involvement in such membrane traffic impairment remain unclear. Recently, sphingosine storage was observed to cause deregulation of lysosomal calcium and consequent endocytic traffic defects in Niemann–Pick type C1 (Lloyd-Evans *et al*, 2008).

In this study, we studied lysosomal membrane properties and function in mouse embryonic fibroblasts (MEFs) derived from mouse models of multiple sulphatase deficiency (MSD) and mucopolysaccharidosis type IIIA (MPS-IIIa), two severe neurodegenerative LSDs in which we previously demonstrated a block of the autophagic pathway resulting in cell and tissue damage (Settembre *et al*, 2008b). We demonstrated that the accumulation of cholesterol in these two LSD models causes an altered organization of the endolysosomal membranes with a significant expansion of regions enriched in this lipid. This directly affects the fusion of lysosomes with endosomes and with autophagic vacuoles. Consistently, loading WT lysosomal membrane with cholesterol mimicked the lysosomal fusion defects observed in LSD cells, whereas lowering cholesterol in the lysosomal membrane from LSD cells efficiently rescued normal lysosomal fusion. Moreover, we observed that the accumulation of cholesterol-enriched membranes in LSD lysosomes sequesters soluble N-ethylmaleimide attachment protein (SNAP) receptor (SNARE) proteins and affects their proper assembly

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and recycling. Our results shed light on the role of cholesterol in LSD pathogenesis providing a mechanistic link between cholesterol accumulation and endolysosomal membrane traffic jam in LSDs.

Results

Lysosomal fusion is impaired in LSDs

We investigated the fidelity of transport to lysosomes of either endosomes and autophagic vesicles in MSD and MPS-IIIa (hereafter referred to as LSDs) mouse embryonic fibroblasts

(MEFs). Figure 1A shows that on epidermal growth factor (EGF) stimulation lysosomal-mediated degradation of EGF receptor (EGFR) was more efficient in wild-type (WT) cells compared with LSD cells. A transport assay was also performed by loading cells with a fluorescently labelled dextran, an inert endocytosed marker. This analysis revealed that after 6 h of chase, the percentage of internalized dextran co-localizing with LAMP1-positive vesicles was significantly higher in WT compared with LSD cells (Figure 1B), indicating that in LSD cells the traffic of membranes to the lysosomal compartment was impaired.

Figure 1 Lysosomal fusion is impaired in LSD cells. (A) EGFR degradation was followed in MSD, MPS-IIIa and WT MEFs by treating the cells with EGF for the indicated time to stimulate EGFR internalization. The cells were then immediately lysed and subjected to anti-EGFR blotting. The amount of remaining EGFR was quantified by densitometry analysis (Image J) of the blot and expressed in the chart as % of the EGFR amount present at time T₀ (100%). The values in the chart represent the mean $\pm$ s.e.m. values of three independent experiments. (B) MSD, MPS-IIIa and WT MEFs cells loaded with dextran (alexafluor-594 conjugated) were labelled with anti-LAMP1 antibody and the percentage of dextran co-localizing with LAMP1 was evaluated. The chart displays merge values (means $\pm$ s.e.m.) that represent the percentage of dextran co-localizing with LAMP1 measured in 15 different cells of triplicate experiments. (C) The rate of lysosome fusion with autophagosomes was monitored in MSD, MPS-IIIa and WT MEFs transfected with a tandem fluorescently tagged LC3 (Kimura *et al*, 2007). The rate of autophagosome maturation reflected the percentage of the LC3 'unfused' (green/red fluorescence ratio) at each time (1 and 3 h) after bafilomycin removal (T₀). The percentage of LC3 'unfused' was displayed versus the value at T₀ (assumed to be 100%). Values are represented as means $\pm$ s.e.m. of triplicate experiments. **P* < 0.05, Student's *t*-test: (A): WT versus MSD and WT versus MPS-IIIa; (B, C): WT versus MSD and WT versus MPS-IIIa at each time point. Scale bar: 10 μ m (B, C).

Subsequently, we examined the progression of lysosome-autophagosome fusion using a tandem fluorescently-tagged autophagosomal marker in which LC3 was engineered with both monomeric red-fluorescent protein (mRFP) and GFP and evaluating the GFP fluorescence loss as a direct measurement of autophagosome fusion (Kimura *et al*, 2007). The validity of this analysis was not affected by the decreased degradation capability of lysosomes in the LSD models analysed as the green—but not the red—fluorescence is rapidly quenched by protonation occurring at the intra-lysosomal acidic pH (pKa value for GFP: 6.0; Kimura *et al*, 2007) and in these LSD models the pH of lysosomes remains below the pKa of GFP (Supplementary Figure 1). Both WT and LSD cells were transfected with mRFP-GFP-LC3 and autophagosome maturation was monitored over a 3-h period. The rate of autophagosome maturation was markedly slower in LSD cells compared with WT cells (Figure 1C). Together, these findings indicate a decreased delivery of cargo to the lysosomes, suggesting an impaired ability of lysosomes to undergo efficient fusion with different target membranes in LSD cells.

Cholesterol accumulates in the endolysosomal membrane of LSDs reducing the efficiency of lysosomal fusion

To investigate the causes of the inefficient lysosomal fusion in LSD cells, we analysed membrane properties and function of lysosomes which were isolated, together with the late endosome fraction, from LSD and WT MEFs using a magnetic chromatography procedure (Dietrich *et al*, 1998; Supplementary Table 1). We observed that the overall amount of cholesterol was significantly increased in the membranes prepared from LSD lysosomes compared with WT (Figure 2A).

The increased levels of cholesterol in the endolysosomal membrane of LSD cells were consistent with Filipin staining showing that cholesterol accumulated inside the endolysosomal vesicles and decorated LAMP1-positive membrane regions (Figure 2B). Importantly, no significant changes in the bulk of phospholipids, the most abundant lipid components of cell membranes, were observed with the exception of an increase in lysobisphosphatidic acid (LBPA), a lipid specifically associated to endolysosomal internal membranes (Figure 2C and D).

To test whether cholesterol accumulation accounted for the lysosomal fusion defects observed in LSD cells, we modulated cholesterol levels in the endolysosomal membranes from both WT and LSD cells and then monitored lysosomal fusion efficiency. The treatment of WT cells with methyl- β -cyclodextrin (M β CD)-complexed cholesterol resulted in cholesterol overload of endolysosomal vesicles and membranes (Figure 3A) and in a decreased rate of both autophagosome maturation (Figure 3B) and lysosomal endocytic transport (Figure 3C; Supplementary Figure 2A). Conversely, depleting cholesterol from LSD cells by using M β CD decreased cholesterol content in the endolysosomal membranes (Figure 3D) and led to a normalization of the rate of both autophagosome maturation (Figure 3E) and lysosomal endocytic transport (Figure 3F; Supplementary Figure 2B). Importantly, M β CD treatment was only effective in extracting the excess of cholesterol because total cholesterol levels in M β CD-treated LSD cells were similar to those measured in WT cells (Filipin staining in Figure 2D; data not shown). These findings indicate that abnormal cholesterol levels in the endolysosomal membrane directly affect the ability of lysosomes to efficiently fuse with target membranes in the cells.

Figure 2 Alterations in lipid composition of endolysosomal membranes from LSD cells. (A) Cholesterol levels were measured in the indicated endolysosomal membrane samples containing equal amount of proteins and expressed as ng of cholesterol per μ g of protein (see Materials and Methods section for details). (B) Filipin staining showing cholesterol accumulation in the endolysosomal compartment of MSD and MPS-III A MEFs (arrowheads and enlarged images). (C, D) Total lipids extracted from the indicated endolysosomal membrane samples (30 μ g of proteins) were either (C) subjected to a phosphate assay to quantify the bulk of phospholipids or (D) separated by TLC. Phospholipids and cholesterol on TLC plates were revealed by molybdenum blue staining. CHOL, cholesterol; LBPA lysobisphosphatidic acid; PC, phosphatidylcholine; PE phosphatidylethanolamine; PI phosphatidylinositol; SM sphingomyelin. (A, C) Values represent the mean $\pm$ s.e.m. values of three independent experiments. * P < 0.05, Student's t -test: (A, C): WT versus MSD and WT versus MPS-III A. Scale bar, 10 μ m (B).

Figure 3 Cholesterol accumulation inhibits lysosomal fusion. Endolysosomal membrane cholesterol measurements and Filipin staining were carried out in either (A) WT MEFs loaded with cholesterol or in MSD and (D) MPS-III A MEFs treated with MβCD. Arrowheads and enlarged images show cholesterol accumulation in endolysosomes of cholesterol-loaded WT MEFs. After treatments the rate of autophagosome maturation (B, E) and the transport of fluorescent dextran to lysosomes (C, F) were also analysed as in Figure 1. WT controls for autophagosome maturation and dextran transport experiments were performed as shown in Figure 1. (A–F) Values represent the mean ± s.e.m. values of three independent experiments. **P* < 0.05, Student's *t*-test: (A, C): WT versus WT + cholesterol; (B): WT versus WT + cholesterol for each time point; (D, F): MSD versus MSD + MβCD and MPS-III A versus MPS-III A + MβCD; (E): MSD versus MSD + MβCD and MPS-III A versus MPS-III A + MβCD for each time point. Scale bar: 10 μm (A, C, D, F).

The organization of endolysosomal membranes is altered in LSD cells

To understand the mechanisms underlying cholesterol-dependent fusion impairment, we investigated whether the accumulation of cholesterol in the endolysosomal membranes of LSD cells led to specific changes in membrane organization that could be relevant for the fusion process. Cholesterol increases lateral heterogeneity of membranes and determines the segregation of a subset of lipids and proteins into ordered microdomains, enriched in cholesterol and glycosphingolipids. It has been proposed that these membrane domains constitute discrete entities termed 'lipid rafts', which mediate important functions in membrane signalling and trafficking (Simons and Ikonen, 1997; Simons and Vaz, 2004; Rajendran and Simons, 2005; Lingwood and Simons, 2010). The components of these cholesterol-enriched regions

are typically resistant to detergents allowing for biochemical coalescence into an insoluble fraction termed detergent-resistant membranes (DRMs), which can be isolated after centrifugation in a sucrose gradient (Lingwood and Simons, 2007) and identified by Flotillin-1 immunostaining. The endolysosomal membranes from LSD and WT cells were processed to isolate the DRMs. Quantitative analysis showed that the percentage of Flotillin-1 associated with DRMs was increased in LSD endolysosomal membranes (Figure 4A), indicating an increased amount of cholesterol-enriched regions in these membrane samples. This was further supported by immunoelectron microscopy (EM) analysis showing that glycosphingolipid GM1, a component of cholesterol-enriched membrane domains, also accumulated in the membranes of LSD endolysosomes (Figure 4B). We also measured membrane order of the isolated membranes using the fluorescent probe

Figure 4 The LSD endolysosomal membrane contains increased amount of cholesterol-enriched regions. **(A)** Endolysosomal membranes from MSD, MPS-III A and WT MEFs were treated with 1% Triton X-114 and loaded on a sucrose gradient. Immunoblots with Flotillin-1 identified DRMs in fractions 2, 3 and 4 (arrows). The fractions at the bottom of the gradient (12 and 13) correspond to high-density detergent soluble fractions, whereas the remaining ones were defined as intermediate fractions (intermediate-I: 5, 6, 7 8; intermediate-II: 9, 10 and 11). The percentage of Flotillin-1 in DRMs was calculated from the densitometric quantification of immunoblots. **(B)** Immuno-EM of GM1 lipid was carried out in WT, MSD and MPS-III A MEFs by staining cells with anti-cholera toxin B antibodies (see Materials and methods section). The number of GM1-positive dots was measured in 25 cells from three independent experiments and displayed as fold to WT. **(C)** Endolysosomal membranes from MSD, MPS-III A and WT MEFs were stained with C-laurdan and subsequently analysed by fluorescence spectrophotometry to calculate the GP value (see Materials and methods section for details). Distribution of cholesterol was also measured throughout the gradient and expressed as percentage of total cholesterol in raft (DRMs) and soluble fractions. **(D)** Equal aliquots from either DRMs or soluble fractions were pooled, the protein content determined and displayed as percentage of total protein in DRM and soluble gradient regions. **(E)** Immunoblotting profiles of the transferrin receptor and LAMP1 in the sucrose gradient. Values represent the mean $\pm$ s.e.m. values of three experiments **(A–D)**. * $P < 0.05$, Student's *t*-test: **(A–C)**: WT versus MSD and WT versus MPS-III A; **(D)**: WT versus MSD and WT versus MPS-III A for each fraction. Scale bar: 0.3 μ m **(B)**.

C-laurdan (Kaiser *et al*, 2009). Notably, despite the overall increase in DRMs and GM1 levels, LSD endolysosomal membranes maintain a membrane order that is similar to that observed in WT cells (Figure 4C). This may be due to a general and proportional build-up of both raft and non-raft membrane regions (i.e. increase in total membrane). This is consistent with previous reports of an expansion of the endolysosomal compartment in LSD cells (Suzuki, 2002).

We also investigated the effect of cholesterol changes on membrane proteins. We observed that in the endolysosomal membranes from LSD cells the amount of DRM-associated proteins was significantly increased, and the amount of protein present in the soluble regions of the gradient concomitantly decreased, with respect to the protein distribution observed in control samples (Figure 4D). This aberrant protein compartmentalization could be restored by M β CD treatment, which leads to a reduction of DRM fraction (Figure 4D).

When we tested whether the increase of DRM proteins reflected a plain recruitment of detergent soluble proteins, we observed that the transferrin receptor, a membrane protein that is normally excluded from DRMs continued to be found exclusively in soluble gradient regions in both WT and LSD cells (Figure 4E). LAMP1 also displayed a similar distribution profile in WT and LSD cells (Figure 4E). Our results suggest a cholesterol-mediated reorganization of a subset of endolysosomal membrane proteins.

Endolysosomal SNARE membrane compartmentalization is highly dependent on cholesterol and is altered in LSD cells

Membrane fusion processes in the endocytic pathways are driven by SNAREs. These are transmembrane proteins, which are able to assemble in high-affinity *trans*-complexes between two opposing membranes to drive the fusion process (Weber *et al*, 1998; Jahn and Scheller, 2006). Previous studies have

shown that plasma membrane SNAREs are functionally organized in clusters integrity of which is dependent on cholesterol (Thiele *et al*, 2000; Chamberlain *et al*, 2001; Lang *et al*, 2001; Puri and Roche, 2006; Lang, 2007). We asked whether the membrane cholesterol abnormalities observed in LSD endolysosomes affect the compartmentalization and function of SNAREs involved in the endocytic membrane traffic pathways. We analysed the lysosomal membrane distribution of VAMP7, Vti1b and syntaxin 7, three post-Golgi SNAREs belonging to different combinatorial set of SNAREs that participate in *trans*-complexes driving the fusion of endolysosomal membranes with either endosome or autophagosomes (Pryor *et al*, 2004; Furuta *et al*, 2010). The results show that these SNAREs become detergent-resistant in LSD cells as we observed that the amount of VAMP7, Vti1b and syntaxin 7 localized to the DRMs was markedly increased at the expense of the SNARE content present in the soluble region of the gradient (Figure 5A and B; Supplementary Figure 3). To test whether the extent of SNARE association to the DRMs was cholesterol dependent, we checked SNARE distribution after either depleting or loading cells with cholesterol. Treatment of LSD cells with M β CD resulted in the dissociation of SNAREs from the DRMs and in an increased localization of SNAREs to soluble and intermediate regions of the gradient, thus restoring a SNARE distribution similar to that observed in WT cells (Figure 5A and B). Conversely, loading WT cells with cholesterol resulted in an increased association of SNAREs with DRM regions, thus mimicking the condition observed in LSD cells (Figure 5A and B).

These data suggest that post-Golgi endolysosomal SNAREs are compartmentalized within the endolysosomal membrane and that this compartmentalization is strictly dependent on cholesterol. This finding was associated with a remarkable enrichment of VAMP7, Vti1b and syntaxin 7 in the endolysosomal membranes of both cholesterol-loaded and LSD cells, which was significantly higher compared with that observed for LAMP1 (Figure 5C and D), distribution of which was not affected by DRM and similar in WT and LSD cells (Figure 4E). However, we observed a limited increase in the amount of the analysed SNAREs in total cell lysates (Figure 5E), whereas LAMP1 showed a more significant increase (Figure 5E), consistent with the expansion of the endolysosomal compartment in LSD cells. This suggests that SNARE accumulation in endolysosomal membranes is the result of an increased cholesterol-mediated sequestration in specific membrane regions, rather than that of slower degradation kinetics due to the reduced degradation capacity of

lysosomes in LSDs. It is likely that the internal localization that we detected is the result of membrane invagination of sequestered/accumulated material on the external membrane (enlarged image in Figure 5D). Importantly, we observed no evidence of altered membrane compartmentalization of non-lysosomal SNAREs. Indeed, SNAP23, a plasma membrane SNARE involved in exocytosis, and Sec22/syntaxin 5, which are involved in ER–Golgi trafficking, showed similar membrane distribution in WT and LSD cells (Figure 5F). Moreover, cholesterol-dependent lysosomal membrane distribution abnormalities affected SNARE proteins specifically and did not affect other crucial components of the membrane traffic apparatus. Indeed, Rab7, a well-established regulator of the endocytic membrane traffic (Zhang *et al*, 2009), is not associated with DRMs and its distribution profile remains unaltered in WT and LSD cells (Figure 5G).

Endolysosomal SNAREs are locked in assembled complexes in LSD cells

The function of SNARE requires an ordered dynamical interaction between different SNAREs with consecutive rounds of assembly, membrane fusion and disassembly of post-fusion SNARE *cis*-complexes (Jahn and Scheller, 2006). Moreover, to maintain membrane identity and ensure new fusion events, post-fusion SNAREs must be trafficked and recycled back to steady-state membrane locations by interacting with specific adaptors of the clathrin vesicular transport (Hirst *et al*, 2004; Miller *et al*, 2007; Tran *et al*, 2007; Pryor *et al*, 2008).

We asked whether the abnormal SNARE sequestration in defined cholesterol-enriched regions of endolysosomal membranes could alter these dynamic interactions, thus affecting proper SNARE function. We first analysed the ability of SNAREs to undergo a correct assembly–disassembly reaction. The amount of assembled SNARE complexes was determined by measuring SNARE complex levels in boiled and non-boiled SDS-treated samples. Immunoblot analysis against Vti1b revealed that the endolysosomal membranes of LSD cells contained higher amount of SDS-resistant complexes compared with WT cells and that the build-up of SNARE complexes occurred largely in the detergent-insoluble fraction (Figure 6A). These data were confirmed by immunoprecipitation analysis demonstrating that in LSD cells anti-Vti1b antibodies immunoprecipitate higher amount of both syntaxin 7 and VAMP7 compared with WT cells (Figure 6B). Importantly, the ER–Golgi syntaxin 5, membrane distribution of which was not affected in LSD cells (Figure 5F), did not accumulate in complexes (Supplementary Figure 4),

Figure 5 SNAREs are sequestered by cholesterol within endolysosomal membranes. (A) VAMP7, Vti1b and syntaxin 7 distributions in endolysosomal membranes from MSD, MPSIIIA and WT MEFs was evaluated by immunoblotting analysis of gradient fractions. To simplify the analysis the DRM, the intermediate-I, the intermediate-II and soluble fractions of the gradient were pooled separately and then subjected to immunoblotting. SNARE distribution was also analysed after loading WT cells with cholesterol and after M β CD treatment. In MSD and MPS-IIIA MEFs, all analysed SNAREs abnormally accumulate in DRMs of lysosomal membranes. Cholesterol modulation results in a change of SNARE distribution. (B) The percentage of each analysed SNARE observed in DRM fractions was quantified from blots (ImageJ densitometry analysis) and displayed as relative amount versus WT. (C, E) Immunoblots and relative quantification showing SNARE protein levels along with LAMP1 protein levels in (C) endolysosomal membranes and (E) total cell lysates from WT (untreated and cholesterol treated) and MSD MEFs (untreated and M β CD treated). In the graphs, the protein levels were displayed as relative amount versus WT. (D) WT and MSD MEFs transfected with GFP–VAMP7 were stained with anti-GFP for immuno-EM. The enlarged image shows internalization of GFP–VAMP7 particles (arrow). (F) Syntaxin 5, Sec22 and SNAP23 distribution in total membrane derived from control WT and MSD MEFs. Syntaxin 5 immunoblot shows two bands (*, 35 kDa and **, 42 kDa) corresponding to the two isoforms of the protein. (G) Distribution profile of Rab7 in WT and MSD lysosomal membranes. Values represent the mean $\pm$ s.e.m. values of three independent experiments (B, C, E). * P < 0.05, Student's *t*-test: (B): WT versus MSD, WT versus MPS-IIIA, WT versus WT + cholesterol, MSD versus MSD + M β CD, and MPS-IIIA versus M β CD for each analysed SNARE; (C, E): WT versus MSD, WT versus WT + cholesterol and MSD versus MSD + M β CD for each analysed protein. Scale bar: 0.3 μ m (D).

indicating that the accumulation of SNARE complexes is specific for endolysosomal SNAREs and is associated with their abnormal enrichment in the DRMs. The overcrowding of endolysosomal SNARE complexes in LSD cells was rescued by cholesterol depletion, whereas cholesterol loading in WT cells resulted in the formation of abnormal complexes (Figure 6A and B). The blotting profile of SDS-resistant

complexes indicated the accumulation of both lower (50–60 kDa; * in Figure 6A) and higher (>80 kDa; ** in Figure 6A) molecular weight complexes that were also decorated by the Vti1b cognate SNARE syntaxin 7 (Figure 6C). These complexes may represent, respectively, SNARE dimers and oligomer/fully assembled *cis*-complexes, or alternatively may reflect nonspecific pairing of SNAREs due to their local

Figure 6 SNAREs are locked in an assembled form in LSD endolysosomal membranes. **(A)** SDS-resistant complexes containing Vti1b were detected by immunoblotting analysis of nonboiled samples corresponding to total, detergent insoluble (DRM) and detergent soluble (Sol.) endo-lysosomal membrane fractions derived from MSD and WT MEFs. The SDS-resistant complexes were also visualized after loading WT MEFs with cholesterol and after treating MSD MEFs with M β CD. Immunoblots revealed the presence of low molecular weight complexes (*, 50–60 kDa) and high molecular weight complexes (**, >80 kDa). The percentage of Vti1b in SDS-resistant complexes in total endolysosomal membranes (bottom-left chart) and the amount of Vti1b-containing SDS-resistant complexes in DRM and soluble fractions (bottom-right chart) were calculated by the densitometric quantification of the correspondent immunoblots (ImageJ). Values represent the mean $\pm$ s.e.m. values of three independent measurements. * P <0.05, Student's t -test: WT versus MSD, WT versus WT+cholesterol and MSD versus MSD+M β CD (bottom-left chart); WT versus MSD, WT versus WT+cholesterol and MSD versus MSD+M β CD for each fraction (bottom-right chart). **(B)** Syntaxin 7 and VAMP7 were co-immunoprecipitated with Vti1b using anti-Vti1b antibodies in WT (untreated or cholesterol treated) and in MSD (not treated or M β CD treated) MEFs. The amount of Vti1b precipitated in each cell line is also shown. **(C)** SDS-resistant complexes are decorated by anti-syntaxin 7 antibodies in total endolysosomal membrane fraction from WT and MSD MEFs. **(D)** Membrane-associated α -SNAP and its release in the cytosol were evaluated by western blot analysis on total cell lysates (total), intracellular membranes recovered after centrifugation from a post-nuclear supernatant fraction (membrane associated) and cell lysates devoid of membranes (cytosolic released) derived from MSD (untreated or M β CD treated) and WT (untreated or cholesterol treated) MEFs.

enrichment in cholesterol membrane microdomains. Notably, the syntaxin 7 blot in Figure 6B showed a shift of the main band present in the lower molecular weight complexes (* in Figure 6C), which was indicative of the accumulation of SNARE homodimers containing either Vti1b or syntaxin 7. The α -SNAP adaptor is an essential cofactor that recruits the N-ethylmaleimide-sensitive factor (NSF) on assembled *cis*-complexes on post-fusion membranes finally allowing SNARE disassembly/ α -SNAP release (Sollner *et al*, 1993; Littleton *et al*, 2001). In addition, the NSF-SNAP system has been demonstrated to operate also on some off-pathway SNARE complexes and on SNARE-assembling intermediates complexes (Hanson *et al*, 1995; McMahon and Sudhof, 1995; Barszczewski *et al*, 2008). We observed that the accumulation of SNARE complexes in both cholesterol-loaded and LSD cells was associated with an increased amount of α -SNAP associated with intracellular membranes and with a concomitant decrease in cytosolic released α -SNAP (Figure 6D). Moreover, the distribution of α -SNAP between membrane-associated and released states changed in response to the depletion of cholesterol from the LSD endolysosomal membrane (Figure 6D). This suggested that the SNARE complexes accumulating in cholesterol-loaded and LSD cells could represent 'dead-end'/intermediate SNARE complexes or post-fusion complexes undergoing inefficient or partial disassembly.

These findings demonstrate an abnormal cholesterol-dependent accumulation of SNARE complexes into the endolysosomal membranes of LSD cells and indicate an imbalance in the SNARE assembly-disassembly functional cycle.

The traffic and recycling of post-Golgi endolysosomal SNAREs is inhibited in LSD cells

The sorting and recycling of post-fusion SNAREs is mediated by specific interaction with dedicated clathrin adaptors. We investigated whether cholesterol-dependent SNARE sequestering within endolysosomal membranes in LSD cells could also affect this process. Co-immunofluorescence analysis showed that VAMP7 and Vti1b co-localized to a larger extent in LSD cells compared with WT cells (yellow merge and quantification of co-localization in Figure 7A) and this co-localization took place mostly in LAMP1-positive structures (white merge in Figure 7A), suggesting that SNARE co-clustering is associated with trapping in the lysosomes in LSD cells. The molecular apparatus responsible for Vti1b recycling is well known. Vti1b is transported from a late endosomal compartment back to an earlier compartment and/or the *trans*-Golgi network (TGN) through the clathrin adaptor epsinR (Hirst *et al*, 2004; Miller *et al*, 2007). We observed that Vti1b co-localized with endolysosomes and TGN in WT cells, whereas in LSD cells Vti1b was retained in the endolysosomal compartment and depleted from the TGN (Supplementary Figure 5).

We then examined whether this could be associated with a decreased efficiency of Vti1b recruitment to epsinR-containing vesicles. In WT cells, 17–20% of Vti1b co-localized with epsinR in a steady-state condition (Figure 7B). In contrast, the extent of Vti1b-epsinR co-localization was markedly decreased in LSD cells (Figure 7B). Notably, these differences did not reflect a significant alteration in epsinR subcellular distribution between WT and LSD cells (data not shown). When cholesterol was increased in WT cells, Vti1b overlap

with epsinR was decreased. Conversely, when LSD cells were depleted of cholesterol the Vti1b overlap with epsinR was increased (Figure 7B). These data indicate that Vti1b recruitment into epsinR-positive vesicles is affected by the extent of cholesterol-mediated sequestration of Vti1b in endolysosomes in LSD cells. To further investigate these findings, we followed the dynamics of Vti1b trafficking route to the TGN in live cells by fluorescence recovery after photobleaching (FRAP) experiments. The fluorescence of transfected GFP-tagged Vti1b (GFP-Vti1b) was photobleached in a TGN juxta-nuclear region and its recovery was tracked for 120–180 s. The recovery of GFP-Vti1b fluorescence was faster in WT cells compared with that observed in LSD cells (Figure 7C), suggesting that in LSD cells the trafficking of GFP-Vti1b from the endolysosomal compartment towards the TGN is impaired due to the smaller fraction of the GFP-Vti1b molecules engaged in epsinR-mediated transport. To verify whether this was due to cholesterol-dependent clustering of Vti1b, we measured GFP-Vti1b FRAP in LSD cells after treatment with M β CD. Depletion of cholesterol increased the mobility of Vti1b (Figure 7C). Conversely, when cholesterol was added to WT cells, FRAP kinetics of GFP-Vti1b became slower and more similar to that observed in LSD cells (Figure 7C). Together, these results demonstrate that the sorting and vesicular transport of post-Golgi SNAREs are impaired in LSD cells and suggest that this might be due to a cholesterol-dependent SNARE clustering that limits SNARE availability to interact with clathrin adaptors.

Discussion

We demonstrated that cholesterol accumulation in endolysosomal membrane changes its organization and severely reduces the ability of lysosomes to efficiently fuse with other membranes. We showed that these cholesterol-dependent abnormalities cause a defect in the fusion of lysosomes with endosomes and autophagosomes in two models of LSDs. We propose that this may represent a common early pathogenic mechanism underlying the endocytic traffic jam observed in these disorders. How the lysosomal primary defect leads to cholesterol accumulation remains to be clarified, although mechanistic connections between these two pathogenic events have been identified in some LSDs (Miedel *et al*, 2008; Walkley and Vanier, 2009).

In our study, we also provide evidence that excessive cholesterol in endolysosomal membrane impairs SNARE function. A stimulatory role of cholesterol in the regulation of various aspects of SNARE function has been reported previously. Indeed, cholesterol-dependent clustering of SNAREs is implicated in defining the exocytotic sites on the plasma membrane in specialized secretory cells and in facilitating the exocytosis of synaptic vesicles (Chamberlain *et al*, 2001; Lang *et al*, 2001; Taverna *et al*, 2004; Gil *et al*, 2005; Puri and Roche, 2006). Cholesterol has been shown to bind to synaptotagmin and promote synaptic vesicle biogenesis (Thiele *et al*, 2000). Moreover, the yeast cholesterol analogue ergosterol was observed to be required for the priming step in yeast vacuole fusion, a process directly dependent on SNARE-mediated fusion (Kato and Wickner, 2001). We argue that the possible deleterious effects of cholesterol accumulation in biological membranes could result from the functional interaction of cholesterol with SNARE apparatus.

Figure 7 Cholesterol levels affect SNARE trafficking. (A) MSD, MPS-III A and WT MEFs were subjected to a triple labelling with anti-VAMP7, anti-Vti1b and anti-LAMP1 antibodies. The merges between VAMP7 and Vti1b (double merges in yellow) and between VAMP7, Vti1b and LAMP1 (triple merges in white) are shown (see also enlarged images showing the extent of co-localization in different regions of the cells). The VAMP7-Vti1b co-localization was quantified in 15 different cells and displayed as % of Vti1b co-localizing with VAMP7 (means ± s.e.m.). (B) Co-localization of Vti1b with epsinR was quantified by double-labelling experiments in MSD and MPS-III A (untreated and MβCD treated) and in control WT (untreated and cholesterol treated) MEFs. The chart displays MSD values (means ± s.e.m.) that represent the percentage of Vti1b co-localizing with epsinR measured in 15 different cells. (C) Vti1b trafficking was monitored by FRAP analysis in WT (untreated and cholesterol treated) and MSD (untreated and MβCD treated) MEFs transfected with GFP-Vti1b (see Materials and methods section for details). FRAP data are displayed as percentage of recovery with respect to the fluorescence before bleach (100%) and are representative of 10 recordings from different cells. A summary of $t_{1/2}$ values is also shown. * $P < 0.05$, Student's t -test: (A): WT versus MSD and WT versus MPS-III A; (B): WT versus MSD, WT versus MPS-III A, WT versus WT+cholesterol, MSD versus MSD+MβCD and MPS-III A versus MPS-III A+MβCD; (C): WT versus MSD, WT versus WT+cholesterol, MSD versus MSD+MβCD. Scale bar: 10 μm (A, B).

Figure 8 A working model. Cholesterol-enriched regions accumulate in the endolysosomal membranes of LSDs sequestering SNAREs in defined domains. This locks SNAREs in assembled complexes and reduces their availability to interact with dedicated adaptor components of vesicle coat, a process needed for SNARE re-distribution between membranes. Consequently, the dysfunction of SNARE cycle decreases the rate of new fusion events.

In this study, we demonstrate that endolysosomal cholesterol levels affect the membrane distribution of post-Golgi SNAREs involved in the fusion of lysosomes with either endosomes or autophagosomes. The excess of cholesterol in the endolysosomal compartment, a condition observed in LSDs and mimicked by loading cells with cholesterol, increases the association of SNAREs with cholesterol-enriched membranes, resulting in the trapping of SNAREs in assembled complexes and in a slower rate of SNARE recycling (see model in Figure 8). Remarkably, a previous study showed that increased association of SNAREs with cholesterol-enriched regions on plasma membrane inhibits SNARE function in exocytosis (Salaun *et al*, 2005). Cholesterol-dependent SNARE dysfunction could be caused by the cholesterol inhibition of SNARE dynamics within endolysosomal membranes, a compartment normally deprived of cholesterol, which could prevent their ability to correctly interact with components of the fusion-recycling machinery. This hypothesis is supported by previous studies showing that cholesterol-mediated subcompartmentalization of the lysosomal membrane protein LAMP-2A negatively affects chaperon-mediated autophagy (Kaushik *et al*, 2006).

In conclusion, we show that cholesterol abnormalities in LSDs determine lysosomal fusion deficiency by affecting proper SNARE function. Recently, we reported the discovery of the CLEAR gene network and of its master gene, *TFEB*, which regulates lysosomal biogenesis and function (Sardiello *et al*, 2009). It would be interesting to investigate whether SNARE-mediated lysosomal fusion can be modulated by *TFEB*.

Our finding also raises the interesting hypothesis that cholesterol-dependent perturbation of SNARE function represents a relevant pathogenic mechanism in other disorders

associated with lysosomal failure, such as Alzheimer's and Huntington's diseases, in which a link between cholesterol accumulation and cellular pathogenesis has previously been documented (Valenza and Cattaneo, 2006). Finally, our data provide a proof of principle for the use of cholesterol reduction as a therapeutic option for the treatment of these disorders.

Materials and methods

Lysosome isolation

Lysosomes were isolated from MEFs by magnetic chromatography using a two-step elution protocol (Dietrich *et al*, 1998). Briefly, subconfluent 150 × 25-mm dishes were treated for 9 h with FeDex medium (Dietrich *et al*, 1998) at 37°C and subsequently maintained in normal Dulbecco's modified Eagle medium (DMEM) for 16 h. Cells were then collected by trypsin treatment, washed in buffer A (250 mM sucrose in 4 mM imidazole/HCl buffer (pH 7.4)) and then resuspended in 750 µl of buffer A⁺ (buffer A with the addition of 5 mM iodoacetamide and protease inhibitors). Cells were broken up by forcing them twice through an 18G needle and five times in a 26G needle and centrifuged at 600g for 5 min. The post-nuclear supernatant (PNS) was loaded on a MiniMACS column equilibrated with 10 ml of buffer A and with the magnet attached. The unbound material was collected by gravity flow (flow-through) and the column washed with 10 ml of TBS (150 mM NaCl, 5 mM Tris-HCl (pH 7.4)). Luminal proteins were eluted by applying a hypotonic buffer B (5 mM Tris-HCl with the same protease inhibitor concentration as in buffer A⁺), whereas lysosomal membrane proteins were eluted by removing the magnet and adding an hypotonic buffer B + 1% Triton X-114.

GP analysis and sucrose gradients

The GP analysis was performed following previously established protocols (Kaiser *et al*, 2009). Briefly, lysosomal membranes were stained for 15 min with 100 nM C-laurdan. Samples were excited at 385 nm, and emission spectra were recorded from 400 to 530 nm. Spectra of unstained samples were subtracted from the sample

labelled with C-laurdan. The GP values were calculated according to following formula: $GP = I_{400 \rightarrow 460} - I_{470 \rightarrow 530} / I_{400 \rightarrow 460} + I_{470 \rightarrow 530}$, where $I_{400 \rightarrow 460}$ and $I_{470 \rightarrow 530}$ are the total fluorescence intensity recorded from 400 to 460 nm and from 470 to 530 nm, respectively. For sucrose gradients, solubilized lysosomal membrane proteins were loaded at the bottom of a 9-ml discontinuous sucrose gradient (40, 35, 30, 25, 20, 15, 10 and 5%) in TNE (50 mM Tris-HCl (pH 7.4), 150 mM NaCl and 5 mM EDTA) with the addition of 1% Triton X-114. Samples were ultracentrifuged at 39 000 r.p.m. for 19 h at 4°C and then 12 aliquots of 750 µl (corresponding to DRMs, intermediate and soluble fractions) were collected from the top of the gradient and processed for cholesterol content, protein concentration and immunoblot analysis. Protein concentration was determined using the Dc Protein Assay kit (Bio-Rad). For immunoblot analysis, proteins from collected gradient fractions were precipitated in methanol/chloroform, resuspended in Laemmli buffer and subjected to SDS-PAGE to be probed with specific antibodies. Densitometry quantification of immunoblotted membranes was performed using the ImageJ program.

Phospholipid and cholesterol assays

Total lipids were obtained from lysosomal membrane samples by a two-step Bligh and Dyer lipid extraction protocol. Dried lipids were resuspended in methanol:chloroform (1:2) and then resolved on a TLC plate (Silica gel 60, MERCK). The developing solvent was methanol:chloroform:ammonia (65:35:5). Phospholipids and cholesterol were detected by Molybdenum blue staining (Sigma).

For phospholipid quantification, we used a phosphate assay protocol. Purified lipids were incubated for 1 h at 190°C with perchloric acid. After addition of ammonium molybdate (1%) and ascorbic acid (4%) the samples were incubated at 37°C for 2 h. The amounts of phospholipids in each sample were calculated by reading absorbance at 800 nm.

Cholesterol content was determined using the Amplex Red Cholesterol Assay kit (Invitrogen) by following the manufacturer's protocol.

Lysosomal pH measurements

We used Lysosensor yellow/blue-dextran (Molecular Probes) as a lysosomal pH indicator and the method described previously for lysosomal pH measurements (Holopainen *et al*, 2001). Briefly, the pH calibration curve was performed using MEFs loaded with Lysosensor-dextran and treated with calibration buffer solutions (pH ranging from 3.5 to 7.0) containing 10 µM Monensin and 10 µM Nigericin. The emission scan was measured using excitation at 360 nm, with both emission and excitation bandwidths set to 4 nm. Subsequently, the fluorescence emission intensity ratios at 451 and 518 nm, respectively, were calculated.

Measurements of WT, MSD and MPS-III A MEFs were performed as described above, with the exception that the cells were resuspended in a buffer containing: 5 mM NaCl, 115 mM KCl, 1.2 mM MgSO₄ (pH 7.76). The emission intensity ratio at 451 nm and 518 nm thus obtained were then converted to an absolute value of lysosomal pH by comparison with the standard curve generated above. The calibration curve was produced as described above and the measured data points (intensity ratio at 451/518) were fitted to a Boltzman equation. The coefficient of determination for the calibration curve was calculated using Microsoft Excel. The calibration curve was then used to calculate the corresponding pH values. The independent-samples *t*-test was used for comparison of means between the different cell lines (WT versus MSD and WT versus MPS-III A). A probability value of $P < 0.05$ was considered to be statistically significant.

Antibodies

The following antibodies were used: rabbit polyclonal anti-LAMP-1 (Sigma), rat monoclonal anti-LAMP-1 (Santa Cruz Biotechnology), mouse monoclonal anti-Vti1b (BD Biosciences), rabbit polyclonal anti-Vti1b (Synaptic System), mouse monoclonal anti-SNARE TI VAMP 1C7 (a kind gift from T Galli, INSERM U950, Paris, France), mouse monoclonal anti-Flotillin (BD Biosciences), mouse monoclonal anti-transferin receptor (Zymed), rabbit polyclonal anti-GFP (Abcam), rabbit polyclonal anti-epsinR (a kind gift from DJ Owen (Miller *et al*, 2007)), rabbit polyclonal anti-golgin 97 (a kind gift from AM De Matteis, TIGEM, Italy), rabbit polyclonal anti-SNAP23 (Synaptic System), rabbit polyclonal anti-Syntaxin 5 (Synaptic System), mouse monoclonal anti- α / β -SNAP (Synaptic System),

anti-Sec22 (a kind gift from AM De Matteis, TIGEM, Italy), rabbit polyclonal anti-Rab7 (Abcam), rabbit polyclonal anti-EGFR (Santa Cruz Biotechnology) and rabbit polyclonal anti-cholera toxin B (Vibrant Lipid Rafts Labeling Kit, Molecular Probes). Secondary horseradish peroxidase-conjugated antibodies (Pierce ECL), secondary antibodies for immunofluorescence, were conjugated to Alexa Fluor dye 488 or 594 and 633 (Molecular Probes).

Transfections and drug treatments

Cells were maintained in DMEM supplemented with 10% FBS and penicillin/streptomycin (normal culture medium). Sub-confluent MEFs were transfected using Lipofectamine™ 2000 (Invitrogen) according to manufacturer's protocols. The following procedures were used for drug treatments: M β CD (Sigma) at the final concentration of 10 mM in normal culture medium for 30 min at 37°C; water-soluble cholesterol (M β CD-complexed cholesterol, Sigma) at the final concentration of 50 µg/ml in normal culture medium for 90 min at 37°C; Bafilomycin A1 (Upstate) at final concentration of 200 nM in normal culture medium for 15 h; and EGF (Sigma) at the final concentration of 100 mg/ml in normal culture medium for various time points (as indicated in the Figure 1a).

Analysis of SNARE complexes

Purified lysosomal membrane samples in Triton X-114 were centrifuged at 15 000 g (30 min at 4°C) to isolate the Triton-insoluble material (DRM fraction) and the Triton-soluble membrane proteins (soluble fraction). Both fractions together with samples containing the total lysosomal membranes were treated with Laemmli buffer (SDS-containing buffer) and then divided in two aliquots. One aliquot was boiled (5 min at 100°C) to disrupt SDS complexes, whereas the other was kept at 4°C (nonboiled samples) before being subjected to SDS-PAGE.

Densitometry quantification of monomeric and complexed form of Vti1b was performed using the ImageJ program.

Immunoprecipitation

Cells were washed in PBS and a post-nuclear supernatant was obtained by scraping the cells in isotonic buffer and centrifuging them at 5000 g for 5 min. One volume of 2 × lysis buffer—50 mM Tris-HCl (pH 7.9), 200 mM NaCl, 1% Triton X-114, 1 mM EDTA, 50 mM HEPES and protease inhibitors (Sigma)—was added to one volume of the supernatant and the samples were then incubated with protein A Sepharose (Sigma) overnight, followed by 3-h incubation with anti-Vti1b antibodies (rabbit polyclonal). The immunoprecipitate was separated by centrifugation, boiled in Laemmli buffer and loaded and onto 5–10% SDS-PAGE.

Plasmids

For the GFP-Vti1b plasmid construction, the corresponding amplified cDNA was cloned in the pEGFP-C3 vector (Clontech). The GFP-VAMP7 was a kind gift from M D'esposito (IGB, Naples, Italy). The GFP-GPI was a kind gift from J Lippincott-Schwartz (NICHD, NIH, Bethesda, MD, USA). The tandem-fluorescent-LC3 (mRFP-GFP-LC3) plasmid was a kind gift from T Yoshimori (Kimura *et al*, 2007).

Immunofluorescence analysis

Cells were washed three times in cold PBS and then fixed in 4% paraformaldehyde (PFA) for 15 min. Fixed cells were washed four times in cold PBS, permeabilized with 0.1% Saponin in blocking solution (0.5% BSA, 50 mM NH₄Cl and 0.02% NaN₃ in PBS) for 30 min and immunolabelled with appropriate primary and secondary antibodies. Cells were then washed four times in cold PBS and mounted in Vectashield mounting medium. For dextran-LAMP-1 co-immunofluorescences, cells were loaded with 100 µg/ml dextran (10 000 MW) conjugated with the Alexa Fluor 594 dye (Molecular Probes) for 2 h, then the dextran was removed and after additional 3 h MEFs were fixed in 4% PFA and subjected to immunostaining with LAMP1. Lipid rafts immunofluorescence was performed using the Vibrant Lipid Raft Labeling Kit (Molecular Probes) according to manufacturer's protocols. Confocal microscopy was performed with a Zeiss LSM 510 microscope equipped with a Zeiss confocal-scanning laser using a × 63 1.4 numerical aperture objective. The percentage of co-localizing fluorescence (merge) was quantified by using the 'co-localization' module of the LSM 3.2 software (Zeiss).

Immuno-electron microscopy

The MEFs were transfected with either GFP-VAMP7 or GFP-GPI. Alternatively, MEFs were treated with cholera toxin B (according to manufacturer's protocols of Vibrant Lipid Raft Labeling Kit, Molecular Probes) to target GM1 ganglioside (a raft lipid). Cells grown were washed with PBS, and fixed in a solution of 4% PFA and 0.1% glutaraldehyde in 0.2 M HEPES buffer (pH 7.4) for 15 min at room temperature and then for additional 30 min in 4% PFA alone. After washing with PBS, cells were incubated for 30 min in blocking solution (50 mM NH_4Cl , 0.1% Saponin and 1% BSA in PBS), and overnight at 4°C with either anti-GFP or anti-cholera toxin B antibody diluted 1:100 in blocking solution. Cells were washed and incubated for 1 h at room temperature with Nanogold-conjugated anti-rabbit IgG Fab fragment diluted 1:100 in blocking solution and processed according to the Nanogold enhancement protocol (Nanoprobes, Yaphank, NY, USA). Stained cells were embedded in Epon-812 and cut. The EM images were acquired from thin sections using an FEI Tecnai-12 electron microscope equipped with an ULTRA VIEW CCD digital camera (FEI, Eindhoven, The Netherlands).

EGFR endocytosis

For EGFR endocytosis analysis, MEFs were starved in DMEM without the addition of FBS for 19 h. After starvation, cells were treated with EGF to stimulate EGFR endocytosis. Cells were then collected at various time points (as indicated in Figure 1a) and lysed in RIPA buffer (50 mM Tris-HCl (pH 7.5), 1% Triton X-100, 150 mM NaCl, 1 mM EDTA and 0.1% Na deoxycholate) with protease inhibitor (Sigma). Protein extracts were subjected to SDS-PAGE and immunoblotted using anti-EGFR antibody.

Analysis of autophagic flux

To monitor the autophagic rate, MEFs were transfected with mRFP-GFP-LC3 that showed a GFP and RFP signal before the fusion with lysosomes, and exhibited only the RFP signal after fusion due to the loss of GFP fluorescence within the acidic lysosomal environment. After 33 h, they were treated with bafilomycin for 15 h to block lysosome-autophagosome fusion. The bafilomycin was then removed from the medium and the extent of lysosome-autophagosome fusion was evaluated after 1 and 3 h by fixing in 4% PFA and measuring the green/red fluorescence ratio (GFP loss) that in the regions corresponding to the vesicles using a homemade MATLAB (The Mathworks) application (F Formiggini).

FRAP analysis

For FRAP experiments, EGFP-Vti1b was transfected in MEFs cells grown on glass-bottomed microwell dishes (Mat-Tek). An area containing EGFP-vti1b-positive endolysosomal clusters was photobleached with 100% of the argon laser power at 488 nm, resulting in

a 70–80% reduction in the fluorescence intensity. The long-range motility of these groups of organelles was negligible. The recovery of fluorescence was monitored over time (300 s) by scanning the bleached area at the conventional (low) laser power to minimize photobleaching during sampling. To analyse the rate of recovery, we compared the fluorescence of the photobleached area to that of an adjacent unbleached area of the same cell with similar fluorescence intensity. For each time point, the fluorescence of the bleached area was normalized to that of the corresponding control (unbleached) area to correct for possible drift of the focal plane or photobleaching incurred during the low light sampling. For each experimental FRAP curve, the $t_{1/2}$ value was calculated by fitting the data with the Boltzmann function. The FRAP experiments were performed at 37°C on Zeiss LSM 510 microscope equipped with a Zeiss confocal-scanning laser using a $\times 63$ 1.4 numerical aperture objective.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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Author contribution: AF designed research, performed experiments, analysed the data and co-wrote the paper; FA performed experiments and contributed to the analysis of data; HJK, AL, CS, NCS and DM performed experiments; AOF contributed with analytic tools; RP contributed with immuno-EM experiments; KS contributed to supervise the data and to write the paper; AB supervised the project and co-wrote the paper.

Conflict of interest

The authors declare that they have no conflict of interest.

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