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Glucosylceramidase mass and subcellular localization are modulated by Cholesterol in Niemann-Pick disease type C

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Running title: Modulation of Glucosylceramidase in NPC cells.

SUMMARY

Niemann-Pick disease type C (NPC) is characterized by the accumulation of cholesterol and sphingolipids in the late endosomal/lysosomal compartment. The mechanism by which the concentration of sphingolipids such as glucosylceramide is increased in this disease is poorly understood. We have presently found that in NPC fibroblasts the cholesterol storage affects the stability of glucosylceramidase (GCase), decreasing its mass and activity; a reduction of cholesterol rises the level of GCase to nearly normal values. GCase is activated and stabilized by Saposin C (Sap C) and anionic phospholipids. Here we show by immunofluorescence microscopy that in normal fibroblasts GCase, Sap C and lysobisphosphatidic acid (LBPA), the most abundant anionic phospholipid in the endolysosomal system, reside in the same intracellular vesicular structures. In contrast, the colocalization of GCase, Sap C and LBPA is markedly impaired in NPC fibroblasts, but can be re-established by cholesterol depletion. These data show for the first time that the level of cholesterol modulates the interaction of GCase with its protein and lipid activators, namely Sap C and LBPA, regulating the GCase activity and stability.

INTRODUCTION

Niemann-Pick disease type C (NPC) is an autosomal recessive neurovisceral lipid storage disorder (1). Most cases of NPC are caused by mutations in the NPC1 gene (2) encoding a protein which possesses a sterol-sensing domain (3). The putative function of NPC1 protein is to facilitate the recycling of lipids from late endosomes/lysosomes to other cellular membranes (4-6). High levels of unesterified lipoprotein-derived cholesterol (Chol) accumulate in NPC1-deficient cells. Although alterations of Chol metabolism play a key role in the pathogenesis of NPC, there is also a more general dysfunction of the intracellular metabolism of lipids such as sphingolipids (SL) (7-10). Spleen and liver of NPC patients accumulate not only Chol, but also glucosylceramide (GC), lactosylceramide, sphingomyelin. Normal concentrations of Chol, but pathological levels of glucosylceramide, lactosylceramide, GM_2-ganglioside and asialo-GM_2 in brain are typical findings (1). These observations indicate that the NPC1 protein may function in Chol and SL homeostasis.

In normal cells the SL are degraded in late endosomes/lysosomes by specific hydrolases. Some of these enzymes need the assistance of activator proteins such as saposins to exert their function (11-13). Saposins are a group of four similar small glycoproteins, Sap A, B, C and D, each of them stimulating the enzymatic degradation of specific SL. In fact, Sap B is required for the degradation of sulfatides by arylsulfatase A and Sap C is required for the degradation of GC by glucosylceramidase (GCase) (14-16). The physiological role of saposins has been unequivocally demonstrated by the observation that SL storage diseases can be caused either by the deficiency of a specific hydrolase or of an individual saposin. For instance, Gaucher disease, a genetic disorder characterized by an extensive GC accumulation within the lysosomes of cells of monocyte/macrophage origin, can be caused by a deficit of either GCase or Sap C (16). In the Sap C-deficient cases of Gaucher disease, normal levels of GCase are unable to degrade GC.

The role of Sap C in the enzymatic GC degradation has been examined in details. In the past we have provided compelling evidence that Sap C, at low pH values mimicking the acidic lysosomal environment, tightly binds to and destabilizes anionic phospholipid-containing membranes (17). Upon affecting the physical organization of these membranes, Sap C promotes the association of GCase with the lipid surface thus favouring the contact between the enzyme and its lipid substrate, GC (18, 19). Anionic phospholipids have a key role in the Sap C-promoted interaction of GCase with membranes; changes in the level and organization of these lipids can affect the topology and activity of GCase (18, 19).

Markedly increased amounts of GC have been found not only in Gaucher disease, but also in visceral tissues and in brains from NPC patients (8, 20). Since SL such as GC are believed to be centrally involved in the pathogenesis of NPC disease (21), the mechanism of their accumulation and the properties of the hydrolases involved in the SL degradation have been extensively investigated. For instance, it has been found that the activities of GCase and sphingomyelinase, are markedly reduced in NPC fibroblasts (22). Cholesterol-mediated regulation of sphingomyelinase activity has been investigated (23, 24), while informations on the regulation mechanism of GCase in NPC cells are not available . It is important to fill this gap, since the accumulation of GC is very pronounced in several NPC tissues. The aim of our present work was to investigate the factors that might influence the GCase activity and stability in NPC cells. The possibility that the function of Sap C, a required cofactor for the enzymatic degradation of GC, might be altered in these cells was also taken into consideration and investigated.

EXPERIMENTAL PROCEDURES

Materials

Complete ^{TM} (protease inhibitor cocktail) was obtained from Roche Molecular Biochemicals. DMEM was from Euroclone Ldt U.K.. [^{35}S]methionine (Tran^{35}S-Label^{TM},1175 Ci/mmol) and methionine/cysteine –deficient DMEM were from ICN Biomedicals, Inc. California. Lipoprotein-deficient bovine serum (LPDS) was obtained from Cocalico Biologicals, Inc. Filipin and Protein A- Sepharose CL-4B were from Sigma Aldrich (St. Louis, MO). Kodak X-Omat Blue films were from Perkin Elmer Life Sciences (Boston, MA). Prolong antifade kit was from Molecular Probes (Eugene, OR, USA). SDS-polyacrylamide gel electrophoresis reagents were from Bio-Rad. ECL Western Blotting reagents were from Amersham Bioscience Buckinghamshire, U.K..

Cell cultures

Two human fibroblast lines with previously described severe NPC1 mutations were used (25). The NPC1a cell line (81057) was homozygous for a Q775P mutation located in the sterol-sensing domain and shown to produce no detectable NPC1 protein by Western blot analysis. The NPC1b cell line (90089, affected sib of reported 87024) was homozygous for a V282fsX299 mutation. Normal and NPC1 fibroblasts were cultured in Dulbecco's modification of Eagle's minimal essential medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, 100 units/ml of penicillin and 100 µg/ml streptomycin. For specific experiments NPC1 cells were first grown in DMEM supplemented with 10% fetal bovine serum (FBS) and then subcultured in fresh medium containing 10% LPDS for the indicated periods of time.

GCase assay

To measure the GCase activity the lipid substrate GC, purified from Gaucher spleens, was utilized (26). GC was labelled with tritium in the glucose moiety (27). The assay mixture contained in a final volume of 0.1 ml: 0.1/0.2 M citrate/phosphate buffer, pH 5.6, 10 µg of cell homogenate, 20 µg of GC supplemented with the [^{3}H]labelled compound to a specific activity of 3000 dpm/nmol , 0.25% taurocholate and 0.05% oleic acid . The assay mixtures were incubated for 1 h at 37ºC. The incubation was terminated by the addition of 0.4 ml of chloroform/methanol (2:1) and 50 µl of a 0.1% glucose solution. After shaking and centrifugation at 4000 rpm, the enzymatically released [^{3}H]glucose present in the aqueous phase was estimated by radioactivity measurements.

Antibodies

Mouse monoclonal (8E4) and rabbit polyclonal anti-GCase antibodies were kindly provided by Dr. H. Aerts, E.C.Slater Institute for Biochemical Research, University of Amsterdam, Netherlands. Rabbit anti-human Sap C antibody was prepared in our laboratory (17). Mouse monoclonal anti-human lysosome-associated membrane protein type 1 (LAMP1) antibody, developed by Dr. J.T. August, was obtained from the Developmental Studies Hybridoma Bank maintained by the University of Iowa (Iowa City, IA). Mouse monoclonal anti-LBPA antibody (6C4) was a generous gift of Dr.Gruenberg, Department of Biochemistry, University of Geneva, Switzerland. The anti-Actin monoclonal antibody was obtained from Sigma.

Western blotting

SDS-PAGE was performed with 10% acrylamide gels (28). After electrophoresis the proteins were electroblotted to polyvinylidene difluoride membranes (Bio-Rad) and GCase

was detected with anti-GCase monoclonal antibody 8E4 using an ECL Western blotting kit according to the manufacturer's instructions (Amersham Bioscience Buckinghamshire, U.K).

Metabolic labeling and immunoprecipitation of GCase or Sap C

Skin fibroblast cultures were grown until they almost reached confluency. Prior to being labeled, the cells were washed twice with ice-cold PBS supplemented with 1 mM $MgCl_2$ and 0.1 mM $CaCl_2$ and starved for 2 h in methionine and cysteine-free medium containing 4% dialyzed FBS. This medium was replaced with the labeling medium (DMEM lacking methionine and cysteine and supplemented with ^{35}S-methionine, 150 µCi/ml, and 4% dialyzed FBS). After 1h incubation the cells were washed three times with DMEM and non radioactive chase medium was added (DMEM containing 4% FBS). The cells were chased for the indicated periods and then harvested and disrupted in lysis buffer (0.5% Triton X-100 and a protease inhibitor mixture tablet/50 ml in 50 mM phosphate buffer, pH 6.5). The suspensions were subjected to brief sonication and centrifuged at 20.000 x g for 30 min. For removing DNA and histones, the supernatants were incubated with 0.03% protamine sulfate for 45 min at 4°C and centrifuged as above. Constant values of total 35S-labeled cellular proteins were utilized for each experimental point. After addition of 0.1% BSA the cell lysates were incubated with rabbit preimmune serum overnight at 4°C and non specific complexes were precipitated with Protein A-Sepharose CL-4B. The clarified supernatants were then incubated either with anti-GCase or anti-Sap C antiserum . Cross-reacting material was precipitated with Protein A-Sepharose CL-4B. The immunocomplexes were washed four times with PBS containing 1% BSA, 1% Triton X-100, 1% SDS, 0.4% sodium deoxycholate and then with only PBS. The washed precipitates were separated by SDS-PAGE. Labeled proteins were detected by fluorography.

Fluorescence Microscopy

For fluorescence microscopy the cells were grown on Labteck chamber slides (Nunc, Naperville, Illinois, USA) and fixed with 4% paraformaldehyde in PBS for 30 min. Cells were then rinsed with PBS, permeabilized with 0.05% saponin for 7 min and incubated with 3% bovine serum albumin for 2 hours.

For intracellular free unesterified cholesterol staining, fixed cells were incubated with filipin solution (0.05% in PBS) for 30 min. The cells were observed with a UV 330-380 filter.

For double immunostaining the cells were incubated for 1 hour with a specific rabbit polyclonal primary antibody (anti-Sap C or polyclonal anti-GCase), rinsed twice with PBS and incubated for 1 hour with the secondary anti-rabbit antibody coniugated with Alexa Fluor 594 (Molecular Probes, Eugene, Oregon, USA). The cells were then rinsed twice with PBS, incubated for 1 hour with a specific mouse monoclonal primary antibody (anti-GCase (8E4) or anti-Lamp1 or anti-LBPA), rinsed twice with PBS and incubated with the secondary anti-mouse antibody coniugated with Alexa Fluor 488 (Molecular Probes, Eugene, Oregon, USA).

Finally, the cells were mounted with ProLong antifade reagent (Molecular Probes) and observed with an Olympus BX52 fluorescence microscope equipped with appropriate filters. The images were acquired using the IAS 2000 software. When specified the fluorescence was viewed by Confocal Laser-Scanning Microscopy (CLSM) using a Leica TCS 4D apparatus, equipped with an argon-kripton laser, double-dichroic splitters (488/568 nm), 520 nm barrier filter for Alexa Fluor-488 (green), and 590 nm barrier filter for Alexa Fluor-594 (red) observations. Image acquisition and processing were conducted by using the SCANware and Multicolor Analysis (Leica Lasertechnik, GmbH, Heidelberg, Germany) and Adobe Photoshop software programs. Signals from different fluorescent probes were taken in parallel, and colocalization was detected in yellow.

Primary antibodies were used at the following dilutions: anti-Sap C (1: 300),

monoclonal anti-GCase (1:300), polyclonal anti-GCase (1:100), anti-Lamp1 (1:200), anti-LBPA (1:80).

RESULTS

GCase activity and mass are reduced in NPC fibroblasts

GCase activity has been reported to be markedly diminished in NPC cells (22). As shown in Fig. 1, the GCase activity was approximately 400 nmol/h/mg of protein in normal fibroblasts, while it was reduced to 75-100 nmol/h/mg protein (about 20% of the normal value) in cell lines from two NPC patients who lacked the NPC1 protein (NPC1a and NPC1b). Thus, much less functional GCase is present in NPC1 fibroblasts as consequence of either a reduction or inactivation of the enzyme protein. To examine the first possibility, the GCase mass was analysed by Western blotting using a monoclonal anti-GCase antibody. The intensity of the enzyme bands in both the NPC1 cell lines was approximately 80% weaker than in normal fibroblasts (inset in Fig. 1) indicating that much less protein was present. Thus, the difference in activity between the control and the mutated cells well correlates with differences in the enzyme mass.

GCase activity and mass are modulated by Chol accumulation.

To address the possibility that the decreased amount of GCase is related to the accumulation of endolysosomal free Chol, the NPC1 cells were grown in LPDS medium. It is known that NPC cells no longer accumulate Chol when cultured for more than two days in lipoprotein-free medium (1). Accordingly, the free Chol level was dramatically reduced upon removal of low density lipoproteins (LDL) as indicated by cytochemical filipin staining of the NPC1 cells (data not shown). The GCase activity increased 3-4 times after seven days of subculture with LPDS (Fig. 2 A) and a parallel increase of the GCase protein was observed (Fig. 2 B). When normal fibroblasts cells are grown in lipoprotein-free medium for seven days we observed a ~ 20% increase of the GCase activity (from about 400 to 450-

480 nmol/h/mg), while in NPC1 cells the increase of activity was ~ 250% (from about 100 to 300-350 nmol/h/mg). Thus, the level of free Chol in the endolysosomal system is able to modulate the level of GCase protein.

Maturation of GCase in NPC1 cells

To investigate at which step of maturation the amount of GCase decreased in the NPC1 cells, the biosynthesis and processing of the enzyme was examined by pulse-chase experiments (Fig. 3). Accordingly with previous findings (29, 30), in normal fibroblasts a GCase precursor form (about 62 kDa) was observed after a pulse of 1 hour . A band at higher molecular weight (about 65 kDa) appeared after a 24 h chase. The fibroblast chased for 72 h contained an additional 58-kDa band of mature GCase. A similar pattern was observed in NPC1 fibroblasts. The densitometric quantitation of the intensity of the bands revealed that the amount of the 62 kDa precursor formed during a 1 h pulse was essentially the same in control and NPC1 cells. In contrast, after a 72 h chase much less GCase was detected in NPC1 than in control fibroblasts. These results indicate that the stability of the mature forms of GCase is markedly decreased in NPC1cells.

Maturation of Sap C in NPC1 cells

Sap C, a small glycoprotein (about 10 kDa), derived from a large molecular weight precursor, prosaposin (65-70 kDa) (31), is the specific activating and stabilizing factor of GCase (11). A possible cause of the GCase instability in NPC1 cells might be a reduced amount of Sap C. To test this hypothesis the biosynthesis and maturation of Sap C have been examined. As shown in Fig. 4, the amount of prosaposin 65-70 kDa forms detected after pulse labelling for 1 h and the amount of Sap C generated after 72 h of chase were nearly the same in normal and NPC1 fibroblasts. Thus, the instability of GCase in NPC1 cells cannot simply

be ascribed to a lack of Sap C. Nevertheless, it must be noted that during a chase of 5 h about 50% of prosaposin was converted to the mature saposin in normal fibroblasts, while only 5-15 % was cleaved in NPC1 cells. This finding, consistent upon repetition, indicates that the prosaposin processing was retarded in the mutated cells.

Subcellular localization of GCase, Sap C and LBPA

Our previous findings showed that the activity of GCase is efficiently expressed only when the enzyme is bound to membranes containing anionic phospholipids (19, 32). Sap C, which preferentially interacts with these lipids (17), promotes in turn the association of GCase with the lipid surface. Accordingly with this model, it can be expected that GCase, Sap C and lysobisphosphatidic acid (LBPA), the most abundant anionic phospholipid of the endolysosomal compartment (33), colocalize in the same regions of the late endosomal/lysosomal membranes in control fibroblasts. As shown in Fig. 5, double-immunostaining revealed a complete colocalization of GCase and Sap C as evident in the merged images. Moreover, all the vesicular structures that contained GCase and Sap C contained also the anionic phospholipid LBPA. The late endosomal/lysosomal localization of GCase and Sap C was confirmed by the complete colocalization of the two proteins with LAMP1, a typical endolysosomal marker (Fig. 6).

To investigate whether an altered subcellular distribution might be responsible of the GCase instability, we performed the same immunofluorescence tests in NPC1 cells. As shown in Fig. 7, extensive storage of free Chol was observed in the two NPC1 cell lines (NPC1a and NPC1b) as visualized by the characteristic staining with filipin. Fig. 7 also shows that some cells staining for Chol were almost devoid of GCase. This observation was quantified by scoring NPC1a and NPC1b cells for GCase staining (n = 20 fields for each cell line). The enzyme was nearly absent in about 50% of the cells, an observation in keeping with the low

amount of GCase found in the fibroblasts homogenates (see Fig.1). Immunofluorescence microscopy furthermore revealed that GCase distributed towards the periphery of vesicles in enlarged rings containing a heavy burden of Chol. The segregation of GCase towards the periphery of vesicular structures was constantly observed in NPC1 fibroblasts.

In cells where a significant amount of GCase was present, double -immunostaining showed the non-coincidence of the GCase distribution with those of LBPA and Sap C (Fig. 8). Also the colocalization of Sap C with LBPA was impaired (Fig. 8). The intracellular distribution of GCase and Sap C was further defined by Laser scanning confocal microscopy. As shown in Fig 9, the contact among GCase and its activating and stabilizing factor, Sap C, is rare in NPC1 cells, while the two proteins completely colocalize in control fibroblasts.

Since the depletion of Chol results in a dramatic increase of both the GCase mass and activity (see Fig. 2), we have investigated whether a reduction of the Chol level could also re-establish the colocalization of GCase with Sap C. The NPC1 cells were cultured for 7 days in medium containing LPDS. After this time the morphology of the cells changed and the filipin staining was no more detectable. As shown in Fig . 10, the decrease of Chol storage actually restored the colocalization of GCase with Sap C.

DISCUSSION

In addition to an impairment in Chol trafficking, the NPC cells are characterized by an extensive endolysosomal accumulation of SL. Previous studies have evidenced that the activity of sphingomyelinase and GCase, which are responsible of the degradation of two SL present at high concentrations in NPC tissues, namely sphingomyelin and GC respectively, are markedly reduced (22). Our present results show that the reduction of GCase activity is paralleled by a decrease of the enzyme mass and that both activity and mass can revert to almost normal levels when the lipoprotein fraction is removed from the culture medium of NPC1 fibroblasts, namely when free Chol within the late endosomal/lysosomal compartment disappears. It is thus evident that Chol can modulate the level of GCase in NPC1 fibroblasts.

The Chol-mediated regulation of sphingomyelinase differs from that of GCase since the high amount of Chol required to knock down the sphingomyelinase activity has a negligible effect on the abundance and size of this enzyme (24). Actually, NPC fibroblasts express about 10% of the sphingomyelinase activity of normal fibroblasts, but possess a normal amount of enzymatic protein. To explain their observations the authors hypothesized that elevated free Chol might alter processing and/or trafficking events critical for sphingomyelinase activity or induce allosteric changes that cause enzyme inactivation (24).

In normal cells GCase is synthesized as a 62 Kda precursor then converted into different molecular forms. In pulse-chase experiments the intensities of the GCase bands appearing after a 24 h chase increase with time, becoming stronger after chase periods of >48h (30). This phenomenon is possibly related to the accessibility and affinity of the different GCase forms for the anti-GCase antibody utilized. Our results showing that in NPC1 fibroblasts a normal amount of the precursor form of GCase is synthesized, while the abundance of the mature forms is low indicate that the decreased GCase level is the consequence of an accelerated degradation of the mature enzyme. Moreover, we observed that the residual

enzyme protein was not uniformly distributed among cells; some NPC1 fibroblasts contained significant amounts of GCase , while others were almost devoid of enzyme protein (see Fig. 7). When present, the enzyme was visualized at the periphery of Chol-filled vesicles. It has recently been reported that the intra endolysosomal membranes are organized as a mosaic of lipid domains with different lipid and protein composition (34). It can be envisaged that the accumulation of Chol, altering the organization of the lipid domains, either brings about a redistribution of GCase towards the more external membranes of the endolysosomal vesicles or increases the susceptibility of GCase present in the core of the vesicles to the protease attack. Interestingly, it has been found that also mutant inactive forms of the NPC1 protein, transiently expressed in CT60 cells (a Chinese hamster ovary cell mutant), are localized in endolysosomal membranes encircling Chol-laden cores (35, 36).

The fast disappearance of GCase in NPC1 fibroblasts suggests that the enzyme is localized in a less protective environment in these cells. It is known that GCase is stabilized when in contact with anionic phospholipids and its activator protein, Sap C (37). Moreover, a recent work indicates that Sap C is required for GCase resistance to proteolytic degradation in the cells (38). The instability of GCase in NPC1 fibroblasts cannot be attributed to a low amount of Sap C, since we found that high levels of prosaposin are synthetized and converted to Sap C in these cells (see Fig. 4), the only difference from control fibroblasts being a delay in Sap C maturation. The slowed down processing of prosaposin suggests that the normal transport of the protein from the endoplasmic reticulum to the late endosomes/lysosomes or/and its proteolysis in these organelles are retarded.

To exert its activating and anti-proteolitic protective function Sap C should be in contact with GCase. Actually, fluorescence microscopy has now shown a complete colocalization of GCase with Sap C in normal fibroblasts. Conversely, in NPC1 fibroblasts most of the GCase-containing structures were not Sap C-positive, indicating that the two proteins preferentially

distribute into distinct vesicular compartments. The occurrence of separate subsets of endolysosomal vesicles with partly different protein and lipid content is well documented (4, 39) . Most likely the localization of GCase on membranes devoid of Sap C decreases the enzyme stability.

As we have previously shown, the localization of GCase on membranes is regulated by several factors the more important being pH, anionic phospholipids and Sap C. In fact, low pH values similar to those of the endolysosomal compartment dramatically increase Sap C hydrophobicity (17, 40). In consequence the saposin associates with and destabilizes anionic phospholipid-containing membranes promoting in turn the association of GCase with the lipid surface (19, 32). The amount and the physical organization of anionic phospholipids have a key role in the Sap C-mediated binding of GCase, to afford the enzymatically active complex. Our present results indicate that GCase neither colocalize with Sap C, nor with LBPA, the main anionic phospholipid of the endolysosomal organelles (33), in NPC1 cells. Most likely the accumulation of Chol within LBPA-rich membranes interferes with the interaction of LBPA with Sap C and thus with GCase, altering in this way the selective localization of the two proteins. It is well known that variations in the membrane lipid composition of defined organelles or of defined membrane regions can modulate the localization and function of membrane-bound proteins (41-43). In this context it is interesting to note that the multifunctional receptor for insulin-like growth factor 2 and for ligands bearing mannose-6-phosphate, that is mainly localized in the trans-Golgi network in normal fibroblasts, is localized in endolysosomal vesicles in NPC fibroblasts as the result of Chol accumulation (33).

The hypothesis that the Chol storage is the cause of the disrupted contact among GCase and Sap C is supported by our finding that a decrease of the Chol content in NPC fibroblasts

not only markedly increases the activity and the amount of GCase (see Fig. 2), but also restores the localization of GCase and Sap C in the same vesicular structures (see Fig. 10).

The mechanism/s underlying the SL accumulation in NPC1 cells have been the subject of intense investigation, but the specific manner in which mutations in the NPC1 protein alter SL metabolism remains to be established. Since the intracellular transport of certain SL is perturbed in NPC cells and this alteration is related to the high level of intracellular Chol (44) it has been envisioned that defects in the NPC1 protein result in the disruption of SL as well as Chol trafficking (39, 44, 45). Our present work shows that, beside a traffic defect, the accumulation of SL might depend on a Chol-induced block of their degradation. The hydrolysis of several SL requires not only specific enzymes but also activators such as anionic phospholipids and/or protein cofactors such as saposins (11) or the GM2 activator (13). According to the present findings, a change of the lipid composition of the endolysosomal membranes might interfere with the interaction of these enzymes with their lipid and protein activators, thus reducing the enzyme activities.

The instability of GCase that we observed in fibroblasts might not be generalized to all NPC1 tissues. For example, normal GCase activity has been observed in NPC liver (22). Most likely the GCase stability might vary according to the variations of the endolysosomal proteolytic systems present in different cell types. Anyhow, it should be stressed that also a normal amount of GCase could not efficiently degrade GC if the contact between the enzyme and its physiological activators is prevented by Chol accumulation. According with this view, changes in the Chol content might modulate the 'in situ' enzyme activity by controlling the organization of the endolysosomal membranes.

In conclusion, our findings indicate for the first time that in NPC1 fibroblasts the amount of GCase protein is markedly decreased and the colocalization of GCase with LBPA and Sap C is impaired as consequence of Chol accumulation. These findings strongly suggest that the

disruption of the complex formed by the enzyme and its stabilizing and activating factors (anionic phospholipids and Sap C) might be the cause of the GCase decreased activity observed in NPC fibroblasts and might partecipate in the GC accumulation observed in NPC tissues.

REFERENCES

1. Patterson, M.C., Vanier, M.T., Suzuki, K., Morris, J.A., Carstea, E., Neufeld, E.B., Blanchette-Mackie, J.E., Pentchev, P.G. (2001) The metabolic and molecular bases of inherited disease (Scriver, C.R., Beaudet, A.L., Sly, W.S. and Valle, D., Eds) pp. 3611-3633, McGraw-Hill, Book Co., NY

2. Carstea, E.D. et al. (1997) Science, 277, 228-231

3. Davies, J.P. and Ioannou, Y.A. (2000) J. Biol. Chem., 275, 24367-24374

4. Neufeld, E.B., Wastney, M., Patel, S., Suresh, S., Cooney, A.M., Dwyer, N.K, Roff, C.F., Ohno, K., Morris, J.A., Carstea, E.D., Incardona, J.P., Strauss III, J.F., Vanier, M.T., Patterson, M.C., Brady, R.O., Pentchev, P.G., and Blanchette-Mackie E.J. (1999) *J. Biol. Chem.*, **274**, 9627-9635

5. Cruz, J.C., Sugii, S., Yu,C. and Chang, T.Y. (2000) *J. Bio. Chem*. **275**, 4013-4021

6. Lange, Y., Rigney, M. and Steck, T. (2000) *J. Biol. Chem*., **275**, 17468-17475

7. Vanier, M.T. and Suzuki, K. (1998) *Brain Pathol.*, **8**, 163-174

8. Vanier, M.T. (1999) *Neurochem. Res*., **24**, 481-489

9. Watanabe, Y, Akaboshi, S., Ishida, G., Takeshima, T., Yano, T., Taniguchi, M., Onho, K. and Nakashima, K. (1998) *Brain Dev.*, **20**, 95-97

10. Taniguchi, M., Shinoda, Y., Ninomiya, H., Vanier, M.T. and Onho, K. (2001) *Brain Dev*., **23**, 414-421

11. O'Brien, J. and Kishimoto, Y. (1991) *FASEB J*., **5**, 301-308

12. Kishimoto, Y., Hiraiwa, M. and O'Brien, J.S. (1992) *J. Lipid Res*., **33**, 1255-1267

13. Furst, W. and Sandhoff, K. (1992) *Biochim. Biophys. Acta*, **1126**, 1-16

14. Wenger, D.A., De Gala, G., Williams, C., Taylor, H.A., Stevenson, R.E., Pruitt, J.R., Miller, J., Garen, P.D. and Balentine, J.D. (1989) *Am. J. Med. Genet.* **33**, 255-265

15. Schlote, W., Harzer, K., Christomanou, H., Paton, B.C., Kustermann-Kuhn, B., Schmid, B., Seeger, J., Bendt, U., Schuster, I. and Langenbeck, U. (1991) *Eur. J. Pediatr*., **150**, 584-591

16. Christomanou, H., Chabas, A., Pampols, T. and Guardiola, A. (1989) *Klin. Wochenschr*., **67**, 999-1003

17. Vaccaro, A.M., Ciaffoni, F., Tatti, M Salvioli, R., Barca, A., Tognozzi, D., and Scerch, C. (1995) *J. Biol. Chem.*, **270**, 30576-30580

18. Vaccaro, A.M., Tatti, M., Ciaffoni, F., Salvioli, R., Barca, A., and Scerch C. (1997) *J. Biol. Chem.*, **272**, 16862-16867

19. Salvioli, R., Tatti, M., , Ciaffoni, F. and Vaccaro, A.M. (2000) *FEBS Lett.*, **472**, 17-21

20. Vanier, M.T. (1983) *Biochim. Biophys. Acta*, **750**, 178-184

21. Zervas, M., Somers, K.L., Thrall, M.A. and Walkley, S.U. (2001) *Curr. Biol.*, **11,** 1283-1287

22. Besley, G.T.N. and Moss, S.E. (1983) *Biochim. Biophys. Acta*, **752**, 54-64

23. Thomas, G.H., Tuck-Muller, C.M., Miller, C.S., Reynolds, L.W. (1989) *J Inherit Metab Dis*, **12**, 139-151

24. Reagan, J.W., Hubbert , M.L., and Shelness, G. (2000) *J. Biol. Chem.*, **275**, 38104-38110

25. Millat, G., Marçais, C., Tomasetto, C., Chikh, K., Fensom, A.H., Harzer, K., Wenger, D.A., Ohno, K. and Vanier, M.T. (2001) *Am. J. Hum. Genet.*, **68**, 1373-1358

26. Vaccaro, A.M., Muscillo, M., Salvioli, R., Tatti, M., Gallozzi, E. and Suzuki, K. (1987) *FEBS Lett.*, **216**, 190-194

27. McMaster, M.C.jr and Radin, N.S. (1977) *J. label. Comp. Radiopharmaceut.* **13**, 353-357

28. Laemmli, U.K. (1970) *Nature*, **227**, 680-685

29. Erickson, A.H., Ginns, E.I. and Barranger, J.A. (1985) *J. Biol. Chem.* **260**, 14319-14324

30. Jonsson, L.M.V., Murray, G.J., Sorrell, S.H., Strijland, A., Aerts, J.F.G.M., Ginns, E.I., Barranger, J.A., Tager, J.M. and Schram, A.W. (1987) *Eur. J. Biochem.*, **164**, 171-179

31. Fujibayashi, S. and Wenger, D.A. (1986) *J. Biol. Chem*., **261**, 15339-15343

32. Vaccaro, A.M., Tatti, M., Ciaffoni, F., Salvioli, R., Maras, B. and Barca, A. (1993) *FEBS Lett*., **336**, 159-162

33. Kobayashi, T., Stang, E., Fang, K.S., de Moerloose, P., Parton, R.G. and Gruenberg, J. (1998) *Nature*, **392**, 193-197

34. Kobayashi, T., Beuchat, M.H., Chevallier, J., Makino, A., Mayran, N., Escola, J.M., Lebrand, C., Cosson, P., Kobayashi, T. and Gruenberg, J. (2002) *J. Biol. Chem.* **35**, 32157-32164

35. Watari, H., Blanchette-Mackie, E.J., Dwyer, N.K., Watari, M., Neufeld, E.B., Patel, S., Pentchev, P.G., and Strauss III, J.F. (1999a) *J. Biol. Chem.*, **274**, 21861-21866

36. Watari, H., Blanchette-Mackie, E.J., Dwyer, N.K., Glick, J.M., Patel, S., Neufeld, E.B., Brady, R.O., Pentchev, P.G., and Strauss III, J.F. (1999b) *Proc. Natl. Acad. Sci. USA*, **96**, 805-810

37. Aerts, J.M.F.G., Sa Miranda, C., Brouwer-Kelder, E.M., Van Weely, S., Barranger, J.A. and Tager J.M. (1990) *Biochim. Biophys. Acta*, **1041**, 55-63

38. Sun, Y., Qi, X. and Grabowski, G.A. (2003) *J. Biol. Chem.*, **278**, 31918-31923

39. Zhang, M., Dwyer, N.K., Neufeld, E.B., Love, D.C., Cooney, A., Comly, M., Patel, S., Watari, H., Strauss III, J.F., Pentchev, P.G., Hanover, J.A., and Blanchette-Mackie E.J. (2001) *J. Biol. Chem.* **276**, 3417-3425

40. Vaccaro, A.M., Salvioli, R., Tatti, M. and Ciaffoni, F. (1999) *Neurochem. Res.*, **24**, 307-314

41. Yeagle, P.L., (1991), *Biochimie*, **73**, 1303-1310

42. Miaczynska, M. and Zerial, M. (2002) *Exp. Cell Res.*, **272**, 8-14

43. Hurley, J.H. and Meyer, T. (2001) *Curr. Opin. Cell Biol*, **13**, 146-152

44. Choudhury, A., Dominguez, M., Puri, V., Sharma, D.K., Narita, K., Wheatley, C. L., Marks, D.L. and Pagano, R.E. (2002) *J. Clin. Investigation*, **109**, 1541-1550

45. Puri, V., Watanabe, R., Dominguez, M., Sun, X., Wheatley, C., Marks, D.L. and Pagano, R.E. (1999) *Nature Cell Biol.*, **1**, 386-388

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LEGENDES OF THE FIGURES

Fig. 1. GCase activity and mass are reduced in NPC1 fibroblasts

The GCase activity in normal and NPC1 fibroblasts (NPC1a and NPC1b) was determined in at least three separate flasks for each cell line. Data represent the means ± SD. The inset shows a representative Western blot of GCase from control and NPC1 fibroblast homogenates. Identical amounts of protein (5 µg) were loaded in each lane . Densitometric quantitation of the bands is also shown.

Fig. 2. Restoration of GCase activity and mass in NPC1 fibroblasts incubated in LPDS medium.

The two NPC1 lines (NPC1a, ? , and NPC1b, ¦) were incubated in LPDS medium for the indicated periods of time (see Methods Section). GCase activity was determined on lysates of cells harvested at the indicated days from time of subculture (A). GCase mass was determined on the same cell lysates by Western blotting (B) . Identical amounts of protein (5µg) were loaded in each lane. The samples were probed for GCase utilizing the monoclonal antibody 8E4. The blots were reprobed for ß-actin to normalize lanes for protein content. The experiments, repeated more than three times, gave similar results.

Fig. 3. Processing of GCase in control and NPC1 fibroblasts.

Control, NPC1a and NPC1b fibroblasts were pulsed for 1h and then chased as indicated. Immunoprecipitation of cell lysates was performed with the anti-GCase monoclonal antibody 8E4. SDS-PAGE and fluorography were carried out as described in Methods. The number on the left refers to the molecula mass (kDa) of albumin standard. The bands were quantitated by densitometry. The experiments, repeated more than two times, gave similar results.

Fig. 4. Processing of prosaposin in control and NPC1 fibroblasts.

Control, NPC1a and NPC1b fibroblasts were pulsed for 1h and then chased as indicated. Immunoprecipitation of cell lysates was performed with anti-Sap C antibody. SDS-PAGE and fluorography were carried out as described in Methods. The numbers on the left refer to the molecula mass (kDa) of standards. The percentage of prosaposin (MW 73-65 kDa) conversion to Sap C (MW 14-10 kDa) was evaluated by the intensities of the corresponding bands quantitated by densitometry. The experiments, repeated more than two times, gave similar results.

Fig. 5. GCase, Sap C and LBPA colocalize in the same vesicular structures in normal fibroblasts

Normal human fibroblasts were double-immunostained for GCase and Sap C (top panels), GCase and LBPA (middle panels) and Sap C and LBPA (bottom panels) as described in Methods. All vesicles containing GCase were also Sap C- and LBPA-positive. The right panels show an enlargement of the region outlined by the boxes in the overlaid panels to better appreciate the complete colocalization of GCase, Sap C and LBPA. Note that GCase was visualized with the monoclonal antibody 8E4 (green, top left panel) or with a polyclonal antibody (red, middle left panel). Bars, 10 µm.

Fig. 6. GCase and Sap C colocalize with LAMP 1 in normal fibroblasts

Normal human fibroblasts were double-immunostained for Lamp 1 and either Sap C (top panels) or GCase (bottom panels) as described in Methods. All vesicles containing Lamp1 were also GCase- and Sap C-positive. Note that GCase was visualized with a polyclonal antibody (red, bottom left panel). Bars, 10 µm.

Fig. 7. GCase and Chol in NPC1 fibroblasts.

NPC1 fibroblasts (NPC1a, top panels and NPC1b, bottom panels) were immunostained for GCase and cytochemically stained with filipin for Chol as described in Methods. Arrowheads highlight cells containing vesicular structures filled with Chol, but almost devoid of GCase. The right panels show an enlargement of the regions outlined by the boxes in the overlaid panels. As better revealed in the magnified images GCase appears as rings at the periphery of Chol-laden vesicular structures. Bars, 10 µm.

Fig. 8. GCase, Sap C and LBPA poorly colocalize in NPC1 fibroblasts

NPC1 fibroblasts were double-immunostained for GCase and Sap C (top panels), GCase and LBPA (middle panels) or for Sap C and LBPA (bottom panels) as described in Methods. The right panels show an enlargement of the regions outlined by the boxes in the overlaid panels to better appreciate the poor colocalization of GCase, Sap C and LBPA. GCase was visualized with a monoclonal antibody (green, left, top panel) or with a polyclonal antibody (red, left, middle panel). As already observed in Fig.7, GCase distributes towards the periphery of the vesicles. Bars, 10 µm.

Fig. 9. Comparison of the GCase and Sap C localization in normal and NPC1 fibroblasts

NPC1 (top panels) and normal (bottom panels) fibroblasts were double-immunostained for GCase (green) and Sap C (red) and observed by Laser scanning microscopy as described in Methods. The right panels show an enlargement of the regions outlined by the boxes in the overlaid panels. The comparison of the overlaid images clearly shows that in normal fibroblasts the intracellular vesicles are yellow, indicating that each contains both GCase and

Sap C, while in NPC1 cells most of the vesicles are either green or red, indicating that the two proteins reside in distinct vesicles. Bars, 10 μm.

Fig. 10. Chol depletion from NPC fibroblasts restores the colocalization between GCase and Sap C

NPC1 fibroblasts were grown for 7 days in medium either supplemented with FBS (left panel) or with LPDS (right panel). The cells were then double-immunostained for GCase (green) and Sap C (red) . The comparison of the overlaid images clearly shows that the incubation with LPDS dramatically increases the number of intracellular yellow vesicles, namely of vesicles that contain both Sap C and GCase. Bars, 10 μm.

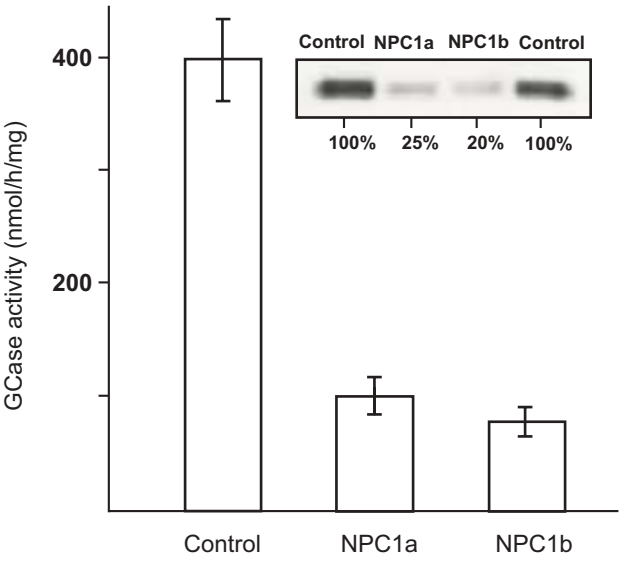

Fig.1

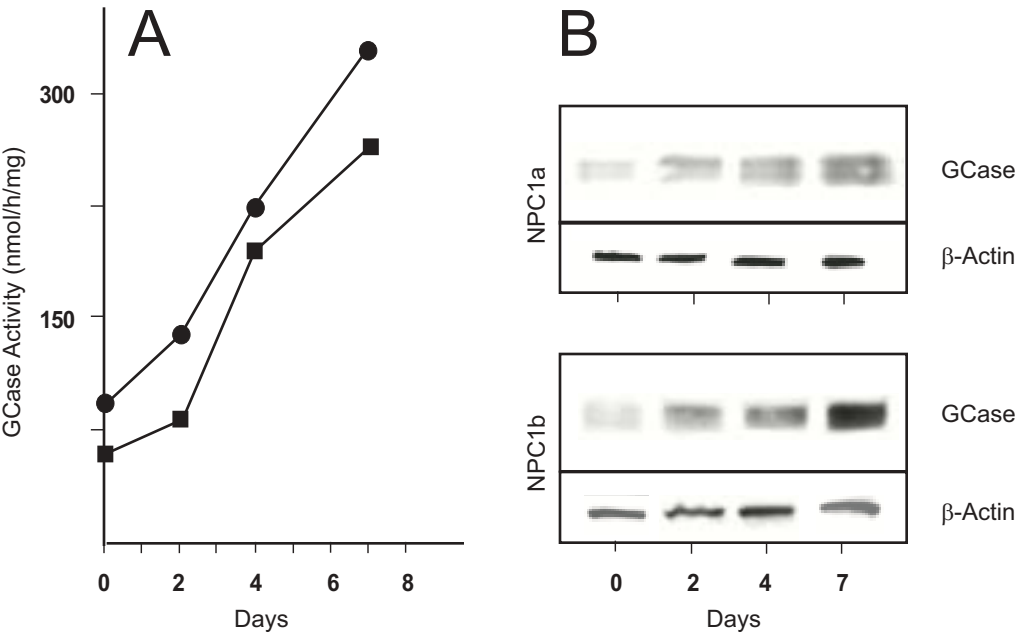

Fig.2

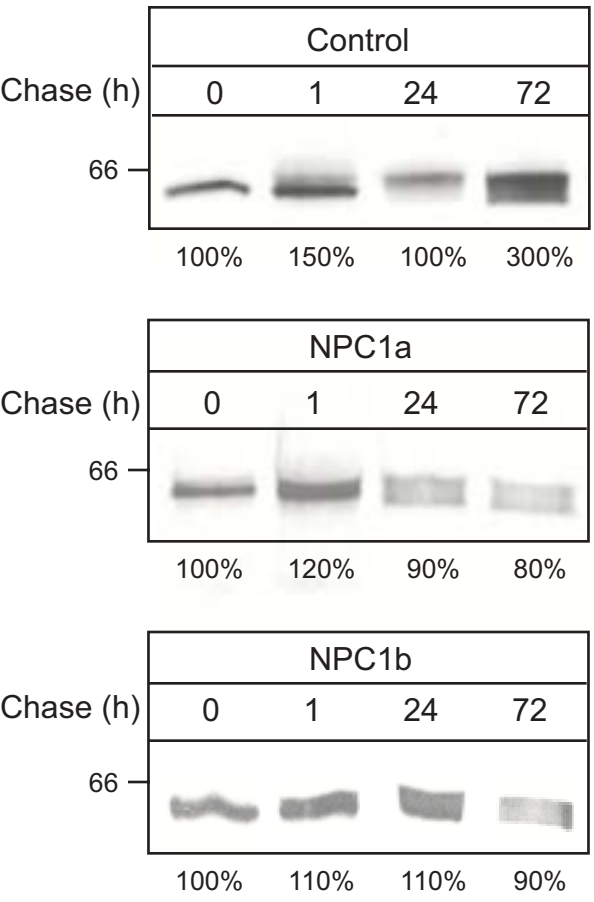

Fig.3

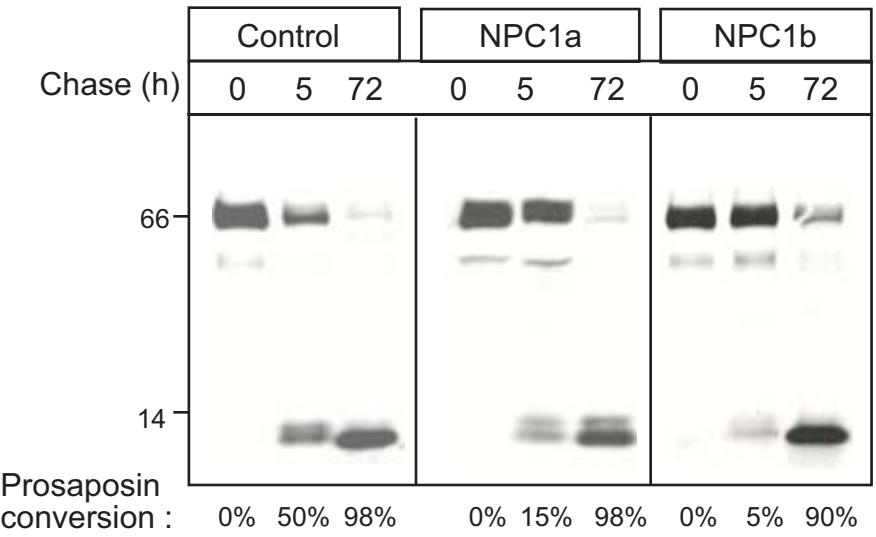

Fig.4

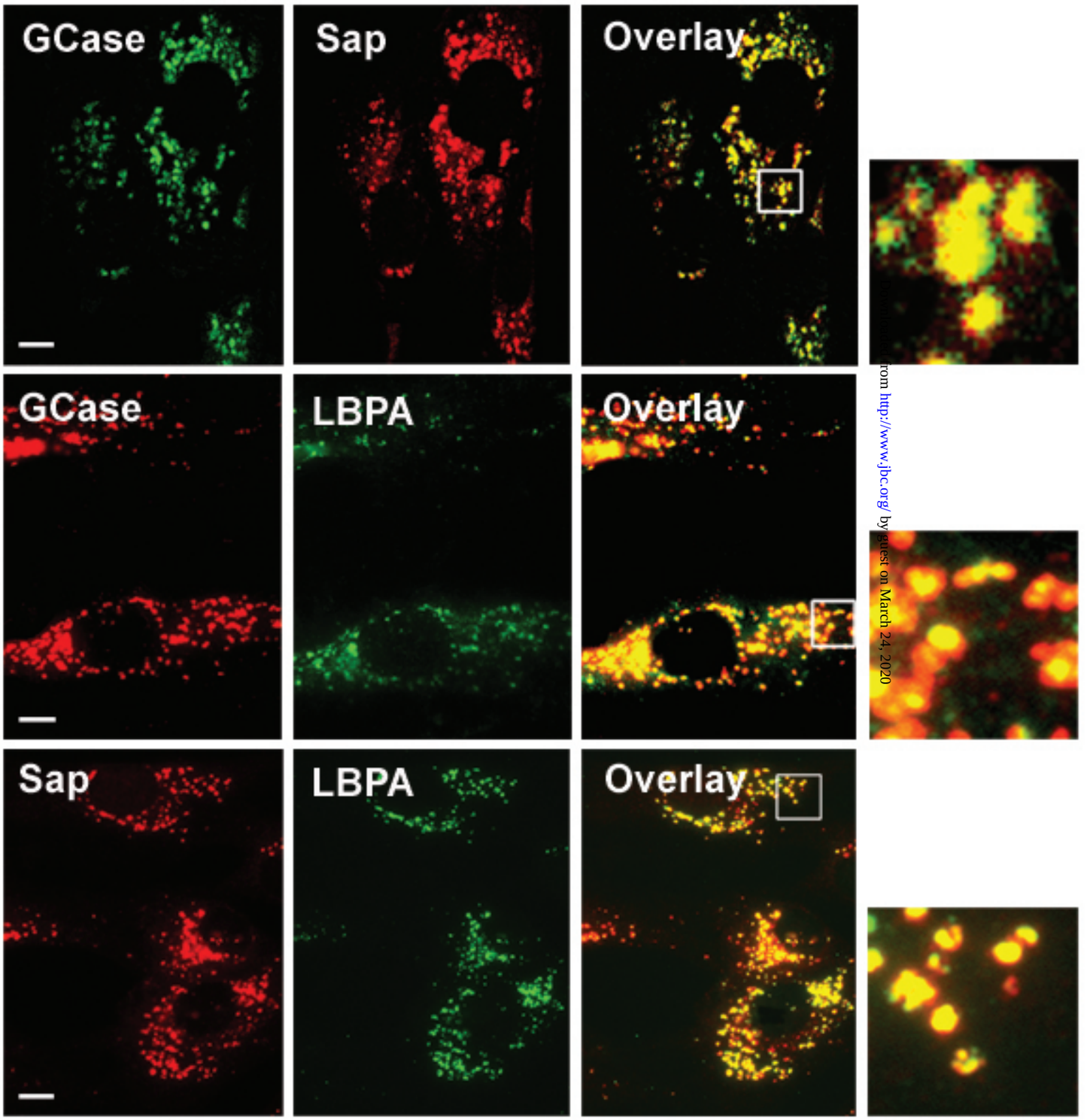

Fig. 5

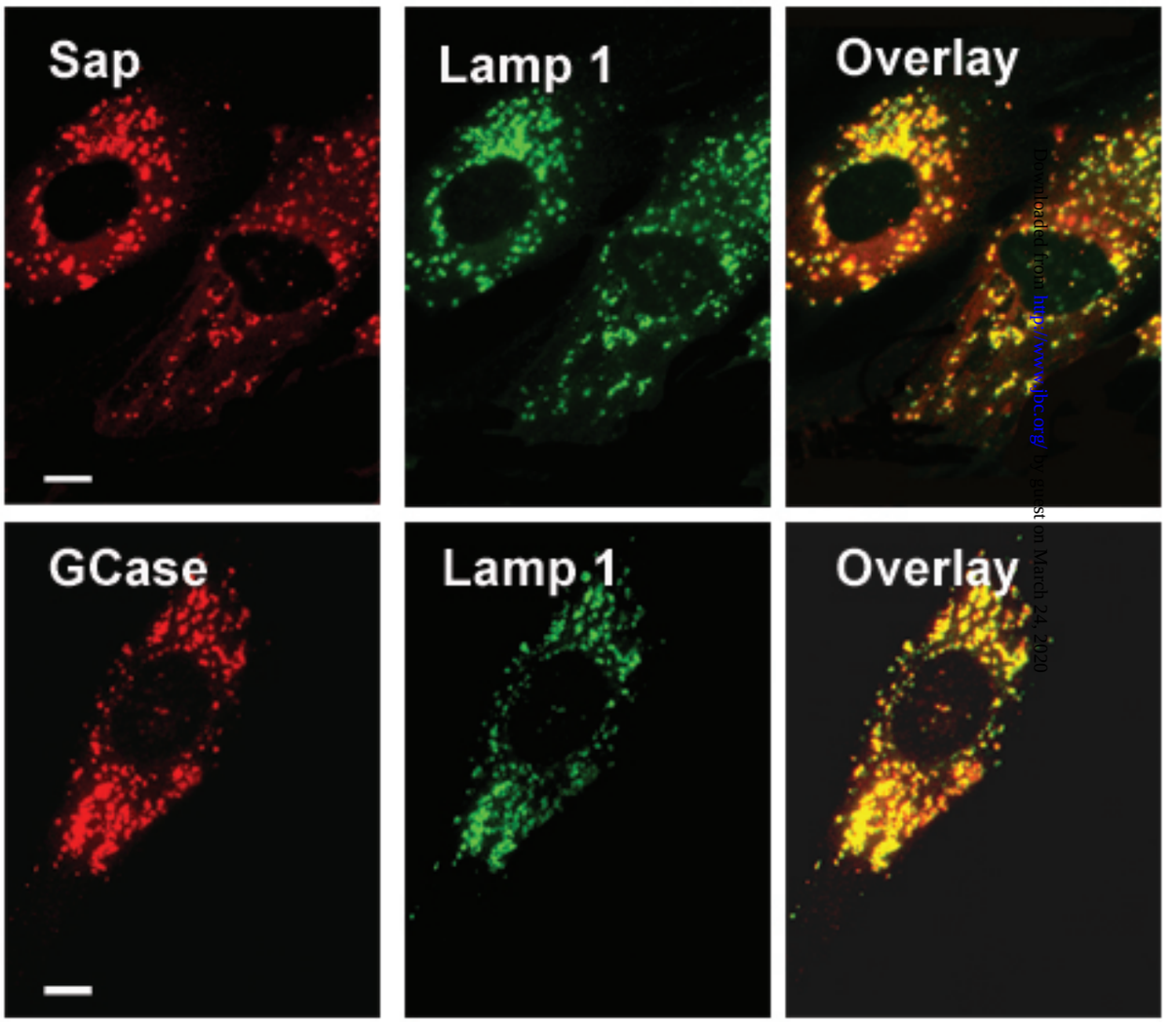

Fig. 6

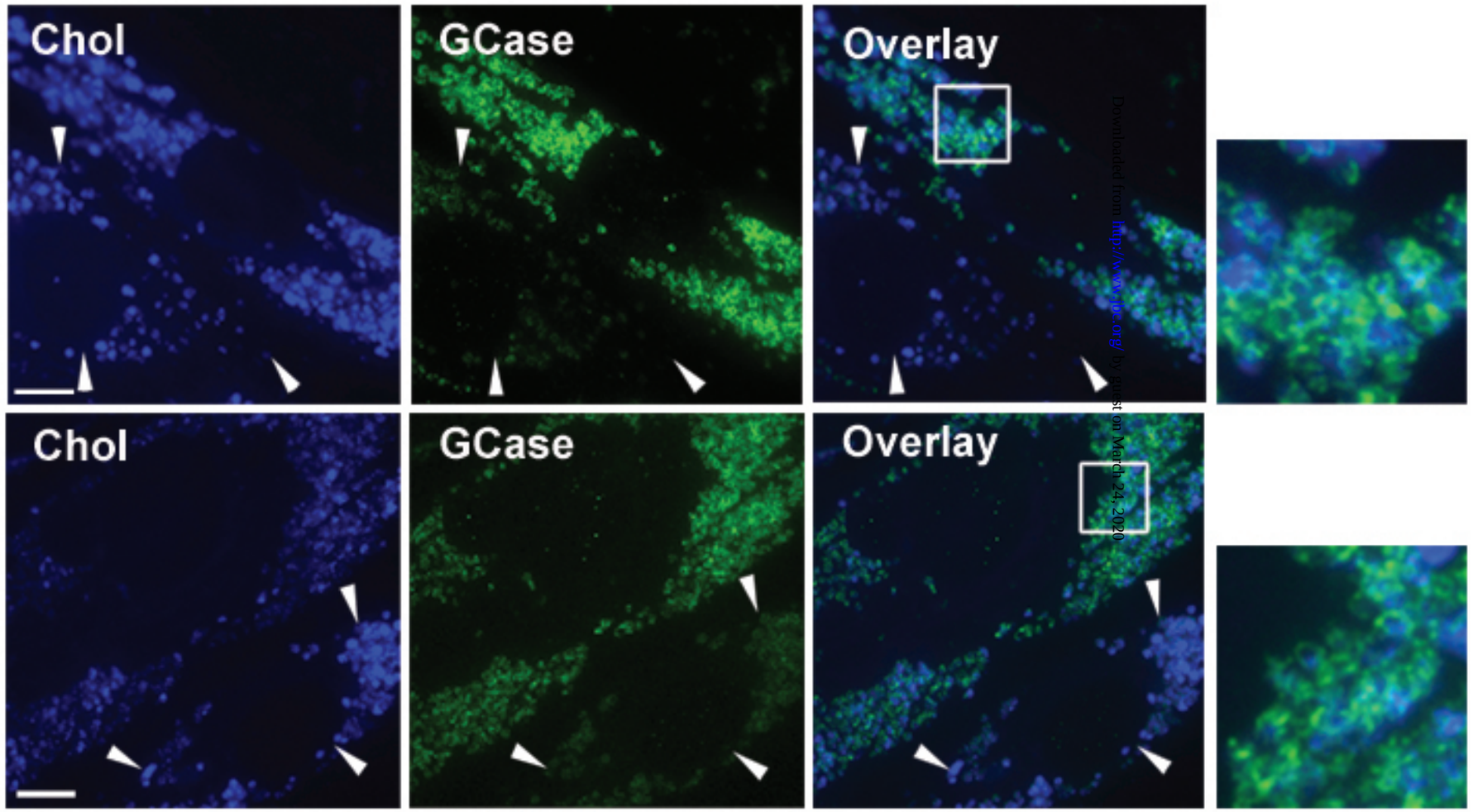

Fig. 7

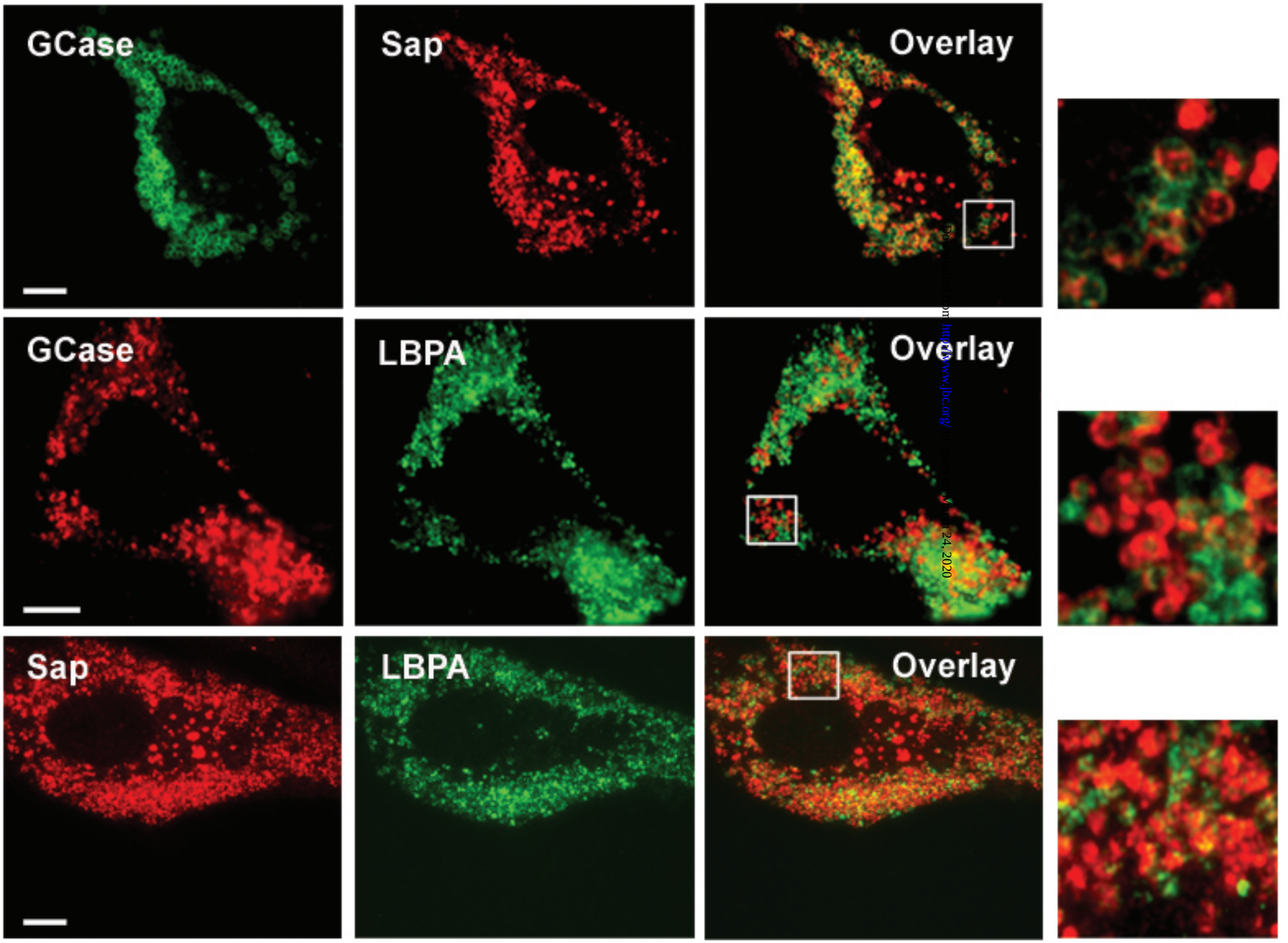

Fig. 8

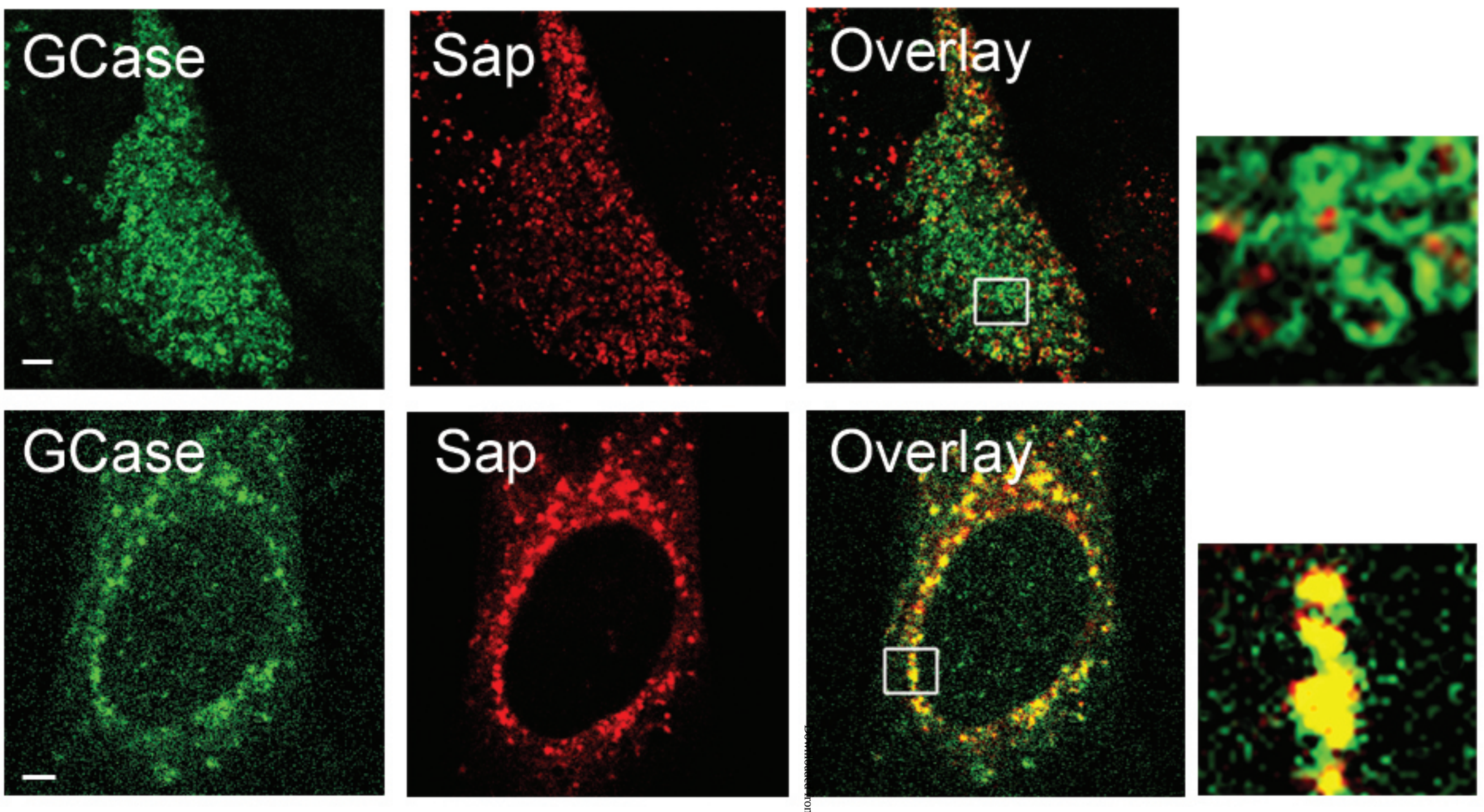

Fig. 9

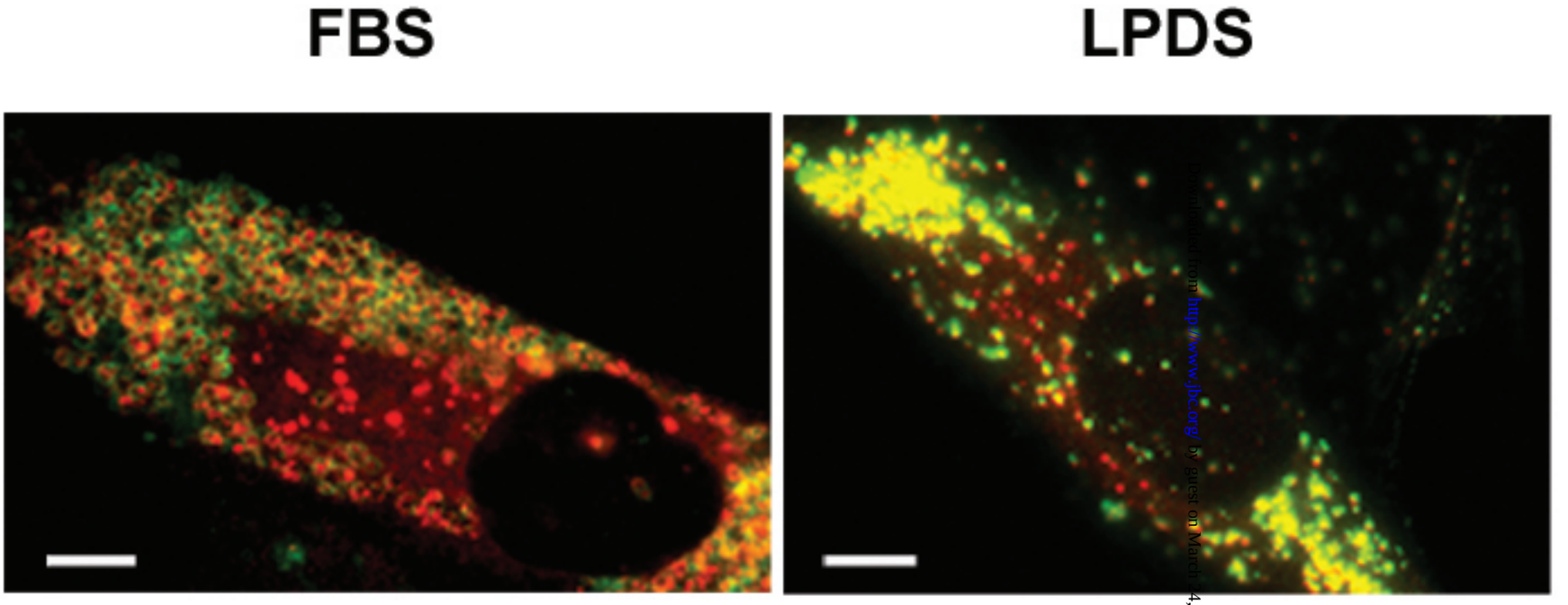

Fig. 10

Glucosylceramidase mass and subcellular localization are modulated by cholesterol in Niemann-Pick disease type C

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