Rab6 and unfolded protein response-mediated proteostasis in Alzheimer’s disease
Elfrink, H.L.

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Rab6 affects lysosomal positioning and function

Hyung L. Elfrink, Rob Zwart, Viola Oorschot, Frank Baas, Judith Klumperman and Wiep Scheper

Manuscript in preparation to be submitted
Abstract

Previously, we showed that Rab6 reduces UPR activation and ER stress toxicity. Our data indicated that Rab6 does not directly affect the UPR signalling, therefore we investigate in this study whether Rab6 affects the function of the major proteolytic system during UPR activation, the autophagy lysosomal pathway. To this end we employed knockdown of Rab6 in HeLa cells. Here we show that reduced Rab6 levels do not affect the processing of LC3, showing that Rab6 does not affect the induction of autophagy. In contrast, we observe a striking relocalization of lysosomal, but not endosomal structures in cells with reduced Rab6 levels. The lysosomes cluster at a juxtanuclear site. Analysis by immuno-electron microscopy confirms our observation that the lysosomes cluster together. These ultrastructural changes are accompanied by a selective change in the glycosylation of LAMP 1 and 2 proteins and increased proteolytic activity indicating disturbed lysosomal function. Interestingly, the lysosomal repositioning that occurs in cells under nutrient or ER stress is disturbed in Rab6 depleted cells. Our data support a role for Rab6 in lysosomal positioning and dynamic repositioning during stress. The results underscore the importance for proper lysosomal function in the maintenance of proteostasis.
Introduction

Disturbed proteostasis is the underlying cause of several human diseases. This is illustrated in neurodegenerative disorders that are characterized by accumulation of aberrant proteins and (apparently ineffective) activation of protein quality control mechanisms. Protein quality control is an intricate interplay of stimulation of proper protein folding and removal of aberrantly folded proteins. The unfolded protein response (UPR) of the endoplasmic reticulum (ER) presents one of the major protein quality control systems in the cell. Activation of the UPR is a homeostatic response that is switched off upon restoration of proteostasis.

An important way to restore disturbed proteostasis caused by stress in the ER is the removal of misfolded proteins by proteolysis. Misfolded ER proteins can be degraded by the ubiquitin proteasome system (UPS) in the cytosol after translocation from the ER to the cytosol, a process called ER associated degradation (ERAD). An alternative system for degradation of ER proteins is via the autophagy/lysosomal pathway (ALP). Autophagy involves the sequestering of material that needs to be degraded by a double membrane structure, followed by fusion with a lysosome and degradation by lysosomal enzymes. The initial autophagophore membrane derives from different compartments, including the ER and Golgi. Our lab has previously shown that during activation of the UPR, the ALP is the preferred pathway for degradation.

We reported previously that the UPR is activated early in Alzheimer's disease (AD) and other tauopathies. In addition, we found that the levels of the small GTPase Rab6 are increased in close correlation with the extent of ER stress in AD brain. Follow-up studies in vitro showed that Rab6 mediates a protective pathway against ER stress. Increased Rab6 levels decrease the output of the UPR, whereas decreased Rab6 levels augment UPR induction. Rab6 does not interfere early in the signalling response of the UPR. Instead, we find that the recovery from an ER stress insult is impaired in the absence of Rab6. Rab6 therefore mediates its effect on the UPR by restoring the proteostasis, via a yet unknown mechanism.

Rab6 is a member of the Ras superfamily that use a GTP/GDP switch to regulate their activity. Rab proteins bind to vesicles in their GTP bound state and assist in different steps of vesicle transport and fusion. They release from membranes upon GTP hydrolysis and after exchange of the GDP for GTP a new cycle can be started. Different Rab proteins are involved in specific trafficking routes. Rab6 has been implicated in trafficking in both secretory and endocytic pathways.

Because UPR activation is persistent in AD and other tauopathies, mechanisms to restore ER proteostasis may provide a potential target for intervention. Therefore in this study we investigated whether Rab6 is involved in the function of the ALP.
Materials and Methods

Materials

Cell culture media and reagents were obtained from Gibco/Invitrogen (Carlsbad, CA, USA) and other chemicals were from Sigma (St. Louis, MO, USA), unless indicated otherwise.

Cell culture and transfection

HeLa cells were cultured in Dulbecco’s modified Eagle medium with GlutaMAX supplemented with 10% (v/v) fetal calf serum (Lonza, Basel, Switzerland), 100 U/mL penicillin and 100 µg/mL streptomycin. Cells were incubated at 37°C, 5% CO₂ and 95% humidity. Cells were plated in a desired wells format at a density of ~8.10³ cells/cm² in complete culture medium. The sequence of the scramble siRNA duplex was (sense: 5’ r(AGUACUGCUUACGAUACGG)d(TT) 3’). The sequences of Rab6 siRNA duplex 1 (sense: 5’ r(GACAUCUUUGAUCACCAGA)d(TT) 3’) and duplex 2 (sense: 5’ r(CACCU-AUCAGGCAACAAUU)d(TT) 3’) were previously published elsewhere. The siRNA duplexes were prepared by Sigma/Proligo. Experimental phenotypes were confirmed with both siRNA duplexes. Cells were transfected with 25 nM siRNA using HiPerFect Transfection Reagent (Qiagen, Venlo, the Netherlands), as indicated by the manufacturer’s protocol, and transfection lasted 72 hours before treatment. Cells were plated at a density of 40.10³ cells/cm² before transfection with 1 µg plasmid DNA (empty vector [mock], myc-tagged Rab6 Wt and -T27N) per well (12 wells format) using TransIT-LT1 transfection reagent (Mirus Bio LLC, Madison, WI, USA) according manufacturer’s protocol.

Cell culture treatments

Cells were treated with 1 µg/mL tunicamycin for 1 or 16 hours, as indicated. Full nutritional starvation was performed with Earle’s balanced salts solution (EBSS; Sigma) preceded by a single EBSS wash step and lasted 2 hours for determination of LC3 processing. To follow the redistribution of lysosomes, a mild nutritional starvation treatment was given by immediate EBSS substitution of complete culture medium without EBSS rinse and treatment lasted 1 hour. Cells were always incubated with DQ-BSA red (100 µg/mL; Molecular Probes/Invitrogen) together with Dextran MW 40,000 488 (20 µg/mL) to take uptake efficiencies in account. Incubation lasted 4 hours prior to (live) fluorescent imaging and FACS analysis. Non-permeabilized cells were incubated with Alexa Fluor 488 conjugated wheat germ agglutinin (WGA-AF488, Molecular probes/Invitrogen; 10 µg/mL in EBSS for 10 minutes) prior to imaging or FACS analyses.

SDS-PAGE and western blotting

Cells were harvested by scraping with a rubber policeman in lysis buffer (1% [v/v] Triton X-100 in phosphate buffered saline [PBS] supplemented with Complete pro-
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tease inhibitors cocktail tablets [Roche]). Protein content of denucleated supernatant (20,000 x g, 5 minutes, 4°C) was determined by Bio-Rad Protein Assay (Bio-Rad, Hercules, CA, USA). Samples were analyzed on polyacrylamide gels for LAMP1 and LAMP2 (6%), amyloid precursor protein (8%), cathepsin D, LAMP1, LIMP2, p62 (10%), Rab6 (12%) and LC3 (18%). Equal amounts of protein were loaded in each lane on a gel. Antibodies and incubation conditions are specified in table 1. Visualization was performed with Lumi-Light Western Blotting Substrate (Roche) on a LAS-3000 imaging system (Fujifilm, Tokyo, Japan).

Immunofluorescence and imaging

Immunofluorescence stainings were performed as previously published. Briefly, cells were fixed (4% [w/v] paraformaldehyde, 4% [w/v] sucrose [Merck], 15 minutes) and permeabilized (0.5% [v/v] Triton X-100, 5 minutes). Coverslips were blocked (5% [v/v] donkey serum, 30 minutes) and incubated with primary (2 hours) and secondary antibody (1 hour) in a dark moist chamber. Washes were performed with 0.05% (v/v) Tween-20. All previous solutions were prepared with PBS. Cells were counterstained with DAPI (1.3 µM, 5 minutes). Coverslips were mounted on glass slides with Prolong Gold (Invitrogen). Antibodies and incubation conditions are listed in table 1. Confocal imaging was performed on a Leica TCS-SP8 confocal scanner mounted on an inverted microscope (Leica Microsystems, Mannheim, Germany). Images were acquired with LAS-AF Software (version 3.0.0). Confocal images were deconvoluted using a theoretical point spread function by Huygens Essential Software (compute engine 4.1.1p1 64b; Scientific Volume Imaging, Hilversum, the Netherlands). Epi-fluorescent images were captured on a Leica DMI 4000 (Leica) and image acquisition was performed with LAS-AF software (version 2.6.0.766).

Quantification of lysosomal structures

The smallest possible circumference i.e. convex hull of the lysosomal structures was determined to quantify the redistribution event. Each main lysosomal structure within a field of view was cropped individually, excluding peripheral lysosomes. The convex hull was determined on LAMP1 positive structures in n=3 independent experiments with each two fields of view using the ImageJ (version 1.46r) plugin Hull and Circle (version 2.0a, 2005/05/24).

FACS analysis

DQ-BSA red and Dextran 488 incubated cell suspensions (1% FCS in PBS) were measured on a BD LSR Fortessa flow cytometer (Becton, Dickinson and company, New Jersey, USA). WGA-AF488 incubated cell suspensions were analyzed on a BD FACSCanto II flow cytometer (BD). Ten-thousand events per sample (gated population ranged from 81% to 91%) were acquired by FACSDiva software (BD Biosciences, version 6.1.3., build 2009, 05 13 13 29) and analyzed using FlowJo software (version 7.6.5, Engine 2.99700).
The procedures for immune-EM analysis are described elsewhere\(^1\). Briefly, cells treated for 3 days with Rab6 or scrambled, siRNA (see Cell culture and transfection) were fixed by addition of either 4% (w/v) paraformaldehyde (PFA) in 0.1 M phosphate buffer (PB) pH 7.4 or a mixture containing 0.4% (w/v) glutaraldhyde (GA; Polysciences Europe GmbH, Eppelheim, Germany) and 4% (w/v) PFA in 0.1 M PB pH 7.4 to an equal volume of culture medium for 10 minutes at room temperature (RT). Subsequently, fixative was replaced for 4% (w/v) PFA in 0.1 M PB or 0.2% (w/v) GA + 2% (w/v) PFA in 0.1 M PB, respectively, for at least 2 hours at RT. Fixed cells were rinsed with PBS, scraped and cell pellets were embedded in 12% gelatin. After overnight infusion in 2.3 M sucrose at 4°C, cells were frozen in liquid nitrogen as described\(^2\). Ultrathin cryosections

### Table 1. Antibodies used for western blot and immunofluorescence

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<th>Antibody</th>
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Incubations for western blot were performed 1:1000 in 5% (w/v) fat-free milk in PBS-T (0.05%, v/v), secondary HRP labeled antibodies were from Dako (Glostrup, Denmark). Incubations for immunofluorescence were performed 1:100 in 5% (v/v) donkey serum in PBS, secondary fluorescent labeled antibodies were from Jackson ImmunoResearch (West Grove, PA, USA). For immuno-electron microscopy, the dilutions for LAMP1 and Cathepsin B were 1:150 and 1:30, respectively. The dilutions for secondary antibodies were 1:300 for rabbit-α-mouse (Nordic) and 1:1500 for rabbit-α-goat (Sigma).\(^1\) Also used for immunofluorescence.\(^2\) Broad range LAMP1 glycoform detection.\(^3\) High molecular mass LAMP1/2 glycoform detection.

The procedures for immune-EM analysis are described elsewhere\(^3\). Briefly, cells treated for 3 days with Rab6 or scrambled, siRNA (see Cell culture and transfection) were fixed by addition of either 4% (w/v) paraformaldehyde (PFA) in 0.1 M phosphate buffer (PB) pH 7.4 or a mixture containing 0.4% (w/v) glutaraldhyde (GA; Polysciences Europe GmbH, Eppelheim, Germany) and 4% (w/v) PFA in 0.1 M PB pH 7.4 to an equal volume of culture medium for 10 minutes at room temperature (RT). Subsequently, fixative was replaced for 4% (w/v) PFA in 0.1 M PB or 0.2% (w/v) GA + 2% (w/v) PFA in 0.1 M PB, respectively, for at least 2 hours at RT. Fixed cells were rinsed with PBS, scraped and cell pellets were embedded in 12% gelatin. After overnight infusion in 2.3 M sucrose at 4°C, cells were frozen in liquid nitrogen as described\(^2\). Ultrathin cryosections
were cut at -120°C using a Leica UCT-FCS ultracryomicrotome (Leica Microsystems), picked up on copper grids with a 1:1 mixture of 2.3 M sucrose in PBS and 2% (w/v) methyl cellulose. Subsequent immunogold labeling was performed as described. Sections were analyzed in a JEOL 1200 EX electron microscope (Tokyo, Japan) at 80 kV. Gold particles were from Microscopy Center Utrecht (produced in-house).

**Peptide-N-glycosidase F and Endopeptidase H treatment**

For both Peptide-N-glycosidase F (PNGase F, Roche) and Endopeptidase H (Endo H, Roche) treatment, cells were harvested in lysis buffer (50 mM, Tris.HCl pH 7.4, 125 mM NaCl, 1% [w/v] SDS, 0.5% [w/v] Deoxycholate(Na), 0.1% Triton X-100, supplemented with Complete protease inhibitor and PhosStop [Roche]). Protein content was measured using the BioRad De Protein Assay (Bio-Rad). Dithiothreitol (20 mM) was added after protein measurement and lysates were denatured at 95°C for 5 minutes.

One volume of PNGase F mixture (50 mU per µg protein in 20 mM EDTA, 2% [v/v] Triton X-100 in PBS) was added to one volume of protein lysate and incubated at 37°C for 24 hours or as indicated.

One volume of Endo H mixture (125 µU per µg protein in 200 mM sodium citrate buffer pH 5.0, 20 mM EDTA, 2% [v/v] Triton X-100) was added to one volume of protein lysate and incubated at 37°C for 24 hours. EndoH reaction mixture pH was neutralized (NaOH) prior to SDS-PAGE sample preparation.

**Spot blotting**

One µg of protein per spot was applied on a dry nitrocellulose membrane (Whatman, Kent, UK). Visualization was performed with Lumi-Light Western Blotting Substrate (Roche) on a LAS-3000 imaging system (Fujifilm). Analyses of the spot blots were performed with Aida Image Analyzer (raytest Isotopenmessgeräte GmbH, Straubenhardt, Germany, version 4.26.038).

**Glycan determination**

The DIG (digoxigenin) Glycan Differentiation Kit (Roche) was used to determine glycan composition of the protein lysates, using the manufacturer’s protocol as a guideline. Briefly, dry nitrocellulose membranes were spotted with protein samples (1 µg per spot). Membranes were incubated with blocking solution, washed (tris buffered saline [TBS] pH 7.4) and equilibrated (1 mM MgCl₂, 1 mM MnCl₂ and 1 mM CaCl₂ in TBS pH 7.4), followed by lectin incubation in equilibration buffer: Galanthus nivalis agglutinin (GNA, 1 µg/mL), Sambucus nigra agglutinin (SNA, 1 µg/mL), Maackia amurensis agglutinin (MAA 5 µg/mL), peanut agglutinin (PNA, 10 µg/mL), and Datura stramonium agglutinin (DSA, 1 µg/mL), as supplied by the kit. For precise carbohydrate recognition see manufacturers manual (Cat. No. 11 210 238 007, version 16.0, November 2010). After lectin incubation membranes were incubated with a DIG antibody, followed by
secondary HRP-labeled antibody incubation (see table 1 for details on the antibodies). Chemiluminescence detection and analyses were performed as described in the SDS-PAGE and western blotting, and spot blotting sections.

**RNA isolation and cDNA synthesis**

The procedures for RNA isolation and cDNA synthesis are described previously\(^1\). Briefly, RNA was isolated using TRIzol Reagent according to the manufacturer's protocol (Invitrogen). cDNA synthesis was performed using a SuperScript II Reverse Transcriptase Kit (Invitrogen) on equal quantities of RNA between samples. Oligo(dT)\(_{12-18}\) primers (125 pmol) were used to prime mRNA poly-A tails.

**Real-Time qPCR**

The procedures for qPCR are described elsewhere\(^1\). Briefly, equal quantities of triplicate cDNA samples were dried in a 384 wells plate. qPCR reactions were performed in a LightCycler 480 system (Roche, Penzberg, Germany). Probe and primer combinations are listed in table 2. Results were analyzed using the LightCycler 480 software (version 1.5.0.39). Data are presented as mean±SD (n=3) from a representative experiment of three.

**Table 2. Primer and probe combinations for qPCR**

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Primers were prepared by Sigma. \(^1\)From Universal ProbeLibrary for Human probes (Roche).

**Statistical analysis**

Statistical analyses were performed using the unpaired two-tailed Student’s T-test and differences were accepted as statistically significant at a level of p≤0.05.
Results

To investigate the involvement of Rab6 in the ALP, we employed siRNA mediated knockdown in HeLa cells, resulting in efficient reduction of Rab6 levels as determined by qPCR, western blotting and immunofluorescence (suppl. fig. 1). As a measure of autophagy induction, the processing of LC3 was assessed on western blot and showed no difference in LC3-II/LC3-I ratio between control and Rab6 knockdown cells (fig. 1, Con). Also when the cells are exposed to autophagy inducing stressors —ER stress induced by tunicamycin (Tm) and nutrient stress induced by Earle’s balanced salts solution (EBSS)— the expected increased LC3-II/I ratio is not different between control and Rab6 knockdown cells (fig. 1, Tm and EBSS). These data indicate that reduction of Rab6 has no profound effect on the induction of autophagy.

Figure 1. LC3 processing is not affected by Rab6 knockdown. Rab6 was downregulated by siRNA in HeLa cells. Autophagy was induced by tunicamycin (Tm; 1µg/mL, 16 h) and Earle’s balanced salts solution (EBSS; 2 h). (A) LC3 processing was visualized by western blot. GAPDH was used as a loading control. A representative blot was shown. Con: control. (B) LC3-II/LC3-I ratios were quantified. Shown are normalized values (mean and SEM) from n=3 independent experiments.

To investigate the ALP downstream of autophagic induction, immunofluorescence using lysosomal markers was employed. Confocal microscopy demonstrates that reduced Rab6 levels result in a striking relocalization of lysosomes. This is observed with both the integral lysosomal membrane protein LAMP1 (fig. 2A) as well as with the luminal hydrolase Cathepsin D (fig. 2B), indicating that Rab6 affects the positioning of lysosomes. In Rab6 knockdown cells lysosomes cluster at a juxtanuclear location which is reflected by a reduction in convex hull by 21% (fig. 2C). Overexpres-
sion of a dominant negative mutant (T27N) of Rab6 also results in juxtanuclear relocalization of lysosomes, confirming that loss of functional Rab6 is responsible for this effect (suppl. fig. 2).

The endosomal markers Rab7 and EEA1 show no strong relocalization in Rab6 knockdown cells (fig. 3). The slightly more juxtanuclear pattern observed for the late endosome marker Rab7 in Rab6 knockdown cells may be explained by partial localization of this marker to lysosomes (fig. 3A). This is supported by the absence of difference between control and Rab6 knockdown cells for the early endosome marker EEA1, which does not co-localize with lysosomes (fig. 3B).

Figure 2. Rab6 downregulation induces lysosomal repositioning. Rab6 was knocked down by siRNA in HeLa cells. Immunofluorescence staining was performed to visualize lysosomes by membrane protein LAMP1 (A) and luminal protein Cathepsin D (B). The nuclei were stained with DAPI. Confocal imaging was performed. Scale bars indicate 10 µm. (C) The convex hull of the lysosomal structures was determined. Shown are mean and SD from n=3 independent experiments with each two fields of view. Statistical difference of p<0.001 is indicated by ***.
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HeLa cells treated with scramble and Rab6 siRNA were analyzed by immune-electron microscopy (EM; fig. 4). In the Golgi region of the HeLa cells, lysosomes show a typical lamellar structure for cells transfected with scrambled siRNA (fig. 4A). Immuno-EM analysis of Rab6 knockdown HeLa cells demonstrates the striking clustering of lysosomes as confirmed by LAMP1 and Cathepsin B double immuno-gold labeling (fig. 4B). In addition, lysosomes are morphologically altered; they are reduced in size (fig. 4B,C). These results from ultrastructural analysis not only strengthen the observation as seen under the confocal, it also adds information about the actual morphology of the lysosomes under Rab6 depletion in HeLa cells.

In conclusion our data indicate that loss of Rab6 function affects the morphology and positioning of lysosomes without affecting autophagic or endocytic pathways to lysosomes.

Rab6 itself does not colocalize strongly with lysosomes, therefore the role of Rab6 in the positioning of the lysosomes is likely to be indirect. Since Rab6 is implicated in maintaining the structural integrity of the Golgi, down regulation of Rab6 may
Figure 4. Rab6 knockdown induces clustering of small sized lysosomes. Electron micrographs of ultrathin cryosections of HeLa cells transfected with scramble (A) or Rab6 (B,C) siRNA. (A) Representative pictures of Golgi (G) region with typical lamellar lysosomes (L) labeled for LAMP1 (10 nm gold particles). (B) Golgi region of Rab6 knockdown cells showing a cluster of small sized lysosomes labeled for LAMP1 (10 nm gold particles) and cathepsin B (15 nm gold particles). (C) Cluster of small-sized, LAMP1-positive (10 nm gold particles) lysosomes in Rab6 knockdown cells. Bars indicate 200 nm.
affect glycosylation. We used different lectins to differentiate between different glycan modifications (fig. 5A). No major global effects on O- or N-glycosylation are observed. Cell surface binding to the fluorescent lectin WGA-AF488 is also not affected by Rab6 knock-down, confirming this result (fig. 5B). Also overall O-GlcNAc modification is not affected (fig. 5C). In contrast, we observed that in Rab6 knockdown cells, the mobility of LAMP1 and LAMP2 on SDS-PAGE is reduced (fig. 6). Removal of all N-glycans with peptide-N-glycosidase F (PNGase F) restores the mobility difference, indicating the N-glycosylation of LAMP proteins is different in Rab6 knockdown cells (fig. 6A). Endopeptidase H (Endo H) treated samples still show a mobility difference, indicating that Rab6 knockdown causes a change in the composition of the complex/hybrid N-glycans (fig. 6B, LAMP1 and LAMP2). Interestingly, Rab6 knockdown does not lead

**Figure 5.** Down regulation of Rab6 has no major effect on overall glycosylation. Rab6 was depleted in HeLa cells. (A) Cell lysates were analyzed for several carbohydrate moieties using different lectins on spot blots. GNA: *Galanthus nivalis* agglutinin, SNA: *Sambucus nigra* agglutinin, MAA: *Maackia amurensis* agglutinin, PNA: Peanut agglutinin, DSA: *Datura stramonium* agglutinin. (B) Non-permeabilized cells were probed with wheat germ agglutinin conjugated to an Alexa 488 fluorescent dye (WGA-AF488) and were analyzed by FACS and confocal imaging. (C) Spot blots with cell lysates were analyzed for O-linked N-acetyl-glucosamine (O-GlcNAc). Shown are results of representative experiments. Bars and error bars indicate mean and standard deviation of triplicates, statistical differences are indicated by * for p<0.05 and ** for p<0.01. Each spot represents 1 µg protein. AU: arbitrary units.
Figure 6. Depletion of Rab6 leads to a mobility shift of LAMP proteins. Rab6 was down regulated by siRNA in HeLa cells. (A) Cell lysates were treated with peptide-N-glycosidase F (PNGase F) for the indicated times and LAMP1 was visualized on western blot. (B) Cell lysates were treated with Endopeptidase H (Endo H) for 24 hours and LAMP1 and LAMP2 were visualized on western blot. Equal amounts of protein were loaded on each gel. Sc: Scramble knockdown, R6: Rab6 knockdown, kDa: kiloDalton.

Figure 7. Down regulation of Rab6 increases the proteolytic activity of lysosomes. Rab6 knockdown was performed in HeLa cells. Cells were treated for 4 h with DQ-BSA red (100 µg/mL). (A) Cells were visualized using epi-fluorescence microscopy. (B) DQ-BSA red fluorescent signal was measured by FACS analysis. Mean and SD are shown from a representative experiment of 4 independent experiments. (C) Degradation of lysosomal substrate p62 was assessed on western blot. A representative experiment is shown, the quantification shows normalized values (mean and SD) from n=3 independent experiments. GAPDH was used as a loading control. Statistical differences are indicated by ** for p<0.01.
Rab6 affects lysosomal positioning and function to differences in glycosylation of Cathepsin D, APP or LIMP-2, suggesting selectivity towards a subset of lysosomal membrane proteins (suppl. fig. 3).

To investigate whether the Rab6 knockdown has implications for lysosomal function, DQ-BSA was used to determine the proteolytic activity. Interestingly, Rab6 depletion is accompanied by increased degradation of the DQ-BSA substrate clearly visible by fluorescence microscopy (fig. 7A) and quantified by FACS analysis (fig. 7B). Because endocytic uptake is not increased by Rab6 knockdown (suppl. fig. 4A) this reflects an actual increase in lysosomal hydrolase activity. This is not caused by increased expression.

**Figure 8.** Rab6 downregulation impairs lysosomal repositioning during nutrient stress and ER stress. Rab6 was downregulated in HeLa cells with siRNA. (A) Cells were treated with Earle’s balanced salt solution (EBSS, 1 h). Lysosomes were visualized using LAMP1 as a marker. (B) Cells were treated with tunicamycin (1 µg/mL, 1 h). Lysosomes were visualized using LAMP1 as a marker. Confocal imaging was performed. Scale bars indicate 10 µm.
of lysosomal components as result of activation of the ALP transcriptional regulator TFEB (suppl. fig. 4B). The steady state levels of the endogenous ALP substrate p62 are decreased in Rab6 knockdown cells, supporting the observed increase in lysosomal activity (fig. 7C).

Repositioning of lysosomes to a juxtanuclear site has been demonstrated to occur during nutrient stress. This is also found in our cells after incubation in EBSS (fig. 8A, scramble). However, in Rab6 knockdown cells, the already juxtanuclear localization of the lysosomes is not changed during EBSS incubation (fig. 8A, Rab6 siRNA). A similar repositioning of lysosomes is observed during ER stress in HeLa cells, and again, this repositioning is disturbed in the Rab6 depleted cells (fig. 8B). Our data indicate that lysosomal repositioning is a common response to cellular stress that is mediated by Rab6.

Discussion

Our previous work suggested a role for Rab6 in ER proteostasis via proteolysis. Because the ALP is the major degradational pathway during ER stress and Rab6 functions via membrane bound compartments, we studied here whether Rab6 is connected to the ALP. The ratio of LC3-II to LC3-I is not affected by Rab6 knockdown at baseline or after autophagy induction by amino acid starvation or ER stress. This is an indication that the initiation of autophagy is not affected by Rab6, although differences in autophagic flux may not be reflected in the LC3-II/-I ratio. In fact, our own data indicate that autophagic flux is increased, as the degradation of the ALP substrate p62 is increased. In contrast, a striking effect on the distribution of lysosomes is observed. Reduction of Rab6 results in redistribution of lysosomes to a juxtanuclear site. This is observed both with the integral membrane protein LAMP1 as well as the luminal hydrolase cathepsin D, indicating this is a relocalization of lysosomes rather than missorting of a lysosomal marker protein. This is further supported by the observation that the late endosome marker Rab7 that also labels a subset of lysosomes is mildly affected, whereas the distribution of the early endosome marker EEA1 is not affected by Rab6 knockdown. The relocalization of the lysosomes is accompanied by increased proteolytic activity, indicated by a DQ-BSA activity assay and the above mentioned reduction in endogenous p62 levels.

Lysosomes are subject to a retrograde repositioning towards the MTOC during nutrient deprivation. We demonstrate that a similar repositioning event occurs during ER stress, which could indicate that lysosomal repositioning is part of a general response to stress situations that require increased lysosomal activity. Interestingly, our data indicate that the stress-induced repositioning of lysosomes is disturbed in Rab6 knockdown cells. It appears that the distribution of the lysosomes in Rab6 knockdown cells already resembles the lysosomal position during stress and is not further affected by exogenous stress. Although the exact function of this dynamic process during cellular stress is not fully elucidated yet, it was shown to play a role in the interaction of lysosomes with regulating signaling molecules, such as the mTORC1 complex. Rab6 knockdown is well tolerated, however, we showed previously that it impairs the recovery from an ER
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stress insult and increases the sensitivity to ER stress toxicity\(^{101}\), which would support a function for lysosomal repositioning in the protection against stress. Interestingly, Ryh1, the Rab6 homologue in S. pombe, was demonstrated to be involved in vacuolar integrity and stress resistance via a TORC2 dependent pathway, and defective Ryh1 function in this respect could be substituted by human Rab6\(^{276}\).

It seems paradoxical that the proteolytic activity of the lysosomes is increased in the Rab6 knockdown cells, where proteostasis is disturbed. However, this may be an ineffective compensatory response to the disturbed repositioning of lysosomes under stress conditions. A master regulator of lysosomal biogenesis is the transcription factor TFEB that is activated under conditions where lysosomal demand is increased, like starvation\(^{277,278}\). We find no evidence for activation of the TFEB induced upregulation of lysosomal biogenesis, indicating a different mechanism is involved in the increased lysosomal activity.

Because Rab6 itself does not colocalize with components of the ALP, the role of Rab6 in the positioning of the lysosomes is likely to be indirect. Rab6 is implicated in maintaining the structural integrity of the Golgi\(^{191,215}\) and active Rab6 is predominantly localized at the trans-Golgi network (TGN). Rab6 has been shown to be involved in several antero- and retrograde trafficking pathways\(^{206,208,238,239}\). Interestingly, Rab6 was shown to assist in the fission of vesicles from the Golgi, which may explain to some extent the broad range of Rab6 mediated trafficking pathways\(^{242}\). Although we cannot exclude that Rab6 has other yet unknown functions as well, it is likely that its role in lysosomal positioning is mediated by its function in vesicle transport. Rab6 knockdown results in cargo transport inhibition at the TGN, accompanied by increased number of Golgi cisternae\(^{216}\), which we also observe in our experiments. The transport defect fits with the presence of lysosomal proteins in the Golgi observed by immuno EM if Rab6 is depleted.

Protein glycosylation is specific for organisms, tissues and cell types\(^{279}\), and this may change in disease states such as cancer and inflammation\(^{279,280}\). The variety of individual N-glycans is vast and indeed glycosylation may present an information dense potential\(^{281}\). Adding to this complexity is so called microheterogeneity, which postulates that one particular glycosylation site on a protein may display a range of glycosylation variations, depending on tissue, cell type or even a particular biochemical state of the cell\(^{281}\). The ER and Golgi are major sites for glycosylation and, in addition, activated Rab6 is recruited to Golgi like structures. Biochemically, Rab6 depletion induces glycosylation differences in a subset of lysosomal proteins. Glycosylation differences are found for LAMP1 and 2, but not for LIMP-2 or Cathepsin D. The mobility shift of LAMP1 and 2 proteins to a higher molecular weight is corroborated by Sun and colleagues: In fact, if anything, as indicated by the slightly retarded migration of LAMP2 in the Rab6 siRNA case, glycosylation was promoted\(^{214}\). Our results strongly suggest that the alteration relates to complex N-glycosylation opposed to high mannose N-glycosylation. This may be caused by addition of entire N-glycan carbohydrate chains or addition/modification of specific carbohydrate moieties on these chains. It was reported previously that polylactosamine modification of LAMP1 and 2 is increased if the retention time in the Golgi is longer\(^{282}\).
We hypothesize that dysfunction of Rab6 leads to altered retention of LAMP proteins or factors that involve glycosylation within this cellular compartment, ultimately affecting the glycosylation of LAMP proteins.

The exact function of LAMP proteins in lysosomal function is not known yet, but it has become clear that the LAMP proteins have overlapping functions that go beyond a structural protein in the lysosome. Knockout of LAMP1 or LAMP2 is well tolerated. However, double knockout of LAMP1 and LAMP2 is embryonic lethal. Interestingly, the proteolytic function of the lysosomes is not affected in the double knockout cells. However, in the LAMP1/LAMP2-/- cells the lysosomes are larger and have a more peripheral localization, which seems to be opposite to the lysosomal phenotype in the Rab6 knockdown cells. It is tempting to speculate that the change in LAMP glycosylation induced by Rab6 is functionally connected to the lysosomal relocalization, different morphology and increased proteolytic activity, but this requires further investigation.

Mechanistically, the role of Rab6 in ALP function may involve as yet unknown regulatory pathways. For example Rab12 was recently shown to regulate autophagy via targeting the amino-acid transporter PAT4 for lysosomal degradation. Our previous work indicated that the membrane association of Rab6 is dependent on presenilin 1, mutations in which cause autosomal dominant variants of AD. Recently presenilin was shown to affect the acidification of the lysosomes and thereby lysosomal function.

The data in this study further underscore the importance of the ALP to restore proteostasis during UPR activation. Therefore, the paradoxical observation that the UPR activation is not resolved by the high Rab6 levels in AD neurons, may relate to the dysfunction of the ALP as observed early in AD pathogenesis. Indeed, in human AD hippocampus, the UPR activation is prominently present in neurons that have disturbances in the ALP. The current study contributes to accumulating evidence that dysfunction of the lysosomal system is a major player and possible target in AD.

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Supplementary figures

Supplemental figure 1. Rab6 is efficiently downregulated in HeLa cells with siRNA. HeLa cells were transfected with scramble and Rab6 siRNA for 72 hours. (A) Rab6 mRNA expression was analyzed by qPCR. Shown are normalized values (mean and SD) of n=5 independent experiments. (B) Rab6 protein expression was analyzed on western blot. Shown is a representative blot and GAPDH was used as a loading control. (C) Rab6 protein expression was analyzed by immunofluorescence. Nuclear counterstain was performed with DAPI. Scale bar indicates 10 µm.

Supplemental figure 2. Dominant negative Rab6 (T27N) induces lysosomal repositioning. Myc-tagged Rab6 wildtype (Wt) and T27N were overexpressed in HeLa cells. Rab6 Wt and -T27N were visualized using the Myc-tag. Lysosomes were visualized with LAMP1. DAPI was used for the nuclear counterstain. Confocal imaging was performed. Scale bar indicates 10 µm. Mock: Empty vector.
Supplementary figure 3. Rab6 selectively affects the glycosylation of LAMP family proteins. Rab6 was down regulated in HeLa cells using siRNA. Cell lysates were analyzed for mobility shifts on western blots in three glycoproteins: cathepsin D (A, CathD), amyloid precursor protein (B, APP) and lysosome integral membrane protein 2 (C, LIMP-2). In addition, cell lysates treated with PNGase and Endo H (24 hours) were analyzed on western blot for LIMP-2 (C). Sc: Scramble, R6: Rab6 siRNA, CathD: cathepsine D, APP: amyloid precursor protein, kDa: kiloDalton.

Supplementary figure 4. Increased lysosomal activity by Rab6 depletion is not caused by increased endosomal uptake or TFEB target upregulation. Rab6 was down regulated in HeLa cells with siRNA. (A) Cells were treated for 4 h with Dextran\textsuperscript{MW40000} 488 (20 µg/mL). (A) The fluorescent signal of Dextran\textsuperscript{MW40000} 488 was measured by FACS analysis. Mean and SD are shown from a representative experiment of 4 independent experiments. (B) Quantitative PCR was performed for the targets of the TFEB transcription factor, LAMP1, cathepsine B (CathB) and cathepsine D (CathD). Normalized values are shown (mean and SD, experimental triplicates) from n=5 of independent experiments and EEF1A was used as a reference. Statistical difference is indicated *** for p< 0.001.