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Supplemental Information

Transcriptional Activation of Lysosomal Exocytosis Promotes Cellular Clearance

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SUPPLEMENTAL INVENTORY

- Supplemental Figures:

Figure S1 refers to main figure 1

Figure S2 refers to main figure 2

Figure S3 refers to main figure 4

Figure S4 refers to main figure 5

Figure S5 refers to main figure 6

- Supplemental figure legends

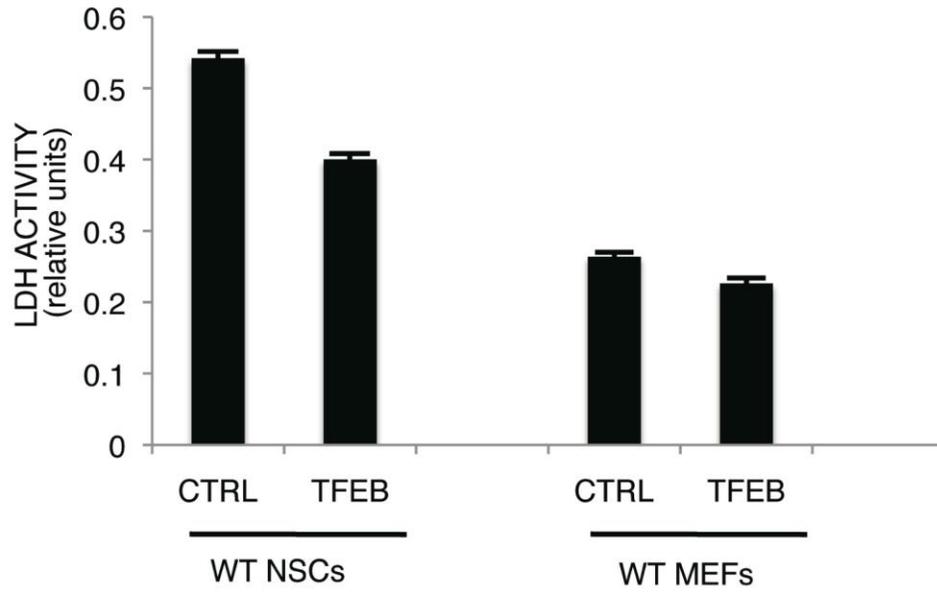
- Supplemental Experimental Procedures:

Full description of the methods used in the paper

- Supplemental References

Figure S1

A



B

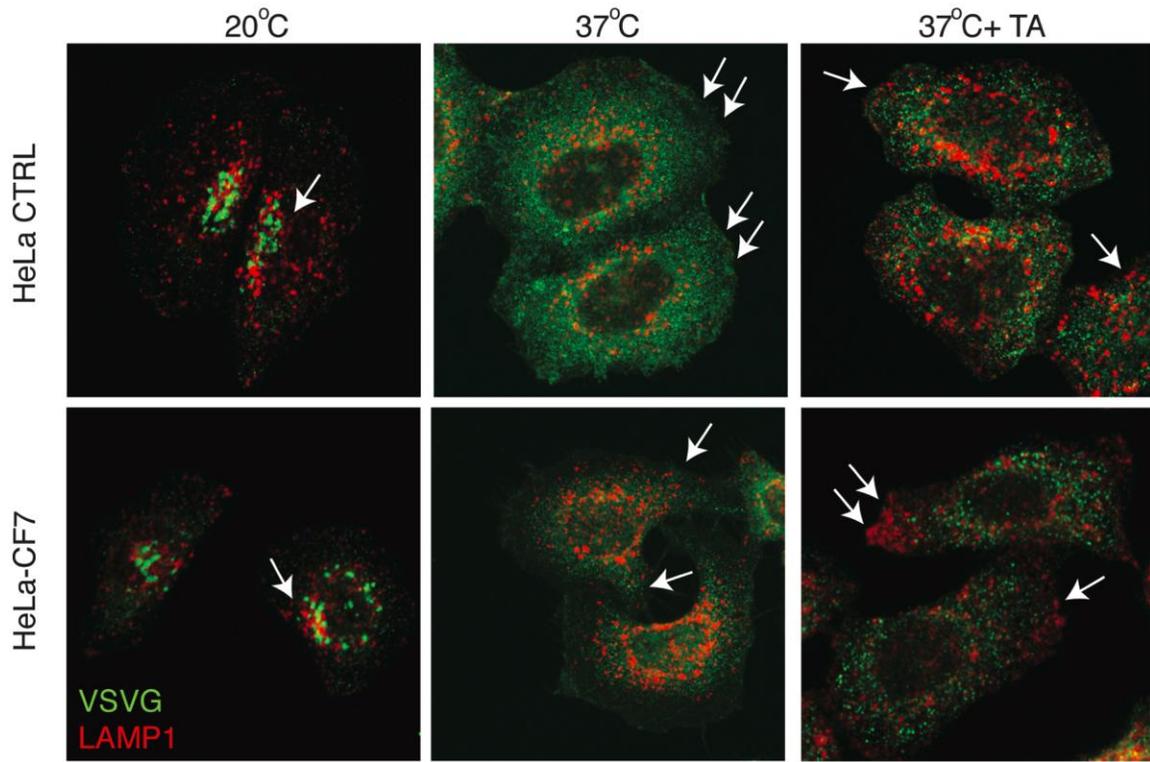


Figure S2

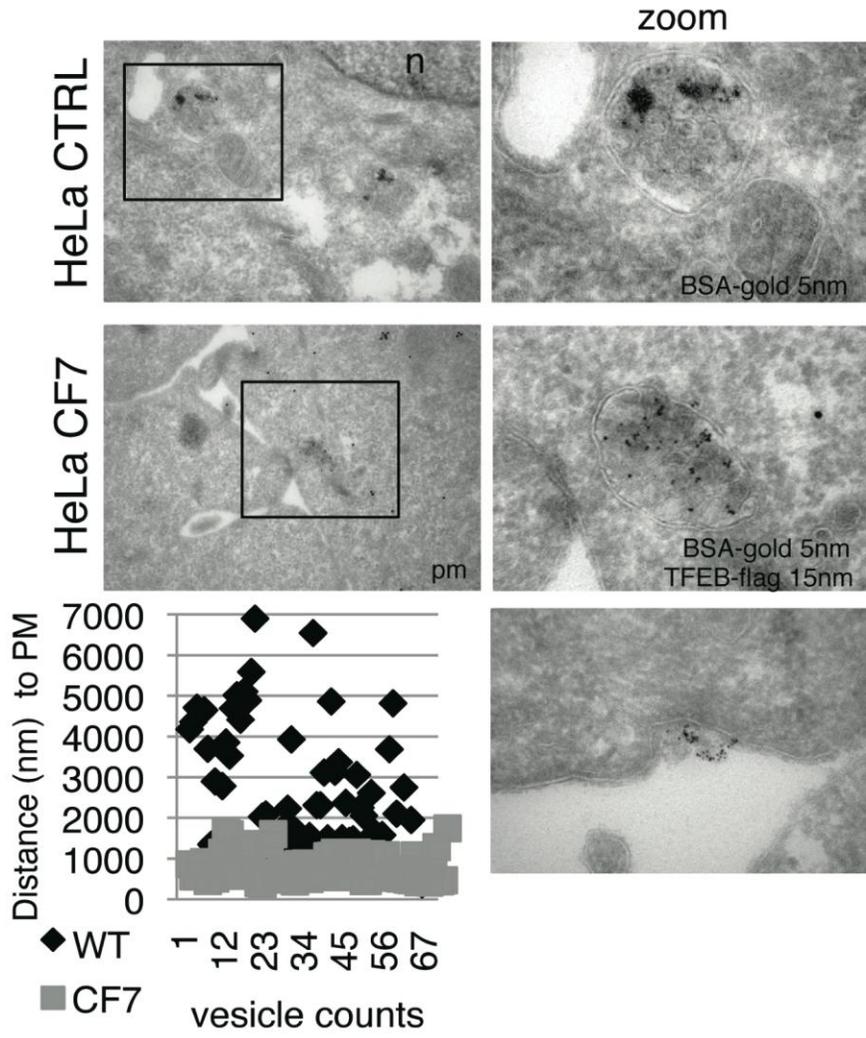


Figure S3

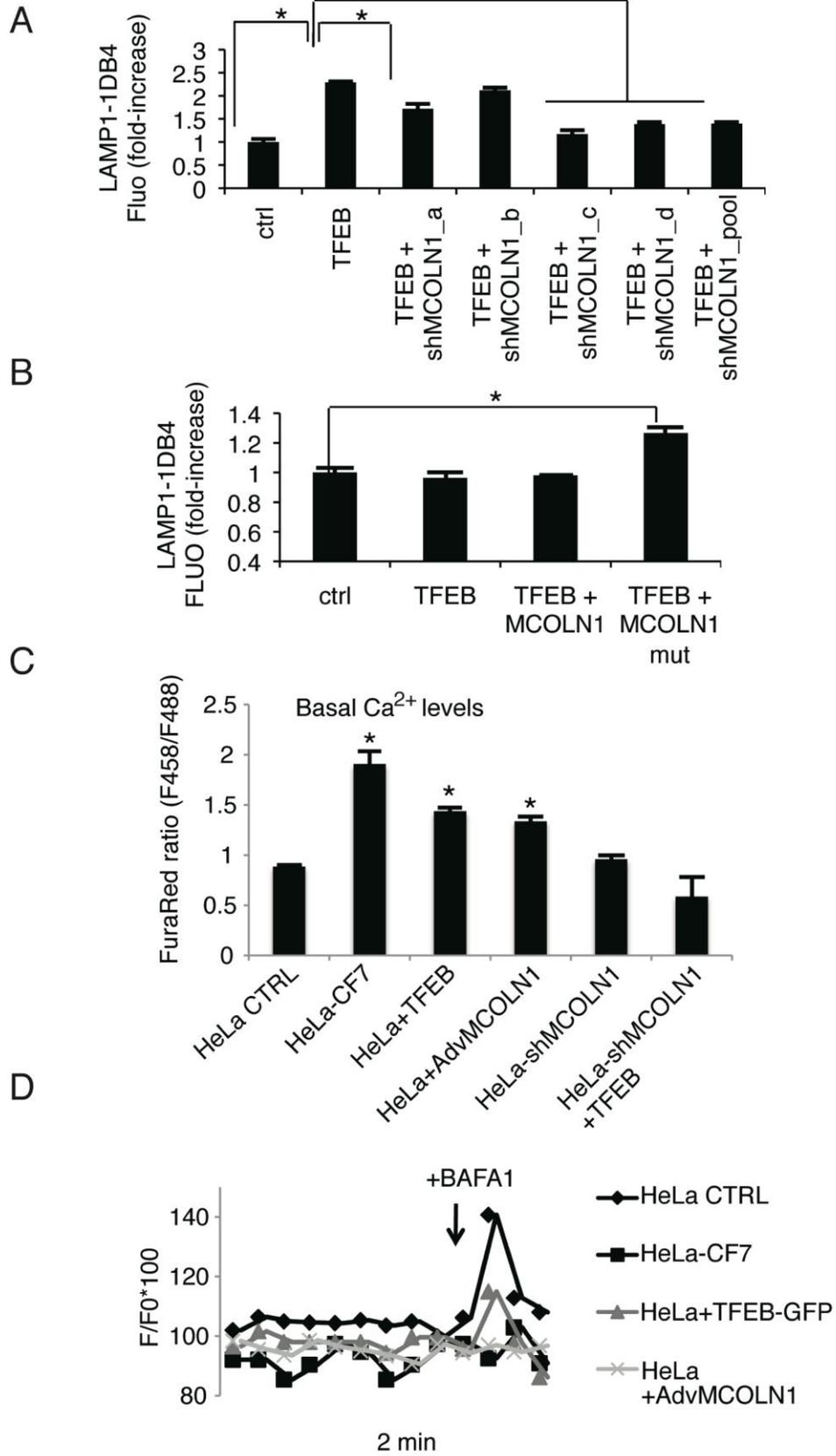


Figure S4

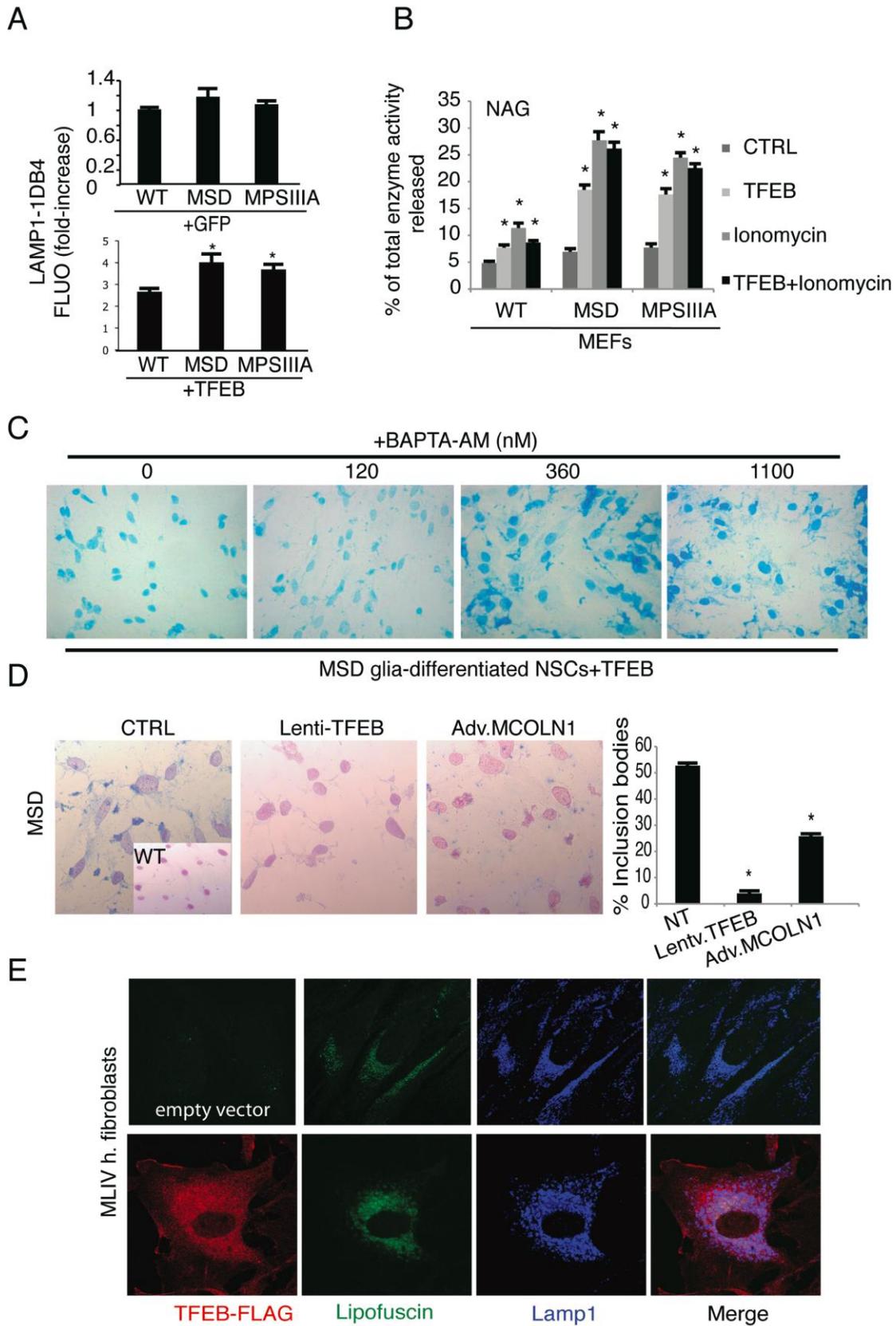
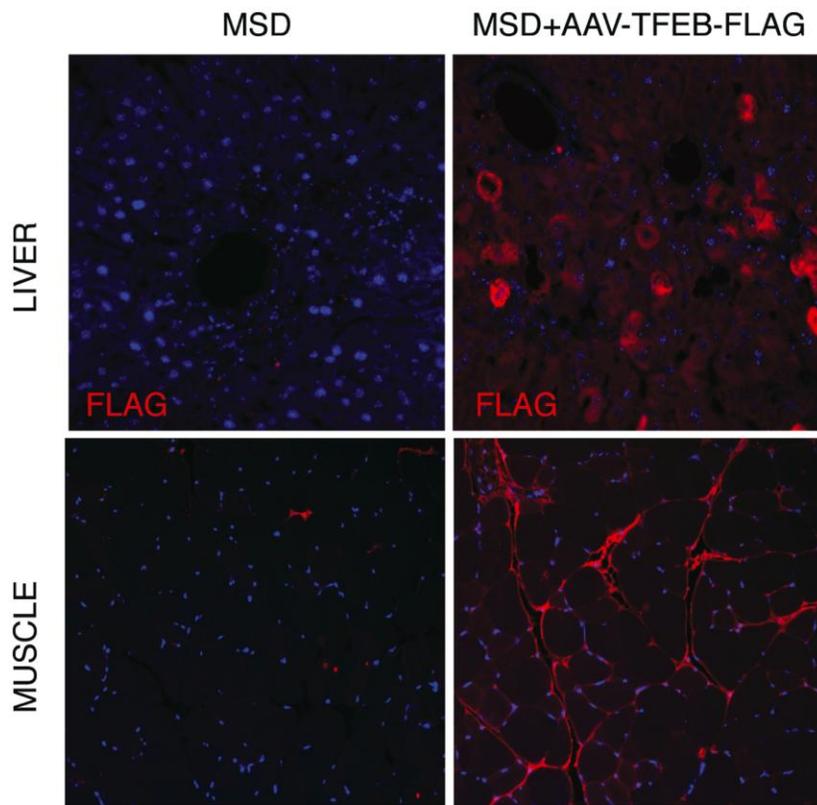


Figure S5



SUPPLEMENTAL FIGURE LEGENDS

Figure S1. (A). TFEB overexpression is not cytotoxic. NSCs and MEFs were transfected either with TFEB or with an empty vector. Supernatants were collected and lactate dehydrogenase (LDH) activity was determined following the manufacturer's manual (Abcam). Experiments were performed in triplicate (data represent mean \pm SEM). (B) HeLa control cells and stable HeLa-CF7 cells were infected with the temperature sensitive strain of vesicular stomatitis virus (VSV) to follow release of its G protein (VSVG) from the 20°C temperature block. In both temperature conditions (20°C and 37°C) the LAMP1 compartment did not colocalize with VSVG, thus indicating that LAMP1 does not escape from the lysosomal route by conventional sorting from TGN to PM. Blocking fusion of Golgi-derived VSVG transport carriers with the PM by the use of tannic acid (TA) resulted in the accumulation of VSVG transport carriers near the PM, as expected. However, no LAMP1 was detected within such VSVG-positive structures, suggesting that LAMP1 was not delivered to the PM from the Golgi in CF7 cells. Data represent mean \pm SEM.

Figure S2. TFEB overexpression reduces the distance of BSA gold-labeled endolysosomes to the PM. HeLa cells (i.e. control, HeLa CTRL and TFEB overexpressing cells, HeLa CF7) were loaded for 30 min with BSA gold (5nm), chased for 120 min in BSA gold-free medium and fixed for immunogold labeling on cryosections. HeLa CTRL and CF7 were then labeled with anti-flag antibody to visualize TFEB. In CF7 cells the endo-lysosomes localize closer to the PM. To quantify this observation, a morphometric analysis was carried out to determine the mean distance of endo-lysosomes to the PM. The histogram shows the relative distribution of endo-lysosomes in CTRL and CF7 HeLa cells (HeLa CTRL 2387nm vs 438nm in HeLa CF7). Representative fields of CTRL and CF7 cells are shown in the top left panels, while the top right panel shows a magnification of the insert. A representative image of an endo-lysosome fused with the PM is shown in the bottom right panel.

Figure S3. (A) The effects of different shRNAs against MCOLN1 on TFEB-mediated induction of lysosomal exocytosis was measured in HeLa cells transfected with a TFEB-expressing vector alone or in conjugation with the indicated shMCOLN1 (single or pool shRNAs). The amount of LAMP1 on PM was assessed by FACS analysis and expressed as fold-increase to ctrl (i.e. non-transfected cells). (B) TFEB-mediated activation of lysosomal exocytosis in HeLa^{shMCOLN1} cells was rescued by a mucopolipin mutant resistant to shRNA. A stable HeLa clone expressing specific shRNA against MCOLN1 (HeLa^{shMCOLN1}) was transfected with the TFEB-expressing vector alone or in conjugation with a plasmid encoding either MCOLN1 wild type or a MCOLN1 mutant resistant to shRNA. The amount of LAMP1 on PM was assessed by FACS analysis and expressed as fold-increase to ctrl (not-transfected cells). (C) Basal cytosolic Ca²⁺ levels were measured by confocal time-lapse microscopy of transfected/infected HeLa cells (see the different groups in the histogram) loaded with FuraRed-AM. Ca²⁺ levels are represented as the ratio of the fluorescence emitted at 660 nm after the excitation with 458 nm (F458) and 488nm (F488). At least 5 cells were measured for each experimental group. (D) TFEB and MCOLN1-overexpressing cells are less sensitive to BafilomycinA1-dependent depletion of lysosomal Ca²⁺ compared with CTRL HeLa cells or HeLa cells depleted of MCOLN1 and overexpressing TFEB. Ca²⁺ levels were measured by loading cells with the ratiometric fluorescent dye FuraRed. After 1.5 min of confocal time-lapse acquisition, cells were treated with 1 μ M Bafilomycin A1 to induce the release of Ca²⁺ from the acidic compartment. Data represent the F458/F488 ratio of each experimental group compared with their basal ratio before stimulation (F/F₀*100). Data represent mean \pm SEM; *, P < 0,05 (A, B, C).

Figure S4. (A) LSD cells are committed to TFEB-mediated lysosomal exocytosis. Wild type, MSD, and MPSIIIA MEFs were transfected with either a GFP or a TFEB expression vector (bi-cystronic TFEB-GFP) and exposure of LAMP1 on the PM was analyzed by flow cytometry. Data represent fold-increase of the levels of LAMP1 on PM in LSD cells, transfected with GFP or TFEB, against WT cells (B) The release of the lysosomal enzyme NAG was determined both in the culture medium and cell lysates

from wild type, MSD, and MPSIIIA MEFs transfected with either TFEB or with an empty vector, with and without stimulation with ionomycin. (C) Ca²⁺ involvement in TFEB-mediated GAG clearance in MSD-NSCs. Alcian blue staining of GAGs was performed on glia-differentiated NSCs isolated from the cerebral cortex of MSD mice and nucleofected with either a TFEB plasmid, or with an empty plasmid. The cells were treated with different concentrations of the Ca²⁺ chelator BAPTA-AM (see figure) for 12 h. (D) MCOLN1 overexpression partially reduces GAG accumulation in MSD cells. Alcian blue staining of GAGs was performed in glia-differentiated MSD NSC cells infected with either a lentiviral vector carrying TFEB or an adenoviral vector encoding MCOLN1. Three representative images were shown for each treatment (non infected cells; CTRL, +lenti-TFEB and + Adv-MCOLN1). (E) TFEB overexpression did not reduce lipofuscin accumulation in human MLIV cells. Human MLIV disease fibroblasts were transfected with either TFEB-FLAG or with an empty vector and analyzed by confocal microscopy. Data represent mean \pm SEM; *, P < 0,05 (A, B, D).

Figure S5. Representative immunofluorescence of liver and muscle sections infected with AAV-TFEB-FLAG. The FLAG was detected using specific anti-FLAG antibodies.

Movie S1. Lysosomal dynamics of a stable HeLa clone expressing the empty vector PCDNA3 (HeLa-PCDNA3). Lysosomal movement was followed by live-imaging in control HeLa-PCDNA3 cells transfected with a vector encoding the lysosomal membrane protein LAMP1 fused to GFP

Movie S2. TFEB increases lysosomal dynamics. The movement of lysosomes was followed by live imaging in a stable HeLa clone expressing TFEB (HeLa-CF7 cells), and transfected with a vector encoding the lysosomal membrane protein LAMP1 fused to GFP

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Cell culture and transfection. HeLa, Cos7 cells, human fibroblasts, and mouse embryonic fibroblasts from mouse models of MLIV (S7), MPS-III A (S7), and MSD were grown in Dulbecco's Modified Eagle's Medium (DMEM, Euroclone), supplemented with 10% heat inactivated Fetal Bovine Serum (FBS, Hyclone). Cells were seeded in six-well plates at 10% confluence before transfection. Transfection was performed by using PolyFect Transfection Reagent (Qiagen) or lipofectamine 2000 Reagent (Invitrogen), according to the manufacturer's protocols. TFEB- 3xFLAG HeLa stable cell lines (CF7 clone) (Sardiello et al., 2009) were maintained in Dulbecco's Modified Eagle's Medium (DMEM, Euroclone), supplemented with 10% heat inactivated Fetal Bovine Serum (FBS, Hyclone) and 1 mg/ml G418 (Sigma). The shMCOLN1 cell line was generated as follows: control and MCOLN1 lentiviral shRNAs were obtained from Sigma (MISSION shRNA Lentiviral Transduction Particles). HeLa cells were infected with MCOLN1-lentivirus (MISSION shRNA TRCN0000083297) together with 8 μ L (8 μ g/ml) of Polybrene. Clones were selected using 1 μ L (1 μ g/mL puromycin. Q-RT-PCR was used to assay for knockdown of MCOLN1. Neuronal progenitor cells were isolated from cortices of WT and MSD and MPSIII A pups (P0) by tissue homogenization using the neural tissue dissociation kit and separation using magnetic sorting of cells expressing the stem cell marker prominin-1 (Miltenyi Biotec Srl). Neuronal progenitor cells were maintained in ESGRO complete medium (Hyclone) in the presence of EGF and FGF2 growth factors (Preprotech). Where indicated, NSCs were differentiated by removing growth factors and incubate in ESGRO medium containing 2% of serum for at least 48h. NSCs were transfected by nucleofection (Amaxa).

Flow Cytometry analysis of surface LAMP1. Confluent cells transfected with TFEB-GFP constructs were trypsinized and washed twice with PBS before incubation with PBS or with 10 μ M ionomycin in PBS for 5 min at 37°C. 10⁶ cells for each assay were centrifuged and resuspended in PBS+1% BSA with anti-rat LAMP1-1DB4 at 4° C for 30 min. Cells were washed in PBS and fixed in 2% paraformaldehyde for 15 min. Anti-LAMP1-1D4B-treated cells were further incubated with Alexa-594 conjugated anti-rat secondary antibodies (Molecular Probes) for 30 min at room temperature. Finally, cells

were washed, resuspended in 0.5 ml PBS, and analyzed on a FACS Aria Flow Cytometer (Becton Dickinson & Co., Mountain View, CA, USA). Forward angle scatter, right angle scatter, and fluorescence intensity were recorded from 50,000 cells whose forward angle scatter fell above a threshold used to distinguish intact GFP-positive cells from both non-transfected or damaged cells.

Immunofluorescence and confocal imaging. Transfected cells were grown on glass coverslips, washed with PBS containing 100 mM MgCl₂ and 100 mM CaCl₂ (PBS/Ca²⁺/Mg²⁺), and fixed with 4% paraformaldehyde (PFA, Sigma) for 10 min. After washing and quenching PFA with 50 mM NH₄Cl for 15 min, cells were washed with PBS and permeabilized in blocking buffer (0.05% saponin/0.2% BSA in PBS/Ca/Mg) for 20 min. Coverslips were then incubated with appropriate primary antibodies and for 1 h with Alexa-594 and Alexa-488 conjugated secondary antibodies (Molecular Probes). The primary antibodies used were: anti-LAMP1 (L1418) (Sigma) and anti-FLAG antibody (clone M2) (Sigma). Coverslips were mounted on glass slides with Vectashield (Vector Laboratories, CA, USA).

Immunofluorescence on tissue sections. Mice tissues were collected after PBS perfusion and fixed with 4% PFA for 12 h at 4°C. Then, tissues were subjected to a sucrose gradient (from 10 to 30%) and incubated over night in 30% sucrose at 4°C. Finally, tissues were embedded in OCT embedding matrix (Kalttek) and snapfrozen in a bath of dry ice and ethanol. Immunofluorescence analyses were performed on 10 µm thick serial cryosections. The specimens were incubated for 1 h with blocking solution (PBS, 0.2% Tween-20) and 10% goat normal serum (Sigma- Aldrich) before incubation over night with the specific primary antibody. The antibodies used were anti-CD-68 (rat anti mouse 1:250; Serotech, Ontario, Canada), anti-GFAP (G-A-5 1:200; Sigma), anti-GAA (generous gift of Dr. Parenti, TIGEMNapoli), and anti-FLAG (clone M2; Sigma). After washing, sections were incubated for 40 min with secondary antibody. The secondary antibodies were purchased from Molecular Probes (Invitrogen, CA, USA). Stained sections were mounted with Vectashield with DAPI (Vector Laboratories, CA, USA).

Images were taken using a confocal microscope (LSM510; Carl Zeiss, Inc.) using a Plan-

Neofluar 63x immersion objective (Carl Zeiss, Inc.). Staining for Surface LAMP1. After the different treatments, MEFs or NSCs cells grown on glass coverslips were incubated in PBS+1% BSA at 4 C for 30 min with anti-rat LAMP1-1DB4 (SantaCruz Biotechnology Inc.). Cells were then fixed with 4% paraformaldehyde for 15 min at 4 C, washed in PBS, and incubated with Alexa- 594 conjugated anti-rat secondary antibodies (Molecular Probes) for 30 min at room temperature. Coverslips were mounted on glass slides with Vectashield (Vector Laboratories, CA, USA). Images were taken using a confocal microscope (LSM510; Carl Zeiss, Inc.) using a Plan-Neofluar 63x immersion objective (Carl Zeiss, Inc.).

Immuno-blot. Cells were lysed in cold lysis buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% TritonX-100) in the presence of protease inhibitors (Sigma) for 30 min on ice. Where indicated nuclear lysates were obtained by using the Nuclear protein extraction kit (Pierce). 10-20 ug of protein samples were separated on SDS-PAGE acrylamide gel and transferred onto nitrocellulose membrane (Amersham Pharmacia Biotech). Primary and (HRP)-conjugated antibodies were diluted in 1% BSA TBS-T. Bands were visualized using the ECL detection reagent (Pierce) and normalized against actin. Proteins were quantified by the Bradford method. Antibodies were used as follows: TFEB (SantaCruz), LAMP1 and anti-beta-Actin (Sigma), beta-1 Integrin (BD Science).

BSA-gold. HeLa control cells and overexpressing TFEB (HeLa CF7) were rinsed, incubated with BSA-gold (OD 5) in serum free-medium for 30min at 37°C and chased in whole medium for 2h in absence of BSA-gold. The cells were then fixed and treated for immunogold labeling on cryosections according to the protocol described previously (Puri et al, 2009). In HeLa CF7 the cells overexpressing Tfeb were identified using a mouse anti-flag antibody (M2 Sigma) followed by a rabbit anti-mouse antibody (Sigma) and protein-A gold (Cell Microscopy Centre, Utrecht, The Netherlands). The samples were observed with Jeol (JEM-1011). Pictures of random fields were taken for both samples and the lysosomes distance from the nucleus was measured using the iTEM software.

Calcium measurements by ratiometric assays. HeLa cells were infected with either control adenovirus (Ad. Null) or adenovirus expressing TFEB (Ad. TFEB-FLAG). After twenty four hours, cells were washed with Calcium Imaging Buffer (CIB) (140 mM NaCl, 4.7 mM KCl, 1 mM CaCl₂, 2.1 mM MgCl₂, 50 mM Hepes, 10 mM glucose, pH 7.4) and loaded with 20 μM Fura-2AM (Invitrogen, Carlsbad, California) in the same buffer for 1h at 37°C. Cells were then extensively washed in CIB and pseudocolor ratiometric images were acquired using an Olympus IX 81 inverted microscope (Tokyo, Japan) equipped with a 20x NA 0.75 UPlanSApo objective, a set of filters (excitation 340 nm and 380 nm and emission 510 nm) and an Andor XON+ EMCCD camera (South Windsor, Connecticut). The acquisition and analysis of the images were done using MetaFluor software (Molecular Devices, Sunnyvale, CA). For Fura-2AM calibration, cells were incubated in either high calcium buffer (140 mM NaCl, 4.7 mM KCl, 2 mM CaCl₂, 1.1 mM MgCl₂, 50 mM Hepes, 10 mM glucose, pH 7.4) containing 10 μM ionomycin or non-calcium buffer (140 mM NaCl, 4.7 mM KCl, 3.1 mM MgCl₂, 2mM EGTA, 50 mM Hepes, 10 mM glucose, pH 7.4) containing 20 μM ionomycin. Fura-2AM ratios (F340/F380) from cells infected with Ad. Null (n=2584; where n represents number of cells) or Ad. TFEB-FLAG (n=2238) were used to calculate the cytoplasmic calcium concentration using the following equation $[Ca^{2+}]_i = K_d \left(\frac{R - R_{\min}}{R_{\max} - R} \right) \left(\frac{S_{f,2}}{S_{b,2}} \right)$ (described in Kao et al., 2010). A K_d of 371 nM for Fura-2AM has been previously calculated (Petr and Wurster, 1997). All the experiments were done in duplicate. Data are expressed as means ±SEM (*P<0.0001).

FuraRed experiments. We loaded TFEB-GFP transfected cells with FuraRed-AM (60 min at 37°C) in Calcium Imaging Buffer according to the protocol described previously (Luciani et al., 2010). Pseudocolor ratiometric images (458 and 488 nm excitation, 610 nm emission) were collected using a confocal microscope equipped with a variable filter wheel. The number of TFEB-GFP positive cells that mobilized calcium was quantified in a minimum of three wells and compared with cells transfected with empty vector. Experiments were repeated a minimum of three times.

Treatments. HeLa cells were loaded with 5mM FuraRed-AM (Molecular Probes) in culture medium at 37°C for 60 min. Cells were washed in Tyrode solution for 10–30min, and the fluorescence intensities at 458 nm (F458) and 488 nm (F488) were recorded using a confocal microscope (LSM510; Carl Zeiss, Inc.) using a Plan- Neofluar 63x immersion objective (Carl Zeiss, Inc.) FuraRed ratios (F458/F488) were used to represent changes in cytosolic Ca²⁺ levels in basal conditions or after the treatment with 1μM of Bafilomycin A1. The EGFP-positive cells were identified by monitoring fluorescence intensity at 488 nm. Flow cytometric calcium flux assay. Cell preparation and loading was performed following the protocol described by Schepers et al., 2009 (Schepers et al., 2009) with some modifications. Briefly, control and shMCOLN1 transfected cells (HeLa, HeLa-CF7, and NSCs) were loaded with Fluo-3AM and FuraRed-AM (Molecular Probes) for 30 min at 37°C. After a wash in PBS–Ca²⁺, the cells were resuspended in the appropriate buffer in accordance to the different conditions and kept at RT until analysis. Ca²⁺-entry is prevented by the use of a PBS–Ca²⁺ buffer containing 1 mM sodium pyruvate, 25 mM HEPES and 5 mM EGTA, the latter to exclude remaining traces of Ca²⁺ contamination. In these fluo3/FuraRed-loaded cells the release of Ca²⁺ from the intracellular stores can be studied. Samples were analysed using the FACS Aria ® flow cytometer (Becton Dickinson). Following preparation the samples were aspirated during 30 s to determine the baseline fluorescence of the fluo-3 and FuraRed. Then, after the addition of ionomycin (5 μM), the acquisition was resumed with changes in [Ca²⁺]_i being recorded over a 150 s time period. Changes in the fluorescence (FL) intensity of the fluo3 and FuraRed were measured on the FL-1 and FL-2 channels. The ratio of baseline Fluo-3/FuraRed was plotted. Data are expressed as means±standard deviation (SD). A p-value of less than 0.05 was considered significant.

Electron microscopy. Control and TFEB-overexpressing cells were washed with PBS, and fixed in 1% glutaraldehyde dissolved in 0.2 M Hepes buffer (pH 7.4) for 30 min at room temperature. After embedding and cutting EM images were acquired from thin sections using a Philips Tecnai-12 electron microscope equipped with an ULTRA VIEW CCD digital camera (Philips, Eindhoven, The Netherlands). Quantification of vacuolization was performed using the AnalySIS software (Soft Imaging Systems GmbH,

Munster, Germany).

Enzymatic activities. Enzyme activities were measured with the appropriate fluorimetric or colorimetric substrates. Specifically, acid phosphatase and β -hexosaminidase activities were measured using the Acid-Phosphatase and β -N-Acetylglucosaminidase assay kit (Sigma), respectively. β -galactosidase was measured by a colorimetric assay using 4-methylumbelliferyl- β -D-galactopyranoside as substrate in 0,5M NaAc buffer pH: 5.0. Lactate de-hydrogenase (LDH) activity was measured using the LDH-Cytotoxicity Assay Kit (Abcam). To measure enzyme activities in the medium, cells were incubated in medium containing 1% BSA (w/v). Protein concentrations were determined using the BCA-assay (Pierce Biotechnology).

Western blot of plasma membrane proteins. Plasma membrane proteins were isolated from cultured MEFs using the Cell Surface Protein Isolation kit (Pierce). The samples obtained were loaded together with correspondent total fraction on SDS-PAGE gel and subjected to western blot analysis using anti-LAMP1 (Sigma), anti- β -actin (Sigma) and anti- β 1-integrin antibodies (BD Bioscience)

Live imaging. WT and CF7 cells were plated on coverslip-bottom MaTek plates (MatTek Cultureware, Ashland MA, USA) and mounted in a perfusion, open and closed cultivation chamber fitted with a Tempcontrol and CO₂-control device (Y module; Carl Zeiss). The imaging was performed on a Carl Zeiss microscope with a LSM 710 confocal attachment using a 63x/1.4 NA Plan Apochromat oil immersion lens. Laser lines at 488 nm (GFP-tagged LAMP1), were used. Laser power was kept at a minimum to minimize photobleaching and photocytotoxicity. The detector pinholes were set to give a 0.536-mm optical slice. Scan rates was 1,96 seconds by using multitracking (line switching) with a line average of 2 and with a delay time of 0 seconds in between scans. Acquisition was performed using ZEISS LSM 710 software. Acquisition times varied from 3 to 5 min. Live imaging analysis. To calculate the trajectory and the mean velocity of vesicles, movies were analyzed by IMAGEJ using the LSM reader and particle tracking plugins. About 100 vesicles (GFP-LAMP1), which could be tracked for at least 10 frames

(approximately 2 min), were chosen for each cell. The results were copied to MS EXCEL, where all further calculations and analyses were performed. The velocity of a particle during each frame was calculated as the distance covered during each frame divided by the total time between frames (the scan time + delay time). The mean of these values was a measure of the velocity of a particle along a particular trajectory.

GAG analysis. Nucleofected NSCs were grown in differentiation ESGRO medium in (Hyclone) in the presence of 7 μ Ci/ml 3H-glucosamine hydrochloride (Perkin Elmer, 37.75 Ci/mmol, Boston, MA, USA) for 24h, washed extensively with PBS and chased for variable times. At each time point cells were harvested, homogenized and subject to chromatography on Sephadex G-25 columns (GE Healthcare, Sweden) to eliminate unincorporated ³H-glucosamine hydrochloride. The amounts of incorporated or secreted radioactivity was measured by liquid scintillation in a Beckman LS6500 counter (Beckman Instruments, Fullerton, CA, USA). Quantitative analysis of GAG accumulation in tissues. The protein extracts were assayed with the dimethylmethylene blue-based spectrophotometry of glycosaminoglycans (de Jong et al., 1989). The samples were read at 520 nm and the GAG concentrations were determined using the heparan sulfate standard curve (Sigma-Aldrich).

Alcian blue staining. After the perfusion of the animals with PBS, the tissues were collected and fixed in methacarn solution (30% chloroform, 60% methanol and 10% acetic acid) for 24 h at 4°C. The next day, the tissues were embedded in paraffin (Sigma-Aldrich) after their dehydration with a 70–100% ethanol gradient. Finally, the tissues were sectioned into 7 mm thick serial sections on a microtome. The tissue sections were stained with 1% Alcian blue (Sigma-Aldrich) in hydrochloric acid. The counterstaining was performed for 2 min with Nuclear-Fast red (Sigma-Aldrich).

SUPPLEMENTAL REFERENCES

Sardiello, M. et al. A gene network regulating lysosomal biogenesis and function. *Science* 325, 473-477 (2009).

Puri, C. Loss of myosin VI no insert isoform (NoI) induces a defect in clathrinmediated

endocytosis and leads to caveolar endocytosis of transferrin receptor. *J Biol Chem* 284, 34998-35014 (2009).

Luciani, A. et al. Defective CFTR induces aggresome formation and lung inflammation in cystic fibrosis through ROS-mediated autophagy inhibition. *Nat Cell Biol* 12, 863-875 (2010).

Schepers, E., Glorieux, G., Dhondt, A., Leybaert, L. & Vanholder, R. Flow cytometric calcium flux assay: evaluation of cytoplasmic calcium kinetics in whole blood leukocytes. *J Immunol Methods* 348, 74-82 (2009).

de Jong, J. G., Wevers, R. A., Laarakkers, C. & Poorthuis, B. J. Dimethylmethylene blue-based spectrophotometry of glycosaminoglycans in untreated urine: a rapid screening procedure for mucopolysaccharidoses. *Clin Chem* 35, 1472-1477 (1989).

Kao JP, Li G, Auston DA. Practical aspects of measuring intracellular calcium signals with fluorescent indicators. *Methods Cell Biol.*; (99):113-52 (2010).

Petr MJ and Wurster RD. Determination of in situ dissociation constant for Fura-2 and quantitation of background fluorescence in astrocyte cell line U373-MG. *Cell Calcium*. 1997 (3):233-240 (1997).