Investigations on the role of impaired lysosomes of macrophages in disease

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Chapter 2

Glucocerebrosidase in cultured cells: extreme sensitivity for medium conditions

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Abstract
Glucocerebrosidase (GCase) is the lysosomal acid β-glucosidase encoded by the GBA gene that degrades the ubiquitous glycosphingolipid glucosylceramide. Inherited GCase deficiency causes the lysosomal storage disorder Gaucher disease. In addition, carriers of an abnormal GBA allele are markedly increased at risk for Parkinson's disease. Newly formed GCase is known to undergo extensive modifications in its four N-glycans en route to and inside the lysosome. These glycan modifications are reflected in changes in apparent molecular weight (MW) of GCase as detected with SDS-PAGE. Fluorescent activity-based probes (ABPs) have been generated that covalently label GCase in reaction-based manner in vivo and in vitro and thus allow sensitive visualization of GCase molecules. Using these ABPs, we studied the life cycle of GCase in cultured fibroblasts and macrophage-like RAW264.7 cells. Specific attention was paid to the impact of the medium. We here report that the pH of culture medium, buffered with compounds such as HEPES, markedly influences processing of GCase and the total cellular enzyme level. The implications of our findings for diagnosis of GD based on measurement of cellular enzyme activity in lysates of cultured cells are described and discussed.

Introduction

Glucocerebrosidase (GCase) is the lysosomal acid β-glucosidase that degrades glucosylceramide (GlcCer). Inherited defects in the *GBA* gene encoding GCase cause the lysosomal storage disorder Gaucher disease (GD).[1,2] More recently, mutations in *GBA* have been shown to pose a marked risk factor for developing Parkinson's disease and Lewy-body dementia, even upon haploinsufficiency.[3,4] A hallmark of GD is lysosomal accumulation of GlcCer in tissue macrophages.[5,6] The lipid-laden macrophages (Gaucher cells) are viable cells and are thought to contribute to the characteristic visceral GD symptoms such as hepatosplenomegaly, thrombocytopenia and anemia.[2,5] Most GD patients do not develop prominent complications in the central nervous system and are designated as type 1. More severe GD cases (type 2 and 3) may develop lethal neurological symptoms and skin abnormalities.[1] With respect to the non-neuronopathic type 1 GD, two types of therapy are currently available: enzyme replacement therapy (ERT), by means of a macrophage-targeted recombinant enzyme, and substrate reduction therapy (SRT), which utilizes inhibitors of GlcCer biosynthesis.[7–12] Both approaches lead to impressive corrections in organomegaly and pancytopenia, which is preceded by corrections in plasma biomarkers of Gaucher disease.[13] The value of alternative therapy for GD is currently being studied, with a focus on gene therapy and a chaperone/activator mediated approach to augment mutant enzyme in patients.[12,14–16]

The availability of effective therapies has boosted the interest in laboratory diagnosis of GD, including (newborn) screening programs.[17,18] Nowadays, the first step in diagnosis is detection of abnormalities in the GBA gene by genome sequencing. Demonstration of impaired GCase activity is subsequently performed by enzyme activity measurement, often by use of the fluorescent substrate 4-methylumbelliferyl-β-glucoside (4MU-β-Glc).[2,13] For this purpose, dried blood spots, white bloods cells and fibroblasts are all used, depending on the laboratory. Specific inhibitors may help to discriminate GCase and that of other β-glucosidases (GBA2 and GBA3) with respect to their contribution to the total activity.[13] Unfortunately, neither genotyping nor the measurement of residual GCase activity in cell lysates accurately predicts onset and progression of GD in individual patients.[19] Although heteroallelic presence of N370S GBA in GD patients is always associated with a non-neuropathic pathology, the disease course may vary markedly in severity, even among siblings.[19,20] Moreover, monozygotic GD twins with different disease severity have been documented.[21,22] It has been speculated that modifier genes and toxic secondary metabolites contribute to the variability in outcome of GCase abnormalities.[12] Onset of GD disease can be sensitively detected by demonstration of elevated Gaucher cell marker proteins in plasma, like chitotriosidase, CCL18 and GPNMB as well as the lipid glucosylsphingosine.[23–25] However, accurate assessment of true residual GCase activity would greatly improve diagnosis and monitoring of GD. Novel cell permeable fluorogenic substrates for *in situ* measurement of GCase activity in cultured cells have recently been developed.[26,27] Other recent tools to detect active GCase molecules *in situ* are fluorescent cyclophellitol-based activity bases probes (ABPs).[28,29] These cell permeable cyclophellitol analogues act as suicide inhibitors that selectively react with GCase by covalent and irreversible binding to its catalytic nucleophile, E340. ABP-labeled GCase molecules can be visualized by microscopy and gel electrophoresis.[28,29] These novel tools allow in depth study of GCase and of factors that could impact on its function.

GCase is synthesized as 497 aa polypeptide containing 4 N-linked glycans.[2] The initially formed enzyme has a molecular weight of 62kDa that subsequently increases to 66-69 kDa by modification of its glycans to sialylated complex type structures.[30] Inside the lysosomes, the local action of neuraminidase, β-galactosidase and β-hexosaminidase cause stepwise reduction to the 58 kDa ('mature') isoform (see also Figure 1C).[31] Although the precise composition of the N-glycans does not impact catalytic activity, N-glycans are essential for correct folding of newly synthesized enzyme molecules in the endoplasmic reticulum.[2] Unlike most other lysosomal hydrolases, GCase in fibroblasts does not acquire mannose-6-phosphate moieties but is transported to lysosomes via binding to LIMP-2 (lysosomal integral membrane protein-2).[32–34] The GCase/LIMP-2 complex is sorted to lysosomes and dissociates upon low luminal pH.[35] Early pulse-chase experiments in fibroblasts showed that [^{35}S]methionine-labeled GCase requires considerable time (several hours) to reach mature lysosomes, where it is degraded by leupeptin-sensitive proteases.[36] GCase is already folded into its active conformation in the ER, as can be detected with fluorogenic substrate and ABP labeling. Therefore, the measured GCase activity in cell lysates does not necessarily reflect actual enzyme capacity in lysosomes.

Recently, we reported how medium conditions impact on lysosomes by using HEPES to buffer the pH of the culture medium.[37] By exploiting the GCase-specific ABPs, we demonstrate that GCase is particularly influenced by medium conditions. The presence of HEPES in the culture medium impaired maturation and reduced proteolytic turnover of GCase in abnormal lysosomes. This eventually resulted in an increase in overall cellular enzyme levels. The implications for diagnosis of GD based on measurement of cellular enzyme activity in lysates of cultured cells are described and discussed.

Results

Impact of medium pH on cellular GCase glycoforms

Murine macrophage-like RAW264.7 cells and human skin fibroblasts were cultured in DMEM and DMEM/F12 medium respectively, at 7% CO2. Different buffers were added to the medium at a final concentration of 50 mM: MES (2-(N-morpholino) ethanesulfonic acid) (pKa = 6.15) or HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) (pKa=7.5). The final medium pH was 7.0, and 7.4 respectively. After a week of culture, cells were harvested and lysed in KPi-buffer supplemented with 0.1% Triton X-100. Active GCase molecules in cell lysates were labelled with fluorescent ABP ME569 and were analysed by SDS-PAGE (**Figure 1**). The cellular abundance of GCase and the GCase glycoform profile was found to be clearly influenced by the medium composition. In fibroblasts and RAW264.7 cells cultured at lower pH, less GCase was labelled with ABP (**Figure 1A**), and less GCase activity was detected (**Figure 1B**). In cells cultured at pH 7.4, in the presence of HEPES, GCase was more abundant, in particular glycoforms with MW of 62-66 kDa (**Figure 1A**). PNGase digestion resulted in the generation of a 52 kDa labelled protein in lysates of both MES and HEPES exposed cells, which confirms that all labelled enzyme is GCase and various MW forms stem from differences in glycans (**Figure 1C**). We also studied cells that were exposed to 50 mM MOPS (3-(N-morpholino) propanesulfonic acid; pKa = 7.15), which buffered the final medium pH at 7.15. As shown in **Supplemental figure 1**, cells cultured at pH 7.15 showed an intermediate GCase profile when compared to the glycan isoform profile of cells cultured at higher and lower medium pH.

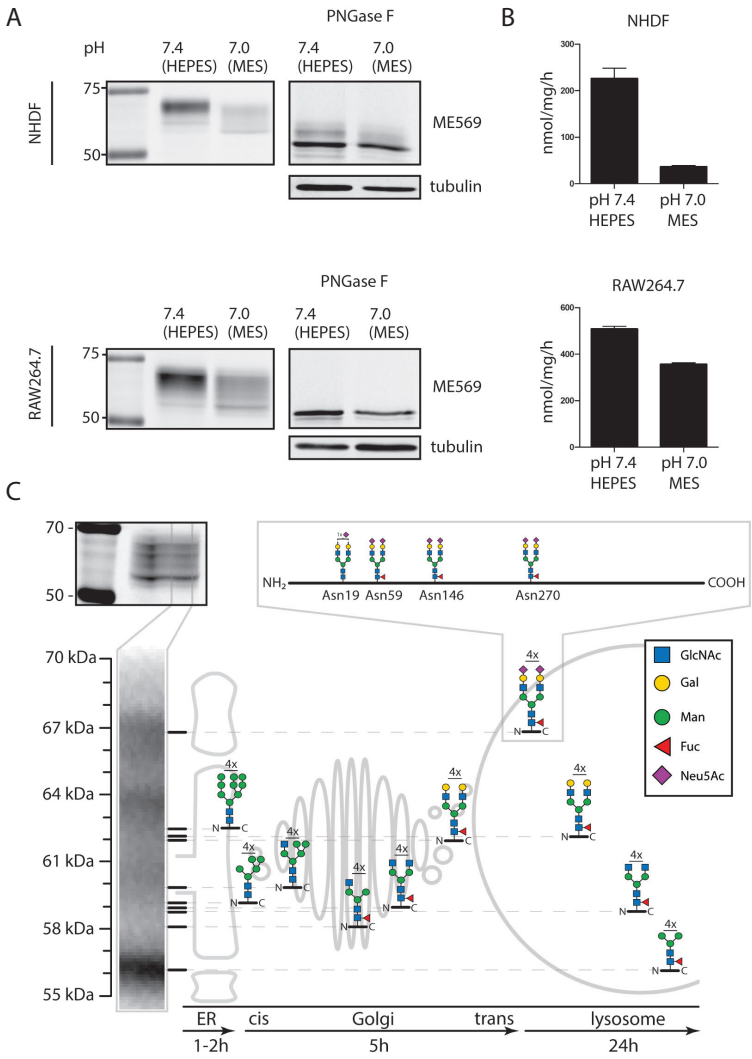


Figure 1. Impact of medium on cellular GCase glycoforms. ***(A)*** *GCase in lysates of skin fibroblasts (NHDF) and RAW264.7 cells was labelled with GCase-specific ABP and subsequently visualized by fluorescence scanning after SDS-PAGE. Labeled GCase was digested with PNGase F to remove N-glycans, as described in M&M.* ***(B)*** *GCase of the same lysates of skin fibroblasts (NHDF) and RAW264.7 cells was measured with 4MU-β-Glc substrate as described in M&M.* ***(C)*** *Scheme depicting processing of GCase glycoforms (adapted from Aerts, thesis).*

Dynamics of induced changes in GCase by medium pH

The induction and reversibility of changes in cellular GCase induced by culture medium composition was investigated more closely. For this, cells (fibroblasts and RAW264.7 cells) were exposed to culture medium containing 50 mM HEPES (medium pH 7.4). In both cell types, GCase with higher MW (reflecting enzyme species with more sialylated complex glycans) accumulated and an increase in overall GCase activity was detected over time (**Figure 2A**). Next, the reversibility of the induced changes in GCase was examined.

Cells were first exposed to medium containing 50 mM HEPES for 3 days. Subsequently, cells were washed and further cultured in the presence of 50 mM MES (medium pH 7.0). At different time points (0-96 hours), cells were harvested and cellular GCase was studied by ABP-labeling and SDS-PAGE, as well as by enzymatic activity measurements (**Figure 2B**). Exposure to a lower medium pH caused a reversal of GCase glycoform profile, which was accompanied by reduced total cellular enzymatic activity (**Figure 2B**). Of note, the correction of GCase in fibroblasts proceeded slower than that in RAW cells (**Figure 2B**). Brightfield microscopy analysis of RAW264.7 cells showed a prominent change in vacuolar morphology upon HEPES induction, which could be reversed through subsequent and prolonged replacement by MES (**Supplemental Figure 2**).

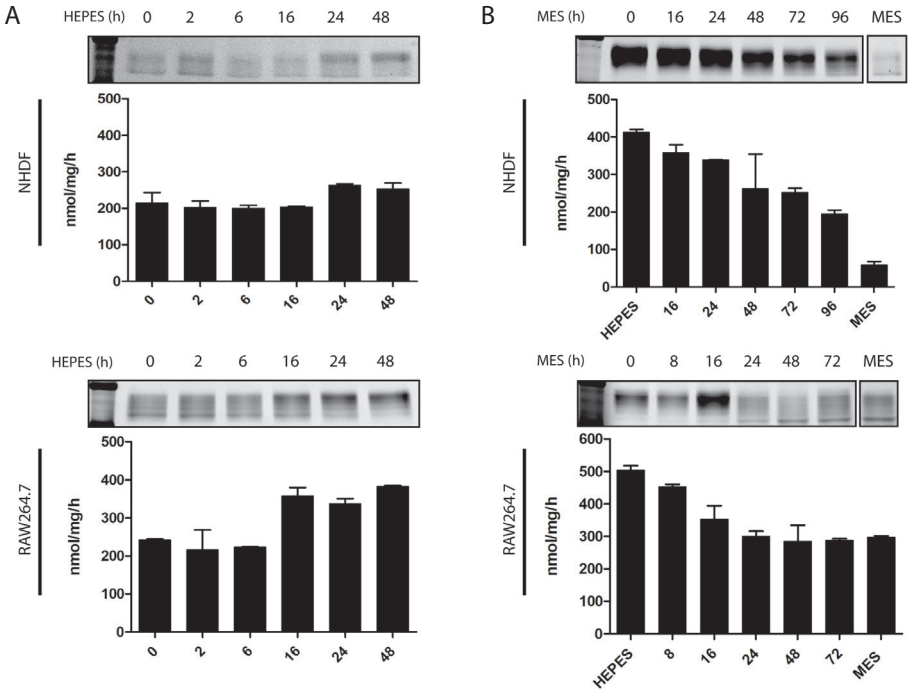


Figure 2. Induction and reversibility of GCase changes by HEPES-containing medium. ***(A)*** *Induction. Skin fibroblasts (NHDF) and RAW264.7 cells were exposed to either 50 mM HEPES or MES, and cellular GCase was monitored in time (0-48 hours) by means of ABP labeling of enzyme in cell lysates and the measurement of enzymatic activity in lysates.* ***(B)*** *Reversibility. Skin fibroblasts (NHDF) and RAW264.7 cells were exposed for 3 days to 50 mM HEPES in the culture medium (pH 7.4). Following washing, cells were cultured in medium containing 50 mM MES (medium pH 7.0), and cellular GCase was monitored in time (0-96 hours) in cell lysates by means of ABP labeling of enzyme molecules and measurement of GCase activity. The last lane to the right represents cells chronically cultured in in the presence of 50 mM MES.*

Life cycle of GCase visualized with ABPs

Two earlier published GCase-specific ABPs conjugated with green and red boron dipyrromethene (BODIPY), MDW933 and MDW941 respectively [28], were used to perform a pulse-chase experiment. Cultured fibroblasts and RAW264.7 cells were first exposed to 100 nM cell permeable MDW933 overnight to irreversibly label all active GCase molecules. Next, cells were extensively washed and subsequently cultured in the presence of red fluorescent MDW941. Detection of MDW941-labeled enzyme allows selective monitoring of *de novo* synthesized GCase in time. The pulse-chase experiments were performed with fibroblasts and RAW264.7 cells cultured in medium containing either 50 mM HEPES or 50 mM MES. Cells were harvested at different time points during the chase period (0-96 h) and cellular GCase was analyzed by SDS-PAGE (**Figure 3**). Incubation of RAW264.7 cells with the red MDW941 probe for extended periods of time resulted in complete labeling of GCase and its inactivation as measured with 4MU-assay, whereas GCase activity was restored 96h after removal of green MDW933 without the MDW941 chase (**Supplemental figure 3**).

During the chase period, MDW941-labeled GCase increased in both types of cells cultured in both media, indicating sustained synthesis of GCase during the various chases (**Figure 3**). Cells cultured with HEPES did not show the transition of 66 kDa GCase to 58 kDa enzyme, a process known to depend on stepwise removal of external sugars from the N-glycans.[31] In contrast, cells cultured with 50 mM MES at pH 7.0 did show detectable formation of 58 kDa GCase after one day of chase (**Figure 3**).

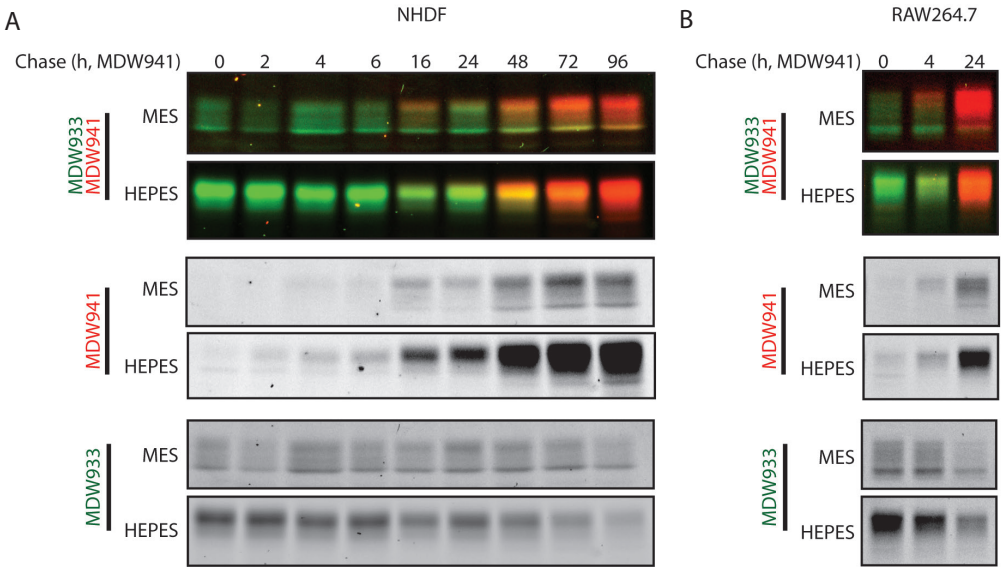


Figure 3. Visualization of GCase isoforms with two distinct ABPs: reduced glycan maturation in HEPES-containing medium. ***(A)*** *Pulse-chase experiments with fibroblasts, performed as described in M&M. Following pre-labeling with MDW933 (green fluorescent, existing GCase), cells were incubated continuously with MDW941 (red fluorescent, newly synthesized GCase) for indicated time periods. Cells were harvested and labeled GCase was visualized following SDS-PAGE.* ***(B)*** *Same experimental set-up was used for GCase studies in RAW 264.7 cells.*

Subcellular localization of GCase in cells cultured in the presence of different buffers.

Immunofluorescence analysis of fibroblasts revealed a punctate pattern for ABP-labeled GCase when cultured in presence of HEPES and MES, that similarly overlapped with immunocytochemically labelled LIMP2, the transporter of GCase (**Figure 4A**). No marked overlap was observed for GCase with the ER protein Calnexin, the Golgi marker Giantin and the early endosome marker EEA1 (Early Endosome Antigen 1) in cells cultured in HEPES-supplemented medium (**Figure 4B**). Localization of labelled GCase was however associated with that of the (endo)lysosome marker LAMP1 (**Figure 4B**). Of note, an increase in LAMP1 positive punctae was observed in fibroblasts cultured in the presence of HEPES, which suggests that more (endo)lysosomes exist upon exposure to HEPES-containing medium (**Supplemental figure 4**).

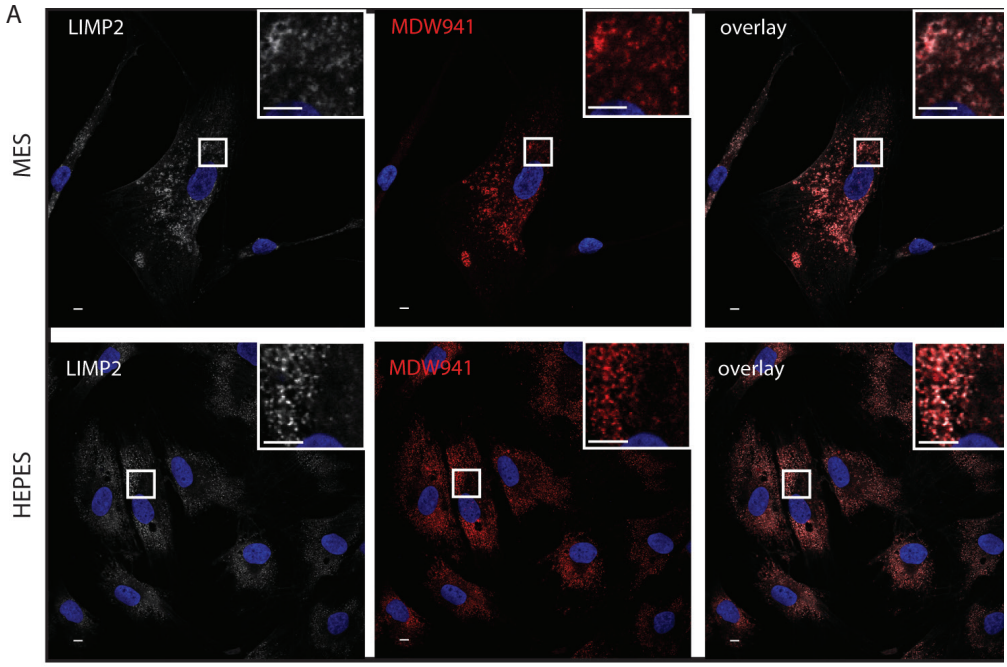
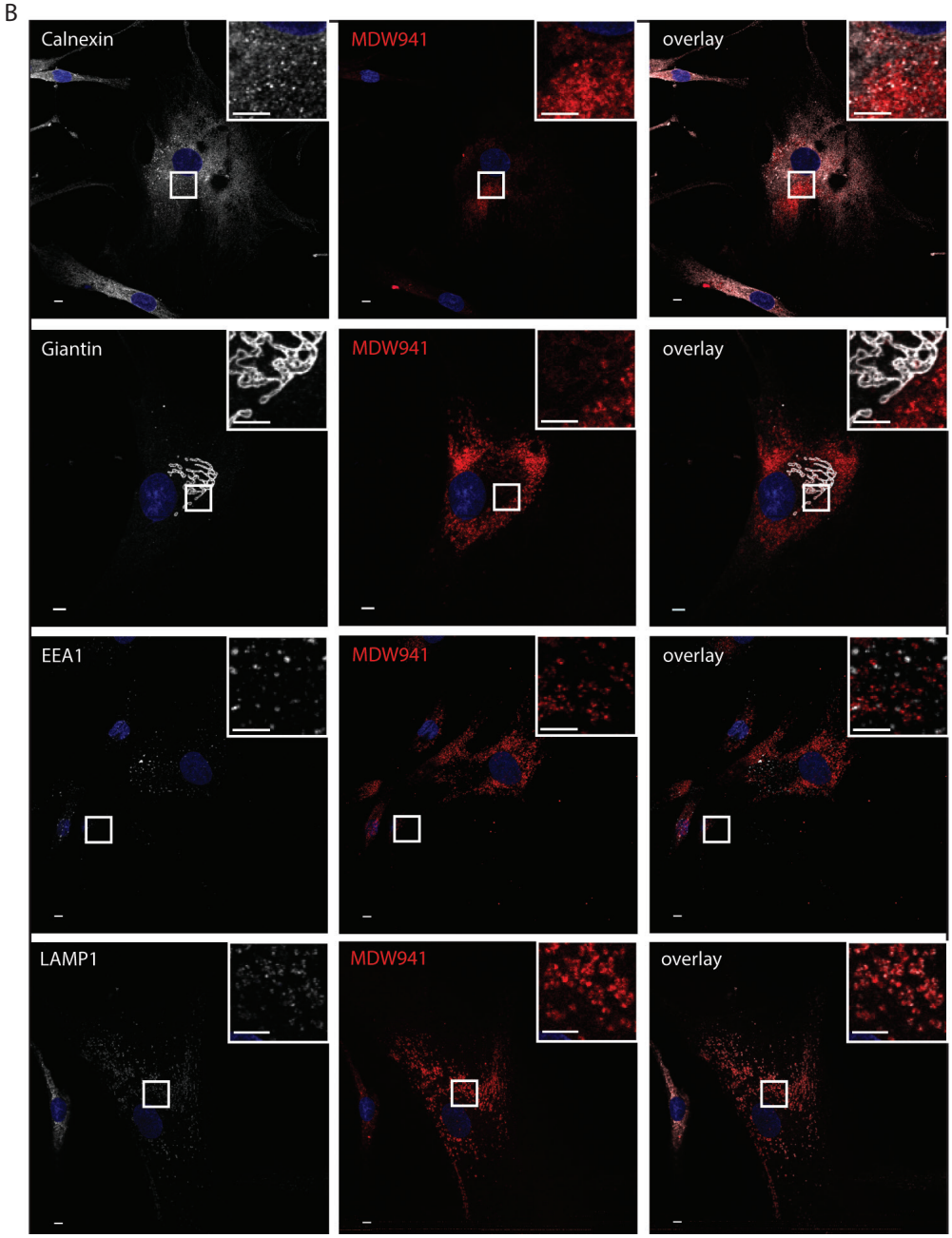


Figure 4. GCase colocalizes with LAMP1- and LIMP2 in HEPES treated fibroblasts. *Cells were pretreated ABP MDW941 to label GCase and subsequently fixed and analyzed by immunohistochemistry to establish overlap of GCase with other marker proteins. **(A)** Visualization of GCase and LIMP2 in fibroblasts cultured in the presence of MES or HEPES. (**B**; **next page ->**) Visualization of GCase and Calnexin, Giantin, EEA1 and LAMP1 in fibroblasts cultured in the presence of HEPES. Scale bar: 10 µm*

Lysosomes are dense organelles and can be separated from ER and Golgi structures based on density difference. Subcellular fractionation was used to separate compartments by means of a continuous Percoll density gradient, as described in M&M. In gradient fractions, the enzyme activities of GCase and the lysosomal enzyme β-hexosaminidase were determined. In the case of MES-exposed RAW264.7 cells, GCase and β-hexosaminidase activities were detected in fractions with high density. These fractions are known to contain mature dense lysosomes (**Figure 5**). GCase β-hexosaminidase activity was virtually absent

in highly dense fractions of cells exposed to HEPES. The lysosomal enzyme activities in compartments with lower density reflect enzymes in pre-lysosomal compartments (ER, Golgi, endolysosomes and immature lysosomes). Similar observations were made for fibroblasts (**Supplemental figure 5**). These findings indicate that HEPES-exposed cells contain on average less dense lysosomes compared to MES-conditioned cells.



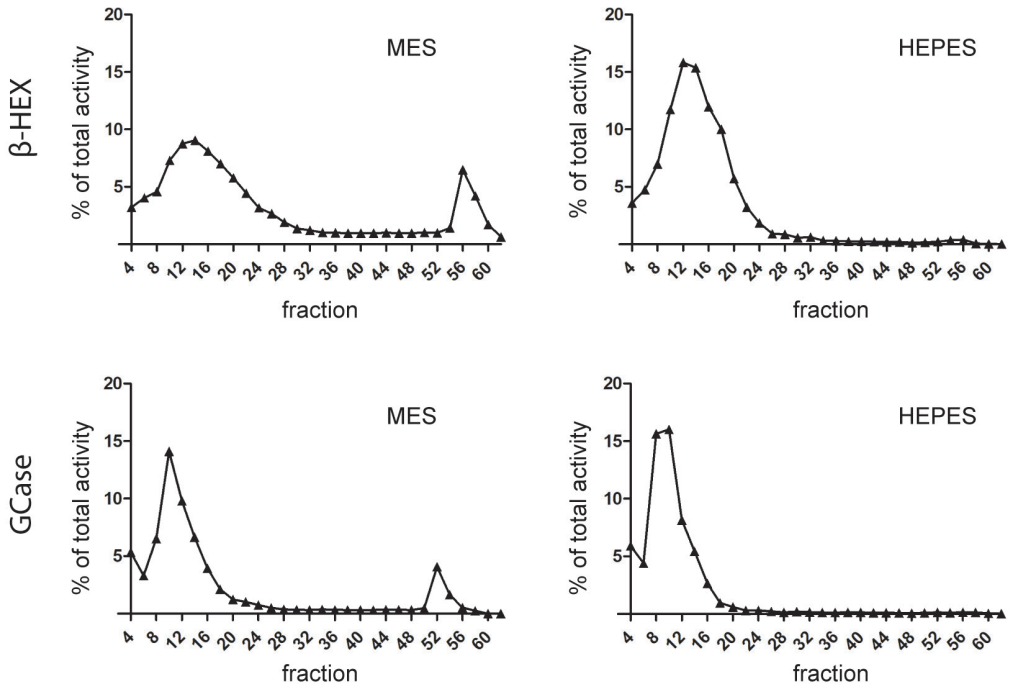


Figure 5. Subcellular fractionation of fibroblasts cultured in the presence of 50 mM HEPES or MES. *Homogenates of RAW264.7 cells treated with HEPES or MES were fractionated and compartments were separated on the basis of density using 49% Percoll centrifugation to generate density gradients. In collected fractions enzymatic activities of GCase and β-hexosaminidase were measured as described in M&M.*

Intralysosomal proteolytic degradation of GCase is known to be potently inhibited by leupeptin, a broad protease inhibitor.[36,38] Consequently, leupeptin induces accumulation of mature 58 kDa GCase upon complete maturation of GCase. The impact of leupeptin on GCase in cells (fibroblasts and RAW264.7) cultured in the presence of MES, MOPS and HEPES was examined. The presence of leupeptin led to accumulation of 58 kDa GCase (**Figure 6A**) and overall GCase activity (**Figure 6B**) in cells in cells cultured with MES and MOPS but not those with HEPES. Thus, even in the presence of leupeptin, HEPES-treated cells do not form the mature 58 kDa form of GCase. This suggests that the machinery leading to the formation of 58 kDa GCase, likely exo-glycosidase activity, is perturbed in these cells.

In summary, the microscopy analyses (**Figure 4**), the outcome of subcellular fractionation experiments (**Figure 5**), and the noted effects of leupeptin (**Figure 6**) all rendered data consistent with the hypothesis that the presence of HEPES in the culture medium affects normal maturation of lysosomes to dense structures, and instead promotes formation of compartments with lower density that contain altered activity of glycosidases and proteases.[37] GCase undergoes prominent trimming of its N-glycans by glycosidases and is degraded relatively rapidly by proteases in mature lysosomes.

Therefore, it is not surprising that GCase is highly sensitive to the presence of HEPES in the medium which causes cellular accumulation of the 66 kDa enzyme.

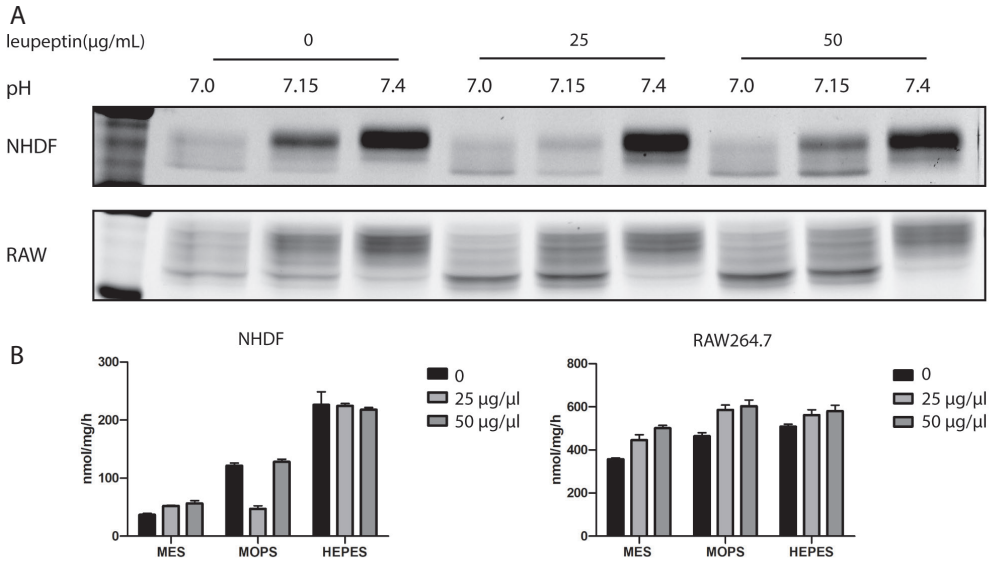


Figure 6. Inhibition of lysosomal cathepsins in increases GCase in cells exposed to MES and MOPS, but not those exposed to HEPES. *Cells (fibroblasts and RAW264.7) were cultured in the presence of 50 mM buffer compound (MES, MOPS or HEPES) in the absence or presence of 0, 25 or 50 µg/mL leupeptin for 48 hours. Cells were harvested and GCase in lysates was visualized by* ***(A)*** *ABP labeling, SDS-PAGE and fluorescence scanning, or* ***(B)*** *by enzymatic GCase activity measurements (as described in M&M).*

Implications for diagnosis of GD using cultured cells.

The use of culture medium with added HEPES is increasingly popular because it allows stable buffering of medium for extended amount of days. Here we show that in cultured cells the enzyme GCase is particularly influenced by the use of this buffer. This has repercussions regarding measurement of enzyme activity levels for diagnostic purposes. **Figure 7** shows the GCase levels (nmol/mg protein/hour) in lysates of fibroblasts from type 1 Gaucher disease patients and normal individuals cultured in the presence of HEPES and MES. The levels for lysates of cells from some patients cultured in the presence of HEPES overlap with values in lysates of cells from normal individuals cultured in the presence of MES. Culturing patient and control cells at different conditions might result in false negatives in GD diagnosis. It also was observed that glucosylsphingosine levels in Gaucher patient-derived fibroblasts were increased when cells were cultured in the presence of 25 mM HEPES, a finding that again points to reduced lysosomal GCase activity under this condition (**Supplemental Table 1**).

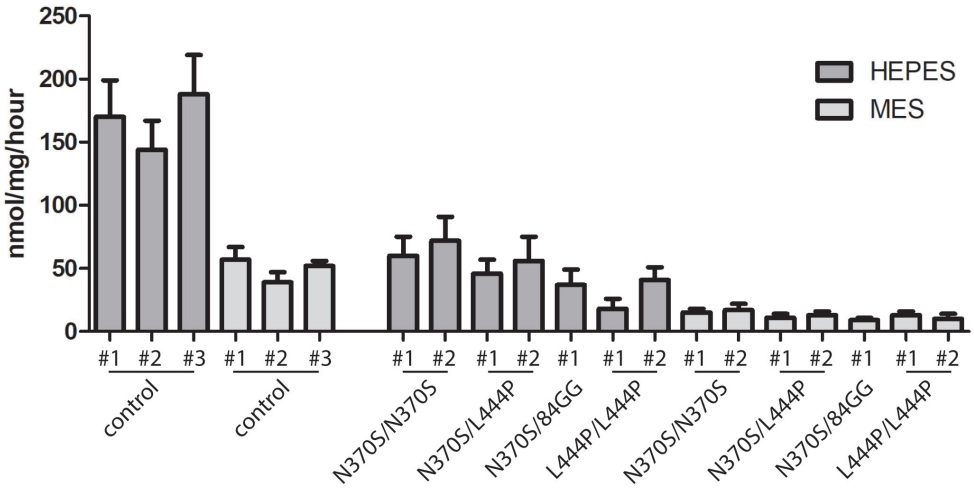


Figure 7. GCase activity level in control and Gaucher fibroblasts cultured in the presence of 25 mM MES or HEPES. *Values expressed a mean ± SD; measurements performed in triplicate.*

Impact of medium on other lysosomal glycosidases

Selective ABPs are nowadays also available for a number of other lysosomal retaining glycosidases like the α-glucosidases (GAA) and β-glucuronidase (GUSB).[39,40] We examined the impact of the culture medium buffers on these enzymes using corresponding ABPs for visualization. **Figure 8** shows that in fibroblasts cultured in the presence of HEPES at a medium pH of 7.4, the ratio of intermediate and mature GAA is altered, pointing to perturbed lysosomes. Likewise, an increase in the intermediate form of GUSB (75 kDa) and a decrease in the mature form (65 kDa) was noted in cells cultured in the presence of HEPES. Proteolytic processing of 95 and 76 kDa GAA and 75 kDa GUSB is thought to largely take place in lysosomes. The findings therefore suggest that the involved proteases in this processing are less active. This explanation was substantiated by the finding that leupeptin treatment did not cause an increase in mature 65 kDa GUSB in fibroblasts cultured in the presence of HEPES (**Supplemental figure 6**). Of note, the intermediate 75 kDa GUSB was increased in cells cultured in the presence of HEPES (**Supplemental figure 6**).

Discussion

In recent times, attractive new tools have become available for investigations on GCase in intact cells. For the purpose of GCase visualization, cell permeable fluorescent activity-based probes have been designed that covalently bind to the catalytic nucleophile E340 of active GCase molecules with extreme specificity, allowing their convenient visualization in intact cells.[28] Based on earlier observations with irreversible inhibitors, the attachment of a bulky group to the pseudo C6 position in substrates confers specificity for GCase over GBA2 and GBA3.[28,29,41,42]

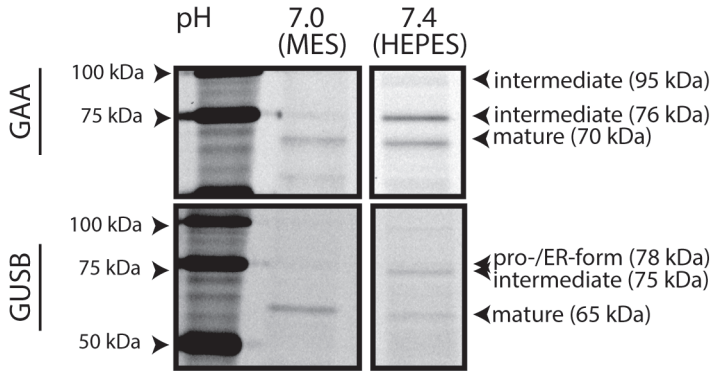


Figure 8. Impact of medium pH on alpha-galactosidase (GAA) and beta-glucuronidase (GUSB) isoforms. *Fibroblasts were cultured in the presence of 50 mM buffer compound (MES or HEPES). Cells were harvested and GAA and GUSB in lysates was visualized by ABP labeling, SDS-PAGE and fluorescence scanning.*

Additional novel GCase-specific substrates have been designed based on this principle, including the cell-permeable fluorescence-quenched substrates for *in situ* measurement of GCase activity[26], or the resorufin substrates for *in vitro* GCase activity and kinetic analysis.[27] Many investigations on GCase make use of cultured cells. Earlier work in our lab revealed that culture conditions may impact on GCase in cells, in particular the addition of the increasingly popular buffer HEPES to culture medium. HEPES maintains the medium at the relative high pH of 7.4.[37] Our present investigation illustrates the marked impact on cellular GCase of the presence of HEPES in the culture medium, both qualitatively and quantitatively.[43] In cells, fibroblasts and macrophage-like RAW264.7 cells alike, exposure to HEPES containing medium causes GCase to steadily accumulate. The accumulating enzyme shows a MW of about 66 kDa, which suggests an abundance of complex-type sialylated glycans. The subsequent intralysosomal conversion to a 58 kDa glycoform by trimming of N-glycans does not seem to occur in cells that are exposed to HEPES. Also, an overall increase in the 66 kDa isoform of GCase is observed in these cells. The observed reduction in lysosomal glycan processing increased abundance of GCase could theoretically be caused by an arrest of the enzyme in the trans-Golgi region. However, microscopy revealed that GCase in HEPES-exposed cells is present in LAMP1-positive compartments and does not overlap in location with Giantin, a Golgi marker and the early endosome marker EEA1. This suggests that the enzyme has passed the Golgi apparatus and does not accumulate in early endosomes. The subcellular distribution of LIMP2 and GCase were also found to overlap in cells cultured in the presence of HEPES. At present, it seems most attractive to assume that mature lysosomes acquire a higher pH upon uptake of HEPES, consequently exhibit lower density and have reduced hydrolase capacities. These include the activities of glycosidases and proteases involved in GCase N-glycan trimming and proteolytic degradation. However, additional explanations cannot be entirely excluded yet. The relatively high medium pH might impact on cytosolic pH, which could in turn impact on lysosome acidification. Indeed, it has been recently reported that STAT3 can promote lysosome acidification via direct interaction with the v-ATPase. The cytosolic pH was found to play a key role in the association of STAT3 with lysosomes.[44] In light of this, recent work by Grinstein and colleagues has

revealed the existence of two pools of lysosomes in cells: highly acid and lytic, perinuclear ones, and less acidic peripheral ones.[45] The two types of lysosomes are similar in v-ATPase composition but nevertheless fundamentally differ in luminal pH.[45] Cytosolic pH has earlier been reported to impact on location of lysosomes in cultured macrophages.[46] Acidification of the cytosol by the presence of acetate in medium was found to cause movement of lysosomes to the cell periphery.[46] At present, we have no indications for a marked difference in distribution of lysosomes in cells cultured in the presence of HEPES compared to those cultured in the presence of MES. It may nevertheless be of relevance to study to which extent medium pH impacts on retrograde and anterograde transport of lysosomes along microtubules.[47]

The change in GCase glycan isoforms in cells exposed to HEPES reveals a striking sensitivity of this glycan composition to lysosomal perturbations. Of note, the mild lysosomal stressor sucrose (80 mM) induced a similar enrichment of the high MW variant of GCase (**Supplemental figure 7**). Sucrose induced vacuolation might negatively impact on lysosomal pH homeostasis and thereby impair GCase maturation in a similar fashion as HEPES. The perturbed vacuolar processing of GCase to the mature 58 kDa isoform was not accompanied by an impaired catalytic function as measured by 4MU-assay. This is in line with previous reports, suggesting that whereas conformational folding of GCase requires occupation of reported glycan sites, the hydrolytic capacity is not dependent on the precise glycan composition.[31] Moreover, buffering medium pH at 7.4 by HEPES induces a prominent increase in total cellular GCase content, as measured in lysates. The majority of GCase in these cells is not present in physiologically mature lysosomes and might therefore be relatively inactive towards substrate. Indeed, we noted that formation of glucosylsphingosine in lysosomes, a measure for impaired degradation of GlcCer, is significantly higher in type 1 GD fibroblasts cultured in the presence of HEPES (**Supplemental Table 1**).[48] It might therefore be prudent to use culture medium with a measured, predefined pH as well as to assess the molecular weight and abundance of cellular GCase when using cultured cells in fundamental research on GCase and related disorders such as GD and PD. This could facilitate comparable and reproducible assessment of cellular GCase functions. Importantly, as pointed out in the results section, during diagnostic demonstration of reduced GCase activity in lysates of cultured cells, special care should be taken that tentative patient cells and normal cells are cultured in identical media.

In conclusion, GCase is remarkably sensitive to presence of HEPES in the culture medium. In view of the major effect of this buffer compound, investigations concerning GCase should better refrain from the use of this medium addition.

Material and Methods

Cell Culture Experiments

RAW264.7 cells (American Type Culture Collection #TIB-71) were cultured in DMEM and normal human dermal fibroblasts cells (NHDFs, Lonza #CC-2511) were cultured in DMEM/F12. Both mediums contained 10% (v/v) fetal calf serum, 1% (w/v) glutamax and 0.2% (w/v) antibiotics (penicillin-streptomycin; all purchased from Thermo Fisher Scientific) at 37°C at 7% CO_2 at controlled humidity. For modulation of medium pH, MES (Sigma, M3671), MOPS (Sigma, M1254) and HEPES (Sigma, H3375) were dissolved and filtered to obtain culture grade stock buffers (1 M). Where mentioned, culture medium was supplemented with culture grade HEPES, MES or MOPS to a final concentration of 50 mM for at least 72h, if not stated otherwise. Stock solutions were titrated so that final pH in medium was 7.0 for MES, 7.20 for MOPS and 7.5 for HEPES. Leupeptin (Sigma, L9783) was added in 25 or 50 µg/mL concentration to medium of cells pre-treated with MES, MOPS or HEPES for 72h and incubated along with the respective buffers for 48 h. Sucrose (Sigma, S7903) was incubated for 24h at a concentration of 80 mM. Gaucher fibroblasts were obtained for fundamental investigations with consent of patients and their GBA genotype was confirmed by sequencing.

Activity-based probe analysis

Cultured cells were lysed in KPi lysis buffer (25 mM K_2HPO4/KH_2PO4, pH 6.5, 0.1% (v/v) Triton X-100) supplemented with protease inhibitors (Roche) and sonicated 5x 1 second with 9 minutes interval (amplitude 25%). Protein concentration was assessed by bicinchoninic acid assay (Thermo Fisher Scientific, 23225) and absorbance measurements (EMax Plus microplate reader, Molecular Devices). Equal protein amounts were labelled with excess of activity-based probe (ABP) conjugated to a fluorescent dye. Labelling of all active GCase molecules in cell homogenates and recombinant GCase (Cerezyme) was performed using 100 nM ABP-ME569 (Cy5).[42] Incubation was performed at 100 nM for 1 h (0.5-1% (v/v) DMSO) on ice. Labelling of acid alpha-glucosidase (GAA) and beta-glucuronidase (GUSB) was performed as described earlier.[41] Shortly, homogenates were prelabelled with 200 nM of β-glc aziridine ABP JJB70 for 30 min at 37 °C, pH 4.0 and 5.0 resp. GAA was subsequently labeled by incubation of 500 nM JJB383 for 30 min at 37 °C, pH 4.0. GUSB labelling was performed through incubation with 200 nM JJB392 for 30 min at 37 °C, pH 5.0. After labelling, 5x Laemlli buffer (50% (v/v) 1 M Tris-HCl, pH 6.8, 50% (v/v) 100% glycerol, 10% (w/v) DTT, 10% (w/v) SDS, 0.01 % (w/v) bromophenol blue) was added and samples were denatured at 95°C. Proteins were resolved by 10% polyacrylamide gel through SDS-PAGE.

PNGase F treatment

Buffer-exchange was performed on GCase-labelled protein homogenate by spin desalting column (Pierce, 89849) and incubated with PNGase F according to the manufacturer's instructions (NEB, P0705S). Shortly, denaturation of protein was performed in denaturing buffer at 100°C for 10 minutes. Subsequent digestion by PNGase F was performed at 37°C for 1h.

Pulse-chase experiment

For *In situ* labelling of GCase in living cells, RAW264.7 cells and NHDFs were cultured overnight in the presence of 100 nM green fluorescent cyclophellitol based ABP (MDW933). Next, cells were thoroughly washed and incubated with 100 nM red fluorescent ABP (MDW941) for different amount of times. Thus, existing GCase is labeled green and newly synthesized GCase is red. Cells were extensively washed, lysed in KPi lysis buffer and equal amounts of protein were analyzed by SDS-PAGE.

In-gel visualization of probes

Detection of fluorescence in wet gel slabs was performed using a Typhoon FLA 9500 fluorescence scanner (GE Healthcare). Green fluorescence (MDW931 and JJB70) was detected using λ_{EX} 473 nm and $\lambda_{EM} \geq$ 510 nm, red fluorescence (MDW941) using λ_{EX} 532 nm and $\lambda_{EM} \geq$ 575 nm and far red fluorescence (ABP-ME569, JJB383, JJB392) using λ_{EX} 635 nm and $\lambda_{EM} \geq$ 665 nm. After imaging, gels were either stained by Coomassie G250 for total protein and scanned on ChemiDoc MP imager (Bio-Rad) or used for western blotting.

Western Blot Analysis

Samples resolved on 10% polyacrylamide gels were transferred to 0.2 µm nitrocellulose membrane (#1704159, Biorad). Blocking of membranes occurred in 5% (w/v) bovine serum albumin (Sigma, A1906) solution in PBS/0.1% Tween-20 (Sigma, P1379) for 1 h at room temperature (RT). Primary antibodies used target GCase (clone 8E4, manufactured in Aerts lab) and tubulin (Cedarlane, CLT 9002). Proteins were detected by using specific secondary conjugated antibodies (Alexa Fluor™ 488/647) (Molecular Probes). Detection of immunoblots was performed using a Typhoon FLA 9500 fluorescence scanner (GE Healthcare).

Labeling of GCase in situ

Functionalized glass coverslips were seeded with NHDF at a confluency of 70% as described and treated with MES or HEPES.[49] Active GCase was labelled by 2 h medium supplementation of 5 nM MDW941. Next, the cells were washed 3x with PBS and fixed with 4% (w/v) formaldehyde (Sigma) in PBS for 20 min at room temperature while kept in the dark. Fixed cells were then washed with PBS and blocked in 5% normal donkey serum (NDS, Jackson Laboratory, 145-017-000-121) for 60 min. Cells were either stained for immunofluorescence microscopy or mounted directly on a microscope slide with ProLong Diamond antifade reagent containing DAPI (Molecular Probes, P36962). Immunofluorescence staining was performed in 2% NDS. Antibodies used were rabbit anti-LAMP-1 (Abcam, AB24170), rabbit-anti-LIMP2 (Novus Biologicals, NB400-129), rabbit-anti-Calnexin (Sigma, C4731), rabbit-anti Giantin (Abcam, AB24586), all at a dilution of 1:400 and rabbit-anti-EEA1 (Cell Signaling, 2411S) at a concentration of 1:200. Secondary antibodies used was Alexa Fluor conjugated IgGs (H + L) donkey anti-rabbit Alexa 488 (Invitrogen). Fluorescence microscopy was performed using a Leica TCS SP8 confocal microscope with a 63×/1.40 numerical aperture (NA) HC Plan Apo CS2 oil immersion objective and equipped with a hybrid detector (HyD).

Enzyme activity assays

Equal protein amounts as assessed by bicinchoninic acid assay were used for enzyme activity assays. GCase activity was assayed using 3.75 mM 4-methylumbelliferyl (4-MU) substrate beta-D-glucopyranoside (44059, Glycosynth) in McIlvaine buffer, pH 5.2, with 0.1% (w/v) BSA, 0.2% (w/v) sodium taurocholate and 0.1% (v/v) Triton X-100. For activity measurements of β-hexosaminidases HexA/B, 5 mM 4MU-β-N-acetyl-glucosaminide at pH 4.5 was used.

Density gradient fractionation

Cultured cells were harvested and washed 2x in PBS and 2x MME buffer (250 mM mannitol, 2 mM EGTA, 5 mM MOPS/Tris pH 7.0) through centrifugation at 1000 g for 5 min. Cells were resuspended in MME buffer and homogenized by 30 strokes using a Dounce homogenizer (B. Braun). The suspension was centrifuged for 2 min at 1000 rpm. The post-nuclear fraction (PSN, supernatant) was transferred to a percoll colum (49% percoll (Sigma, P1644), 250 mM mannitol, 2.5 mM MOPS-Tris, HCl titrated to pH 7.0) on top of a cushion of 2.5 M Sucrose (Sigma). Ultracentrifugation of the column was performed at 30,000 g in a SW 41 Ti swinging bucket rotor (Beckman). Optimal density-based fractionation was verified by Density Marker Beads (Pharmacia, 17-0459-01). After centrifugation, fractions of 250 µl were obtained, weighed, and used for enzyme activity measurements.

Measurement of glucosylsphingosine

Levels of glucosylsphingosine in cultured fibroblasts were determined as earlier described.[50,51] Briefly, samples were analyzed by mass spectrometry (ESI-LC-MS/MS), along with a ^{13}C-isotope encoded internal standard.[50]

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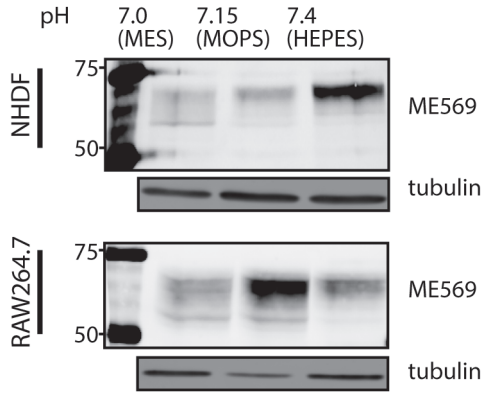
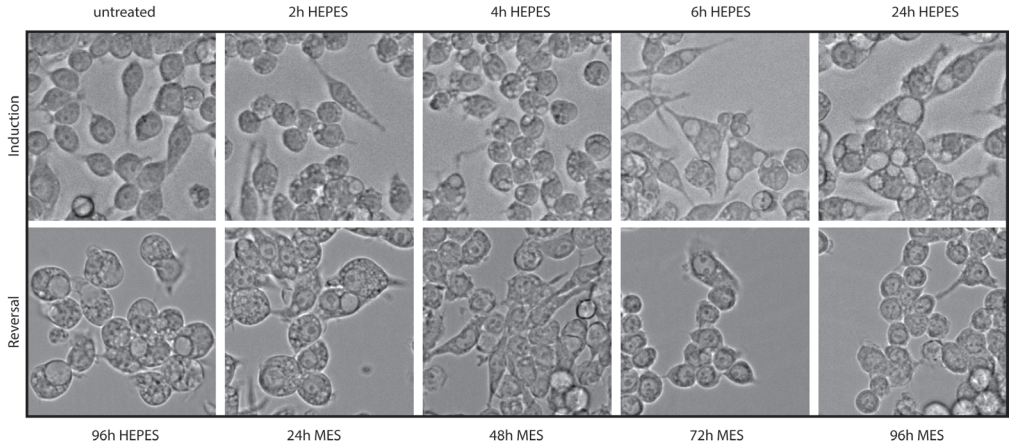
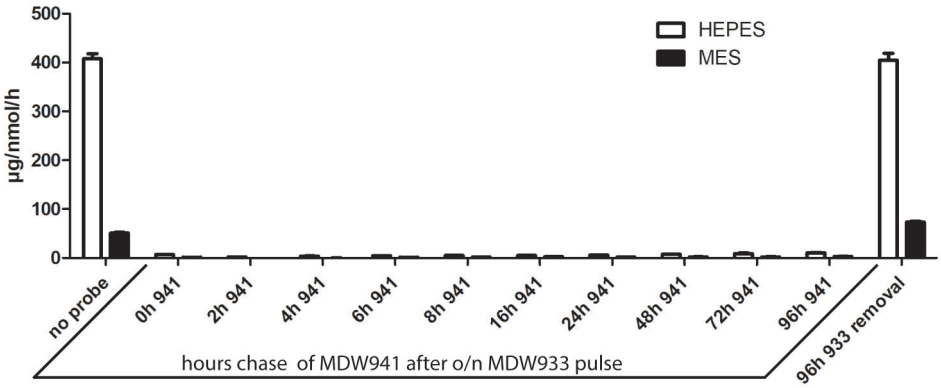
Supplemental Figures



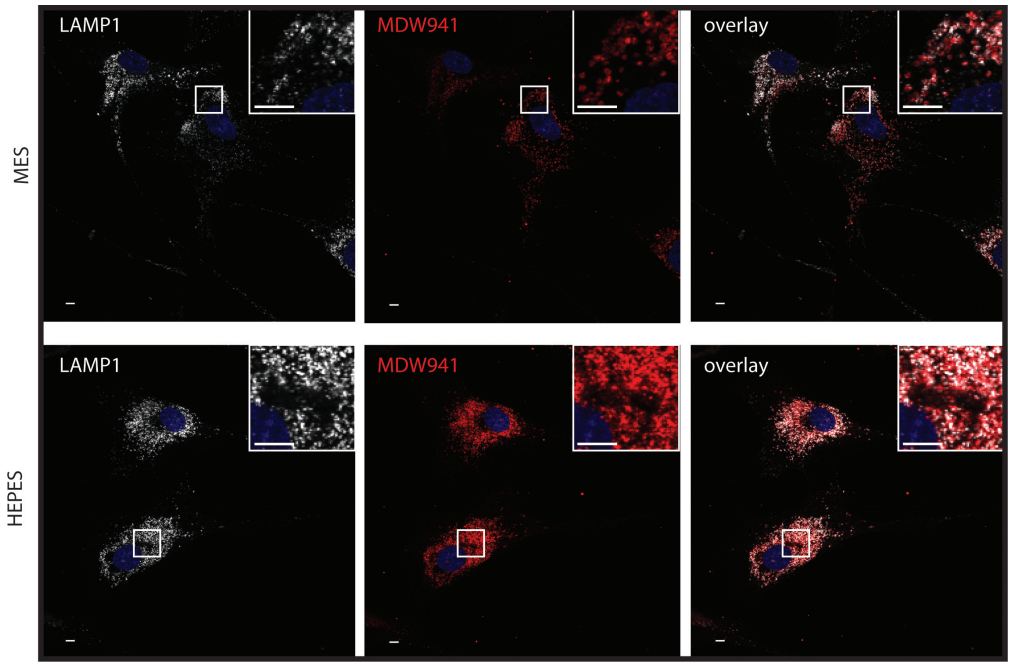
Supplemental figure 1. Impact of the presence of 50 mM MES, MOPS and HEPES in medium on GCase in cultured fibroblasts and RAW264.7 cells. *GCase in aliquots of the same lysates was labelled with GCase-specific ABP and subsequently visualized by fluorescence scanning after SDS-PAGE.*



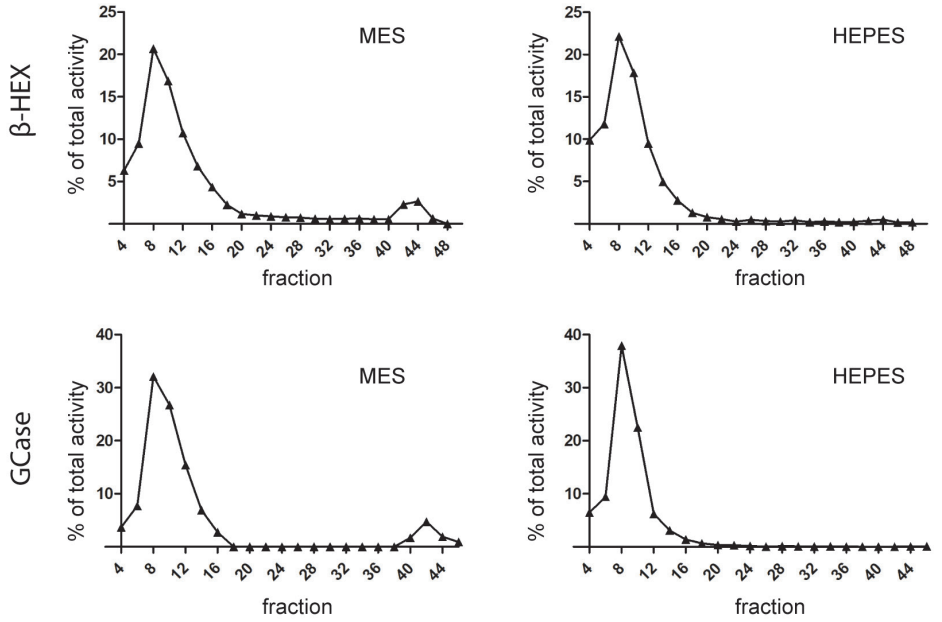
Supplemental figure 2. Reversible vacuolization upon HEPES exposure of RAW264.7 cells over time. *RAW264.7 cells were cultured in the presence of HEPES (50mM) for 0-96h and visualized over time by phase contrast microscopy. Cells were subsequently washed, and medium was replaced by MES (50mM) containing medium for 96h and captured by phase contrast microscopy.*



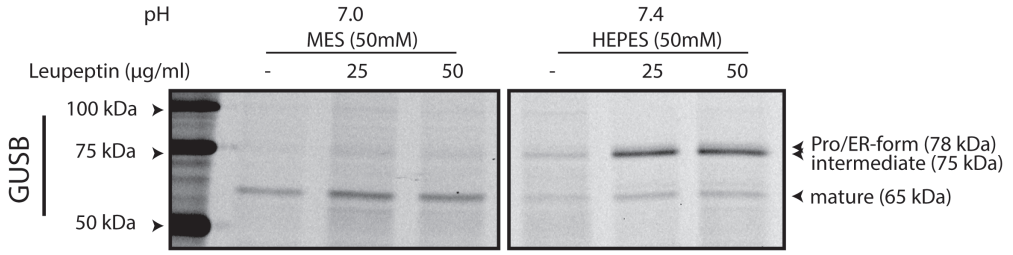
Supplemental figure 3. Inhibition of GCase in pulse (MDW933)-chase (MDW941) experiments as measured by 4MU-assay. *Pulse-chase experiments with HEPES and MES treated RAW264.7 cells, performed as described in M&M. Following pre-labeling with MDW933 (green fluorescent), cells were incubated continuously with MDW941 (red fluorescent) for indicated time periods. Basal GCase activity was assessed in samples left untreated with probes. Recovery of GCase after block was assessed by applying pulse (MDW933) and subsequent culturing without probe for 96h. Cells were harvested and lysed for enzyme activity measurements using fluorogenic substrate for GCase as described in M&M.*



Supplemental figure 4. Immunofluorescence analysis of LAMP1 in fibroblasts exposed to MES or HEPES. *Cells were pretreated ABP MDW941 to label GCase and subsequently fixed and analyzed by immunohistochemistry to establish overlap of GCase with other marker proteins. Scale bar: 10µm*



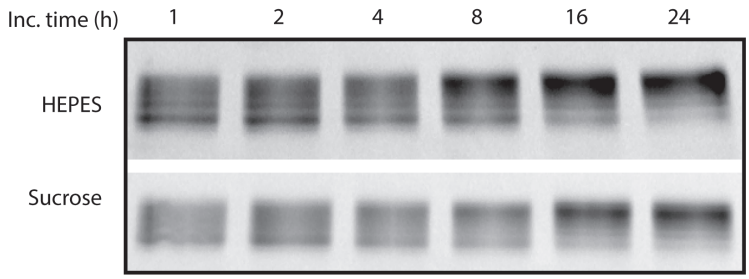
Supplemental figure 5. Subcellular fractionation of fibroblasts cultured in the presence of 50 mM HEPES or MES. *Normal human derived fibroblasts (NHDFs) were fractionated and compartments were separated on the basis of density using 49% Percoll centrifugation to generate density gradients. In collected fractions enzymatic activities of GCase and β-hexosaminidase were measured as described in M&M.*



Supplemental figure 6. Impact of leupeptin and medium pH on β-glucuronidase (GUSB). *Fibroblasts were cultured in the presence of 50 mM buffer compound (MES or HEPES) simultaneously with 72h hour incubation with different concentrations leupeptin. Cells were harvested and GAA and GUSB in lysates was visualized by ABP labeling, SDS-PAGE and fluorescence scanning.*

Supplemental Table 1. Glucosylsphingosine (GlcSph) content of fibroblasts cultured in the absence or presence of 25 mM HEPES. *Values expressed as mean ± SD on three independent cell cultures and triplicate measurements.*

GlcSph (pmol/mg total protein of cell lysate)		
Fibroblasts	**no HEPES**	**+ 25 mM HEPES (pH 7.4)**
Control wt GBA	<0.3	<0.3
Control wt GBA	<0.3	<0.3
N370S/L444P GBA	1.3 ± 0.4	4.2 ± 1.2
N370S/L444P GBA	0.9 ± 0.3	3.3 ± 0.6
N370 S/N370S GBA	0.7 ± 0.3	1.4 ± 0.5
L444P/L444P GBA	2.5 ± 0.4	2.4 ± 0.8



Supplemental figure 7. Sucrose induced changes in GCase glycan isoform profile. *RAW264.7 cells were cultured in the presence of 80 mM Sucrose for different amounts of time. Cells were harvested and GCase in lysates was visualized by ABP labeling, SDS-PAGE and fluorescence scanning.*