GM130 gain-of-function induces cell pathology in a model of lysosomal storage disease

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Cell pathology in lysosomal storage diseases is characterized by the formation of distended vacuoles with characteristics of lysosomes. Our previous studies in mucopolysaccharidosis type IIIB (MPSIIIB), a disease in which a genetic defect induces the accumulation of undigested heparan sulfate (HS) fragments, led to the hypothesis that abnormal lysosome formation was related to events occurring at the Golgi level. We reproduced the enzyme defect of MPSIIIB in HeLa cells using tetracycline-inducible expression of shRNAs directed against α-N-acetylglucosaminidase (NAGLU) and addressed this hypothesis. HeLa cells deprived of NAGLU accumulated abnormal lysosomes. The Golgi matrix protein GM130 was over-expressed. The cis- and medial-Golgi compartments were distended, elongated and formed circularized ribbons. The Golgi microtubule network was enlarged with increased amounts of AKAP450, a partner of GM130 controlling this network. GM130 down-regulation prevented pathology in HeLa cells deprived of NAGLU, whereas GM130 over-expression in control HeLa cells mimicked the pathology of deprived cells. We concluded that abnormal lysosomes forming in cells accumulating HS fragments were the consequence of GM130 gain-of-function and subsequent alterations of the Golgi ribbon architecture. These results indicate that GM130 functions are modulated by HS glycosaminoglycans and therefore possibly controlled by extracellular cues.

INTRODUCTION

Lysosomal storage diseases (LSDs) are genetic defects affecting the catalytic activity of lysosomal hydrolases, or co-factors necessary for the function of these enzymes, or lysosomal membrane transporters. They result in the accumulation of incompletely digested macromolecule fragments in cells and their environment. A hallmark of LSDs is the formation of vacuoles that accumulate in the cytosol, the nature of which is unclear. Mucopolysaccharidosis type IIIB (MPSIIIB) is an LSD in which the deficiency of α-N-acetylglucosaminidase (NAGLU) results in the accumulation of undigested heparan sulfate (HS) glycosaminoglycans (1). Affected children suffer progressive and severe mental retardation and die prematurely (1). Vacuoles present in MPSIIIB-affected cells are acidic and possess characteristics of lysosomes, like the presence of the lysosomal-associated membrane protein 1 (LAMP1) in the limiting membrane and the presence of lysosomal hydrolases in the lumen (2). However, according to their size, deficient mobility and highly heterogeneous content, these vacuoles are not normal lysosomes. In MPSIIIB mouse cortical neurons, vacuoles are not connected to the endocytic or macroautophagy pathways, which are normally efficient in these cells, and do not possess markers of late endosomes or autophagosomes (2). In contrast to normal lysosomes, the cis–Golgi matrix protein GM130 is present in vacuoles membranes. Abnormal lysosomes are associated with enlarged and disorganized Golgi apparatus, and increased GM130 levels (2). Similar observations were made in affected neurons derived from MPSIIIB patient induced pluripotent stem cells (iPSc) (3). These features are consistent with the hypothesis that abnormal lysosomes accumulating in MPSIIIB cells are caused by events affecting early stages of lysosome biogenesis.

GM130 is a cytosolic coiled-coil protein anchored to Golgi membranes (4). Interactions of GM130 with various partner proteins mediate multiple functions with cardinal roles in cell biology (5). In combination with p115 and GRASP65, GM130 functions as a tethering factor for pre-Golgi carriers.

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undergoing fusion with cis–Golgi cisternae and regulates the fusion of Golgi cisternae into elongated Golgi ribbons (6–8). Binding of GM130 to the A-kinase anchoring protein AKAP450 participates in the regulation of microtubule nucleation on cis-Golgi membranes and to the formation of Golgi-derived microtubule network (9). Interaction of GM130 with Stk25 (also named YSK1), a protein important for neuron polarization, controls the morphology and subcellular distribution of the Golgi apparatus (10). GM130 also controls Golgi disassembly and re-assembly during mitosis, and the organization of the centrosome through binding to Tuba, a guanine exchange factor for Cdc42 (11,12). These various functions converge to confer important roles to GM130 in the control of Golgi size and morphology and in the control of cell polarity and oriented migration.

We investigated the determinants of vacuole formation and Golgi defects associated with MPSIIIB using HeLa cells in which the inducible expression of specific shRNAs directed against NAGLU induced complete depletion of this enzyme and subsequently the accumulation of undegraded HS glycosaminoglycans. NAGLU-depleted HeLa cells faithfully recapitulated defects associated with MPSIIIB, as previously observed in patient iPSC and in mouse or human affected neurons. NAGLU-depleted HeLa cells and non-depleted controls could be conveniently manipulated to inducing GM130 overexpression or down-regulation. These experiments provided evidence for an implication of GM130 in Golgi alterations and vacuole formation associated with NAGLU depletion. They indicate that HS glycosaminoglycans affect regulatory pathways controlling GM130 functions.

RESULTS

Acute NAGLU depletion rapidly induces storage and cell vacuolation

Typical cell features associated with MPSIIIB consist of the storage of partially digested HS glycosaminoglycans, secondary accumulation of glycosphingolipids (13) and formation of intracellular vacuoles expressing lysosomal markers. We examined the installation of these disorders in HeLa cells acutely depleted of the lysosomal enzyme NAGLU, the missing enzyme in MPSIIIB. Five siRNAs were investigated that all induced transient down-regulation of NAGLU in HeLa cells. One sequence was selected for the construction of a tetracycline-inducible shRNA expression plasmid. Plasmid DNA was stably introduced in HeLa cells. Figure 1A and B shows drastic reduction in NAGLU mRNA levels (80–90%) and complete depletion of NAGLU catalytic activity in three independent cell clones upon tetracycline treatment. Down-regulation was maximal 7 days after induction and persisted for 1 week, after which revertant cells having recovered NAGLU activity overgrew the cultures. This result indicates that NAGLU expression conferred a selective advantage over NAGLU-depleted cells.

The consequences of NAGLU depletion were examined in one cell clone (HeLa shNAGLU cells) 7 days after tetracycline induction. Control cells producing normal NAGLU levels consisted of parental HeLa cells, HeLa shNAGLU cells not exposed to tetracycline and HeLa cells stably expressing a scrambled shRNA (HeLa scrambled cells) upon tetracycline treatment. Control cells gave similar results and are thereafter referred to as non-depleted cells.

Glycosaminoglycan levels were measured in extracts of non-depleted and NAGLU-depleted cells using metabolic labeling with [3H]-glucosamine. NAGLU-depleted cells contained higher amounts of glycosaminoglycans than non-depleted cells (5.3-fold, Fig. 1C), indicating accumulation of NAGLU substrate molecules. Consistently, immuno-staining with anti-HS antibodies revealed more abundant and larger intracellular vesicles loaded with HS in NAGLU-depleted than in non-depleted cells (31 ± 1.5 versus 18 ± 0.5 vesicles per cell; 8.6 versus 3.4% of vesicles larger than 0.2 μm²; Fig. 1D). HS signal at the cell surface and immediate environment was increased in NAGLU-depleted cells (Fig. 1E), suggesting increased levels in the extra-cellular matrix. NAGLU-depleted cells also stored glycosphingolipids, as shown by immuno-staining for the ganglioside GM3 (Fig. 1F). They overexpressed LAMP1 (Fig. 1G) and contained numerous large intracellular vacuoles positive for LAMP1 (Fig. 1D and F). Vacuoles were acidic and contained the lysosomal hydrolase α-L-iduronidase fused to a fluorescent tag (GFP-IDA, Fig. 2A and B, NAGLU-depleted). Vacuoles did not express the late endosome marker mannose-6-phosphate (M6P) receptor, or the early endosome antigen 1 (EEA1) protein (Fig. 2C and D, NAGLU-depleted). Lipidated LC3 protein (LC3-II), a marker of activated macro-autophagy, was not detected in NAGLU-depleted cells (Fig. 2E, NAGLU-depleted). Cell vacuolation was visible by phase-contrast light microscopy (Fig. 1H). Electron microscopy revealed highly heterogeneous vacule contents, including clear material, internal debris, electro-dense aggregates and membrane stacks (Fig. 1H). These aspects were highly reminiscent of storage lesions observed in MPSIIIB human (3) or mouse (2) brain cells. In NAGLU-depleted HeLa cells, GM130 staining was more intense than in non-depleted cells (Fig. 1F). LAMP1-positive vesicles co-expressed markers of the pre-Golgi and cis-Golgi compartments, including sec23 (a COPII component), GM130 or GRASP65 (Fig. 1F and Supplementary Material, Fig. S1A). Immuno-gold electron microscopy confirmed that GM130 and LAMP1 were co-localized in storage vesicle limiting membranes (Supplementary Material, Fig. S1B) and showed that doubly stained vesicles were more abundant in NAGLU-depleted cells than in non-depleted cells (4.5 ± 0.5 versus 0.8 ± 0.2 doubly stained vesicle per cell, n = 20 cells, P < 0.001, Student’s t-test). Thus, HeLa cells acutely depleted of the lysosomal enzyme NAGLU rapidly accumulated primary and secondary storage products and developed cellular lesions fully recapitulating the pathology previously described in chronically deficient MPSIIIB neurons (2,3).

Co-labeling of NAGLU-depleted cells showed very limited co-localization of LAMP1, HS and GM3 signals (Fig. 1D and F), suggesting storage in different organelles, as reported in other cell types (14). In contrast, GM130 and GM3 signals mostly overlapped in large vesicular structures (71% of GM3-positive vesicles larger than 1 μm² were also positive for GM130 and co-localized signals represented 29% of the vesicle surface, Fig. 1F). This result suggested that GM3, which is synthesized in the cis-Golgi (15–17), accumulated in structures related to this compartment.
NAGLU depletion is associated with alteration of the Golgi complex

We previously observed alterations of the Golgi ribbon architecture in MPSIIIB mouse cortical neurons (2) and in human MPSIIIB patient iPSc and iPSc-derived neurons (3). Immuno-labeling of NAGLU-depleted HeLa cells showed high expression of the Golgi-specific GM130 protein indicating expansion of Golgi structures, when compared with non-depleted cells (Fig. 3A). Consistently, western blot showed a higher expression of GM130 in NAGLU-depleted than in non-depleted cells (1.5-fold, \( P < 0.05 \), Fig. 3B). GM130 mRNA levels were also increased, as determined by quantitative reverse transcription-polymerase chain reaction (RT–PCR) (Fig. 3C). To study further Golgi alterations, the cis-Golgi marker GM130 (4) was used in combination with markers of other Golgi compartments, including giantin—a cis/medial-Golgi marker (18), mannosidase II—a medial-Golgi marker (19), and golgin97 and p230—two markers of the trans-Golgi network (20,21). Immuno-labeling revealed increased expression of all markers analyzed, consistently with increased Golgi size (Fig. 3D). An area of overlap was observed between GM130 and each marker examined, which presumably represents partial co-localization of the proteins. The possibility that the region of overlap might also represent the failure to resolve close, but distinct fluorescent signals cannot be excluded. This observation is in line with previous immunofluorescence microscopy studies showing that although GM130 predominantly localizes to the cis-Golgi, it partially overlaps with medial and trans-Golgi markers (4). GM130 co-localized extensively with giantin and mannosidase II, but showed limited overlap with golgin97 or p230, consistent with the known locations of the proteins that were analyzed. In each case, quantification of co-localized signals showed increased values in NAGLU-depleted cells, when compared with non-depleted cells (data not shown). When protein-specific signals were quantified after excluding the area of overlap, compartment-specific differences were observed (Fig. 3D, see graphs). Whereas giantin and mannosidase II-specific signals were increased in NAGLU-depleted cells, when compared with non-depleted cells, golgin97 and p230-specific signals were unchanged. These results indicate that at least part of the trans-Golgi compartment was not affected, and suggest selective expansion of the cis- and medial-Golgi compartments. Brefeldin A inhibits anterograde transport from the endoplasmic reticulum to the Golgi and induces collapse of normal Golgi stacks (22,23). In NAGLU-depleted cells, the partial resistance of giantin-labeled structures to brefeldin A was reminiscent of observations previously made in MPSIIIB mouse neurons (2), and supported the hypothesis of altered Golgi dynamics (Supplementary Material, Fig. S2).

Examination of Golgi structures in non-depleted cells by electron microscopy showed linear ribbons formed with regular and thin cisternae stacks (Fig. 3Ea). In contrast, Golgi structures in NAGLU-depleted cells consisted of irregular and incorrectly aligned stacks of abnormally elongated Golgi ribbons (Fig. 3Eb). Quantification of ribbon length revealed significant increase when compared with non-depleted cells (1.22 ± 0.07 versus 0.72 ± 0.03 \( \mu m \), \( P < 0.001 \)) (Fig. 3Eh). Elongated abnormal ribbons exhibited marked tendency to bending, occasionally forming horseshoe-like structures (Fig. 3Ee,f), or even circular structures resembling vesicles with internal membrane stacks (Fig. 3Eg). Quantification of curvature confirmed abnormal ribbon morphology (125.6 ± 3.9 versus 159.8 ± 1.7 degree angle, \( P < 0.001 \)) (Fig. 3Eh). Cisternae were also frequently distended (Fig. 3Ec). Clear storage vesicles apposed to abnormal Golgi structures (Fig. 3Ed) suggested that cisternal distensions could give rise to distended vesicles.

As GM130 is involved in lengthening and maintenance of the Golgi ribbon (8,24), these results suggest that increased GM130 levels induced expansion and disorganization of Golgi structures in NAGLU-depleted HeLa cells.

Golgi expansion in NAGLU-depleted HeLa cells is related to GM130

To determine whether Golgi defects associated with NAGLU depletion were related to GM130, we performed GM130 gain-of-function and loss-of-function experiments in both non-depleted controls and NAGLU-depleted cells.

Golgi size was measured using giantin immuno-staining in cells transiently transfected with either FLAG-tagged GM130 expression plasmid to increase GM130 levels (11), or specific siRNAs aimed at down-regulating GM130 expression (8). DNA transfection was performed after 4 days of treatment with tetracycline, before the occurrence of cell defects. Cells were examined at day 7 and compared with untransfected cells present on the same cover slip (Fig. 4A). In non-depleted cells, giantin staining was reduced in GM130-negative cells when compared with untransfected cells, consistently with previous observations of Golgi fragmentation in the absence of GM130 (8,24). Non-depleted cells expressing FLAG-tagged GM130 showed increased giantin staining indicating Golgi expansion. In NAGLU-depleted cells, GM130 depletion decreased giantin staining, indicating efficient Golgi fragmentation, and FLAG-tagged GM130 expression increased giantin staining, suggesting further Golgi expansion.

Golgi morphology was examined by electron microscopy in cells stably expressing FLAG-tagged GM130 (Fig. 4B). In non-depleted cells, FLAG-tagged GM130 expression was associated with elongation and bending of Golgi ribbons, resulting in aspects very similar to those observed in NAGLU-depleted cells (Figs 3E and 4B). Quantification of Golgi length and curvature showed significantly modified values, when compared with non-depleted cells transfected with control DNA (Fig. 4B). In NAGLU-depleted cells, FLAG-tagged GM130 expression aggravated further Golgi alterations, when compared with NAGLU-depleted cells transfected with control DNA (Fig. 4B). These results show that GM130 over-expression induced Golgi expansion and disorganization in normal cells and therefore strongly suggest that Golgi defects observed in NAGLU-depleted cells were related to increased GM130 levels.

NAGLU depletion is associated with expansion of Golgi microtubule network

Golgi ribbon formation requires Golgi-derived microtubules (25). Microtubule nucleation on Golgi membranes is facilitated by the recruitment of \( \gamma \)-tubulin and \( \gamma \)-TuRC complex to the cis-Golgi via the interaction of GM130 with...
Figure 1. NAGLU-depleted HeLa cells provide an accurate model of MPSIIIB. (A) NAGLU mRNA levels normalized to ARPO mRNA (59) were determined by quantitative RT–PCR and expressed as percentage versus non-depleted cells (depicted as 100%, grey line). (B) NAGLU enzymatic activity was determined by fluorimetric assays. Clone 3 (most robust NAGLU inhibition) was used for subsequent analyses. (C) Glycosaminoglycan content was measured by metabolic labeling with [3H]-Glucosamine. (D) Cells were immuno-labeled with the HepSS-1 anti-HS antibodies (55) (green), in combination with anti-LAMP1 antibodies (purple). (E) Cells were immuno-labeled with the 10E4 anti-HS antibodies (55,60) (green). (F) Cells were immuno-labeled with anti-GM3 ganglioside (green), anti-LAMP1 (purple) and anti-GM130 (red) antibodies. (D, E, F) Nuclei were counterstained in blue. Scale bars, 10 μm. Apotome views are shown. NAGLU-depleted cells showed more abundant and distended LAMP1-positive vesicles, larger and more numerous intracellular HS-positive dots, more intense extracellular HS staining and larger and more intense GM3-positive vesicles when compared with non-depleted cells. HS and GM3 showed little co-localization with...
AKAP450 (9,26,27). Golgi-nucleated microtubules are highly acetylated and stable (28). Immuno-staining revealed a larger and denser network of acetylated microtubules in NAGLU-depleted cells, when compared with non-depleted cells (Fig. 5A). Increased amount of acetylated microtubules was confirmed by western blot (Fig. 5B). This microtubule subset extended from the giantin-positive Golgi area toward the cell edge, indicating more active microtubule nucleation at the Golgi (Fig. 5C). Consistently, expansion of Golgi-derived microtubules in NAGLU-depleted cells was associated with larger and more intense AKAP450 signal (Fig. 5D). AKAP450 localizes both at the Golgi and at the centrosome (29,30). Whereas the AKAP450-positive area essentially co-localized with the centrosome marker pericentrin in non-depleted cells (Fig. 5D), it was wider in NAGLU-depleted cells, extending over the entire giantin-positive Golgi region (Fig. 5E). These results are consistent with enhanced microtubule nucleation at the cis-Golgi in NAGLU-depleted cells, a phenomenon that can be promoted by increased amounts of GM130–AKAP450 complexes.

Cell vacuolation is mediated by GM130

We next examined whether accumulation of LAMP1-positive vesicles, increased levels of LAMP1 and cell vacuolation in NAGLU-depleted cells were related to GM130.

As described above, GM130 was either transiently depleted with specific siRNAs, or transiently over-expressed using a FLAG-tagged GM130 expression plasmid. LAMP1 signals were quantified by immuno-staining in transfected cells and compared with untransfected cells present on the same cover slip (Fig. 6A). In non-depleted cells, LAMP1 staining was reduced when GM130 expression was abrogated, and increased when FLAG-tagged GM130 was expressed. In NAGLU-depleted cells, FLAG-tagged GM130 expression did not increase LAMP1 signal further. NAGLU-depleted cells in which GM130 was down-regulated showed a drastic reduction in LAMP1 staining, albeit still higher than in normal cells.

Cells stably expressing a lentiviral vector encoding shRNAs directed against GM130, the FLAG-tagged GM130 expression plasmid or control DNA were used to examine the consequences of permanent down-regulation, or over-expression of GM130. Western blot analysis confirmed GM130 down-regulation or over-expression and provided evidence for a link between GM130 and LAMP1 expression levels (Fig. 6B). Indeed, LAMP1 protein levels dropped down below normal levels in both non-depleted and NAGLU-depleted HeLa cells when GM130 expression was down-regulated. When FLAG-tagged GM130 was expressed, LAMP1 levels slightly increased in non-depleted cells, but did not increase further in NAGLU-depleted cells.

These cells were further examined for the presence of vacuoles. Non-depleted cells over-expressing GM130 accumulated distended intracellular vacuoles with typical characteristics of abnormal lysosomes. As observed in NAGLU-depleted cells, these vacuoles were positive for LAMP1, contained the lysosomal enzyme IDUA-GFP and were acidic, but did not express the M6P receptor or EEA1, and cell extracts did not contain LC3-II (Fig. 2, non-depleted+GM130-FLAG). Ultrastructural examination revealed vacuoles resembling those accumulating in NAGLU-depleted cells (Figs 1H and 6C). In some cases, their aspect was highly evocative of Golgi-derived structures (Fig. 6Cd). GM130 down-regulation in NAGLU-depleted cells was associated with an almost total absence of vacuoles (Fig. 6Cc), whereas abundant vacuoles were observed in NAGLU-depleted cells expressing control DNA (data not shown). Vacuoles accumulating in NAGLU-depleted cells over-expressing GM130 presented the same characteristics and morphology as those accumulating in NAGLU-depleted cells expressing control DNA (data not shown).

Taken together, these results indicate that the formation of distended LAMP1-positive intracellular vacuoles, as observed in NAGLU-depleted cells, was mediated by the augmentation of GM130 levels.

DISCUSSION

Induction of the expression of shRNA directed against NAGLU mRNAs by tetracycline resulted in an abrupt deprivation of this enzyme in HeLa cells. After 7 days, NAGLU-depleted cells expressed disorders recapitulating cell alterations associated with MPSIIIB in mice (2) or humans (3). These disorders included the primary storage of HS glycosaminoglycans, the substrate of the missing enzyme, the secondary accumulation of GM3 gangliosides and the formation of distended intracellular vacuoles decorated with the lysosomal marker LAMP1. Rapid installation of cell disorders contrasted with slow neurological deterioration in affected individuals and animal models, indicating that cell damages precede clinical expression of the disease. They suggest acute toxic effects of undigested HS glycosaminoglycans fragments on cardinal cell functions. Consistently, our results showed that they affect GM130 functions, a molecule with multiple biological roles, including intracellular trafficking, Golgi dynamics, cell polarity and oriented cell migration (5).

Observations in NAGLU-depleted HeLa cells are relevant to MPSIIIB cell pathology

Three independent NAGLU-depleted HeLa cell clones exhibited phenotypic changes, when compared with non-depleted control cells. Controls consisted of HeLa cells containing...
NAGLU-specific shRNA sequences, which did not receive tetracycline, or HeLa cells containing non-specific scrambled shRNA, which were exposed to tetracycline. These cells were undistinguishable from parental HeLa cells. Phenotypic changes in NAGLU-depleted cells were therefore unlikely due to clonal selection, shRNA insertion in host cell DNA, tetracycline treatment or non-specific effects on mRNA processing.

Phenotypic changes in NAGLU-depleted HeLa cells faithfully reproduced typical MPSIIIB cell disorders. HS glycosaminoglycan and GM3 ganglioside storages are invariably observed in humans (1), mice (31) or dogs (32,33) with MPSIIIB. LAMP1-positive intracellular vacuoles are present in various tissues of the MPSIIIB mouse, including in neurons of the entorhinal (34) and rostral cortex (2). Ultrastructural examination of mouse brain sections (31,35), as well as post-mortem studies of human brain samples (36–38), revealed typical features also present in cultured MPSIIIB mouse cortical neurons (2), MPSIIIB patient-derived iPSc and neurons differentiated from these iPSc (3). These features include the co-existence in the same cell of vacuoles with light granular content and vacuoles with electron dense, often multi-lamellar materials. Alterations of Golgi morphology were initially described in MPSIIIB mouse neurons (2) and also found in neurons derived from MSPIIIB patient iPSc (3). The increased expression of GM130 and the presence of GM130 immuno-reactivity in vacuole limiting membranes were initially observed in MPSIIIB mouse neurons (2). The presence of all these defects in NAGLU-depleted HeLa cells suggests that these cells recruited pathogenic mechanisms related to MPSIIIB.

Figure 2. Vesicles with characteristics of abnormal lysosomes are not related to the endocytosis or autophagy pathways. (A–D) The identity of vesicles accumulating in NAGLU-depleted cells stably expressing control DNA (upper panel), and in non-depleted cells stably over-expressing FLAG-tagged GM130 (lower panel) was analyzed. (A) Cells were transduced with organelle lights lysosome-RFP, which targets LAMP1 (purple) and with HIV-IDUA-GFP (green). Nuclei were counterstained in blue. Merged confocal images revealed co-localization of the two markers in the same vesicles. Scale bars, 10 μm. (B) Cells transduced with HIV-IDUA-GFP (green) were stained with LysoTracker (purple). Nuclei were counterstained in blue. Merged confocal images revealed that vesicles expressing IDUA were acidic. Scale bars, 10 μm. (C) Cells were immuno-labeled with anti-LAMP1 antibodies (purple) and anti-M6P receptor antibodies (green). Nuclei were counterstained in blue. Merged apotome images show that LAMP1 signals did not colocalize with the late endosome marker M6P receptor. Scale bars, 5 μm. (D) Cells were immuno-labeled with anti-LAMP1 antibodies (purple) and anti-EEA1 antibodies (green). Nuclei were counterstained in blue. Merged apotome images show that LAMP1 signals did not colocalize with the early endosomal vesicle marker EEA1. Scale bars, 5 μm. (E) LC3-I (16 kDa band) to LC3-II (14 kDa band) conversion was analyzed by western blot on total protein extracts from NAGLU-depleted cells stably expressing control DNA and non-depleted cells stably expressing FLAG-tagged GM130. LC3-II was barely elevated in all experimental conditions, in contrast to a positive control (asterisk) provided by normal cells treated with rapamycin (250 nmol/l) and leupeptin (50 μmol/l).

Phenotypic alterations in NAGLU-depleted HeLa cells are related to GM130 over-expression

A characteristic feature of NAGLU-depleted HeLa cells was the increased size of the Golgi. Enlarged Golgi apparatus expanding into neurites was observed in mouse and human MPSIIIB neurons (2,3). Experimental GM130 over-expression increased Golgi size in control HeLa cells. Since GM130 is over-expressed in MPSIIIB neurons, we concluded that Golgi expansion in these cells was related to GM130 over-expression. This conclusion is in line with previous reports, indicating that GM130 controls Golgi extension. In neurons, the balance between two opposite signaling pathways, one involving Reelin and the other one involving GM130 interaction with YSK1, regulates Golgi dispersion into dendrites (10). In ldlG cells, a chinese hamster ovary mutant cell line that lacks GM130, the length of Golgi cisternae increased...
GM130-mediated Golgi expansion may result from increased cisternal membrane fusion triggered by GM130–GRASP65 complexes (8). It may also result from the expansion of tangential Golgi-derived microtubules bound to cis-Golgi membranes triggered by AKAP450-GM130 complexes (9,25).
The proliferation and swelling of vacuoles with characteristics of abnormal lysosomes is a hallmark of LSDs (39). It is prominent in various cell types in the MPSIIIB mouse model (31,35) and in MPSIIIB patient neurons. Vacuoles are characterized by their large size, the expression of the lysosomal marker LAMP1 in the limiting membranes and their heterogeneous contents. Vacuoles typical of LSD were observed in NAGLU-depleted HeLa cells. We provided

**Figure 4.** GM130 over-expression induces Golgi complex alterations in non-depleted HeLa cells. (A) Cultures transiently transfected with FLAG-tagged GM130 DNA construct (left panel), or siRNAs directed against GM130 (right panel) were immuno-labeled with anti-giantin (purple) antibodies. Anti-FLAG (green) antibodies identified cells over-expressing GM130. Anti-GM130 (green) antibodies identified GM130-expressing and GM130-depleted cells. Nuclei were counterstained in blue. When compared with non-transfected cells visible on the same field, merged apotome views showed higher giantin staining in cells positive for FLAG tag (arrowheads), and lower giantin staining in cells negative for GM130 (asterisks). For single labeling, see Supplementary Material, Figure S3. Scale bars, 10 μm. Giantin signal was quantified in at least 100 cells in three independent experiments (graph). Values are means ± SEM; *P < 0.05, **P < 0.01, ***P < 0.001, Student’s t-test. (B) Golgi complex morphology was analyzed by electron microscopy in normal cells stably over-expressing FLAG-tagged GM130. Pictures show elongated Golgi ribbons (left panel), marked Golgi bending (middle panel) and horseshoe-like Golgi structures (right panel). Scale bars, 0.2 μm. Quantification of Golgi length and bending was performed in comparison with cultures receiving control DNA (graphs). Values are means ± SEM from quantifications in at least 40 individual Golgi apparatus; **P < 0.01, ***P < 0.001, Student’s t-test.
evidence that their formation resulted from GM130 over-expression: they were cleared from NAGLU-depleted HeLa cells when GM130 expression was down-regulated; they were formed in control HeLa cells when GM130 was over-expressed. Ultrastructural aspects let us consider the possibility that at least some of these vacuoles directly emanated from abnormal Golgi structures. Evocative images consisted of dense vacuoles apparently resulting from bent Golgi ribbons forming horseshoe-like structures that subsequently circularized and closed up with stacks of compacted membranes entrapped in the lumen.

**GM130 over-expression causes sorting defects in the cis–medial-Golgi compartments**

Golgi alterations in NAGLU-depleted cells prevailed in early Golgi compartments but did not affect the trans-Golgi. A well-established role for microtubules and motor proteins moving...
on these tracks is to provide tensile forces required for membrane curvature and fission, which participate to vesicle sorting in the Golgi (40–42). Exacerbated microtubule anchoring and nucleation on Golgi membranes in NAGLU-depleted HeLa cells may enhance membrane deformation in the cis- and medial compartments and contribute to generate abnormal Golgi-derived vesicles. The presence of vesicles positive for the lysosomal marker LAMP1 and for early Golgi markers suggests that the phenomenon principally affected compartments destined to lysosome biogenesis.

Figure 6. Cell vacuolation is mediated by GM130 expression levels. (A) Cultures transiently transfected with FLAG-tagged GM130 DNA construct (upper panels), or siRNAs directed against GM130 (lower panels) were immuno-labeled with anti-LAMP1 (purple) antibodies. Anti-FLAG (green) antibodies identified cells over-expressing GM130. Anti-GM130 (green) antibodies identified GM130-expressing and GM130-depleted cells. Nuclei were counterstained in blue. When compared with untransfected cells visible on the same field, merged apotome views showed higher LAMP1 staining in cells positive for FLAG tag (arrowheads, upper panels), and lower LAMP1 staining in cells negative for GM130 (asterisks, lower panels). For single labeling, see Supplementary Material, Figure S5. Scale bars, 10 μm. LAMP1 signal was quantified (graph). Values are means ± SEM from signals quantified in at least 150 cells in three independent experiments; **P < 0.001, Student’s t-test. (B) Total proteins were extracted from cells stably over-expressing control DNA, FLAG-tagged GM130 DNA (left panel) or shRNA against GM130 (right panel) and analyzed by western blot for GM130 (130 kDa band), LAMP1 (110 kDa band) and actin (42 kDa band) expression levels. LAMP1 levels were expressed relative to actin (graph). Values are means ± SEM from three independent experiments; ***P < 0.01, **P < 0.001, Mann–Whitney test. (C) Cells were processed for electron microscopy. Low magnification images (upper row) showed extensive vacuolation in non-depleted cells over-expressing GM130 (a). In contrast, vacuoles were observed neither in non-depleted (b) nor in NAGLU-depleted (c) cells when GM130 was down-regulated. High magnifications of vacuolar structures observed in non-depleted cells over-expressing GM130 (bottom row) showed numerous vesicles with internal membranes. Morphology was evocative of circular Golgi-like structures (d), typical multi-lamellar vesicles (e) or dark electron-dense fingerprints formed by tightly compacted membranes (f, see insert). Other vesicles were clear (g), or contained heterogeneous granular or fibrillar material (h). Scale bars (a–c) 2 μm; (d–h) 0.1 μm.
The absence of M6P receptor indicates that abnormally formed vesicles were misrouted before they reached the trans-Golgi network. They therefore presumably lacked components necessary for fusion with the endo-lysosomal system, such as clathrin adaptor proteins. This defect can generate a dead-end compartment. Altered response to brefeldin A in NAGLU-depleted HeLa cells and in mouse cortical neurons (2) is consistent with abnormal sorting in proximal Golgi apparatus (43). The accumulation of vesicles positive for GM130, negative for LAMP1 and loaded with GM3 gangliosides may also be a consequence of sorting defects in proximal Golgi compartments, where glycosphingolipid synthesis is initiated. Whereas GM3 ganglioside synthesis principally takes place in proximal Golgi compartments, GM2 ganglioside synthesis is carried out in distal Golgi compartments (15–17). The accumulation of GM3-positive vesicles in NAGLU-depleted HeLa cells, and the predominant overload of GM3 in MPSIIIB mouse (35), dog (32) and children (44) brains, when compared with GM2, may therefore arise as a consequence of sorting defects in proximal Golgi compartments. Our previous observations of normal glycosylation pattern of the lysosomal hydrolase α-L-iduronidase and normal trafficking of ts045VSV-G in MPSIIIB mouse neurons suggested that Golgi defects were nevertheless compatible with unaltered trafficking along the secretory pathway.

**GM130 over-expression is a consequence of deficient HS degradation**

In MPSIIIB model animals (35) or cultured cells (2,45,46), correction of NAGLU deficiency through genetic trans-complementation indicated that the unique primary cause of cell defects associated with the disease is the defective clearance of undigested HS glycosaminoglycans. Our results show that GM130 is a target of HS glycosaminoglycan pathogenicity.

How do HS affect GM130 functions? HS proteoglycans present at the cell surface and in the extracellular matrix (ECM) bind fibrillar proteins of the matrix and cell surface receptors, including integrins and fibroblast growth factor receptors (FGF-R) (47–49). Modified levels of mRNAs coding for ECM components, integrins and FGF-R were observed in MPSIIIB patient iPSc-derived neural stem cells (3). Expression of these mRNAs was also modified in NAGLU-depleted HeLa cells (data not shown), suggesting alteration of cell sensing of the environment with possible consequences on cell adhesion, proliferation, polarization and migration (50–52). GM130 is also involved in the control of several of these cardinal cell functions (10,53,54). Although links between integrin or FGF-R signaling and GM130 functions have not been established, our results raise the possibility that GM130 acted as a mediator of activation pathways recruited by extra-cellular cues.

**MATERIALS AND METHODS**

**Antibodies**

Antibodies (dilutions for immunofluorescence): mouse mAb IgG1 anti-LAMP1 (clone H4A3, 1:200, SouthernBiotech, Birmingham, AL, USA), mouse mAb IgG1 anti-GM130 (clone 35, 1:200, BD Biosciences, Erembodegem, Belgium), rabbit polyclonal anti-GM130 (1:200, Sigma, Lyon, France), rabbit polyclonal anti-Sec23 (COPII, 1:300, Affinity BioReagents, Golden, CO, USA), rabbit polyclonal anti-GRASP65 (1:500, Novus Biologicals, Cambridge, UK), rabbit polyclonal anti-giantin (1:500, Covance, Emeryville, CA, USA), mouse mAb IgG1 anti-golgin97 (clone CDF4, 1:200, Molecular Probes, Eugene, OR, USA), mouse mAb IgG1 anti-p230 (1:200, BD Biosciences), rabbit polyclonal anti-mannosidase II (1:200, Millipore, Molsheim, France), mouse mAb IgM anti-HS clones HepSS-1 and 10E4, which recognize different epitopes on saccharide chains (55) (1:200, Seikagaku, Tokyo, Japan), mouse mAb IgM anti-GM3 (clone GMR6, 1:100, Seikagaku), mouse mAb IgG2a anti-M6P receptor (1:200, AbCam, Cambridge, UK), rabbit polyclonal anti-EEA1 (1:200, Affinity BioReagents), rabbit polyclonal anti-pericentrin (1:1000, AbCam), rat mAb IgG2a anti-α-tubulin (clone YL1/2, 1:200, AbD Serotec, Düsseldorf, Germany), mouse mAb IgG2b anti-acetylated-α-tubulin (clone 6-11B-1, 1:100, Invitrogen, Cergy-Pontoise, France), mouse mAb IgG1 anti-AKAP450 (1:200, BD Biosciences), rabbit polyclonal anti-FLAG (1:200, Sigma) and mouse mAb IgG1 anti-FLAG (clone M2, 1:500, Sigma). Secondary antibodies conjugated to Alexafluor® or DyLight™ were from Molecular Probes (Invitrogen) and Jackson ImmunoResearch Laboratories (West Grove, PA, USA).

**Molecular biology**

Five siRNAs targeting the human NAGLU mRNA sequence were assayed on HeLa cells. The two best sequences with respect to NAGLU mRNA depletion were selected for shRNA construction. Two scrambled control sequences with similar nucleotide compositions were designed. Selected sequences were introduced in the BLOCK-iTTM Inducible pENTRTM/H1/TO Entry Vector (Invitrogen), according to the manufacturer’s instructions. Selected clones expressed the following NAGLU-specific target sequence: 5′-GCCA GAAAGGATTCCTATTTCAAGAGATAGACCACTTGG TTCTGCG-3′. The corresponding scrambled sequence was 5′-GGCACGGAACCGGTATATTTCAAGAGATATACCG TCGTTGCTGCG-3′. The loop sequence is indicated in bold. GM130 RNA interference (RNAi) was done using previously described siRNA oligos, and a scrambled siRNA duplex as a negative control (8). All DNA and RNA oligos were purchased from Eurogentec. The pFLAG-GM130 plasmid was a kind gift from Dr C. Sutterlin, University of California, Irvine, CA, USA. Control plasmid consisted in the empty pCMV-Tag2 backbone. GM130 shRNA lentiviral particles and control shRNA lentiviral particles were from Santa Cruz (Heidelberg, Germany).

**Cell culture and transfections**

The cell line, cell culture reagents, media and Dulbecco’s phosphate buffered saline (DPBS) were obtained from Invitrogen. Fetal bovine serum (FBS) was from Sigma. The HeLa T-Rex cell line expressing a tetracycline repressor was maintained in MEM medium complemented with 10% FBS, 2 mM...
g-glutamine, 100 μg/ml penicillin/streptomycin, 5 μg/ml blasticidin and non-essential amino acids. We used FuGENE® 6 (Roche, Meylan, France) for plasmid transfections, and Lipofectamine™ RNAiMAX (Invitrogen) for siRNA transfections, according to the manufacturer’s instructions. To generate stable cell lines in which the expression of NAGLU can be turned off using tetracycline-inducible shRNA expression, HeLa T-Rex cells were plated in six-well plates and transfected with the inducible pENTRTM/H1/TO plasmids. Forty-eight hours after transfection, the dishes were split, diluted 1:100, 1:500, 1:2000 in 10 cm dishes and zeocin was added to the medium at a concentration of 200 μg/ml. Following a 14-day selection period, zeocin-resistant isolated colonies were picked and propagated. To induce NAGLU depletion, tetracycline was added into the culture medium at a concentration of 1 μg/ml. Negative controls consisted in non-induced cells, providing an internal control for each clone, and tetracycline-induced scrambled controls.

In transient transfection experiments, transfections were performed at day 4 after initiation of tetracycline treatment and cells were grown for 3 days after transfection before analysis. To generate stable cell populations expressing the FLAG-tagged GM130 plasmid construct, cell transfection was followed by G418 selection for 10 days. Negative control consisted of G418-resistant populations transfected with the empty plasmid backbone. Control shRNA and GM130 shRNA lentiviral particle transduction was performed following the manufacturer’s recommendations. Following transduction, stable populations were selected via puromycin dihydrochloride selection. Selected cells were treated with tetracycline for 7 days.

Quantitative RT–PCR
Total RNA was extracted with Trizol (Invitrogen) at different time points after tetracycline induction according to the manufacturer’s instructions. One microgram of RNA was reverse transcribed as previously described (56). Reverse transcription of the RNAs was followed by Q-PCR performed on a 7300 Real Time PCR System (Applied Biosystems, Foster city, CA, USA) with 100 ng of cDNA. For the amplification of NAGLU and ARPO (endogenous control) cDNAs, reaction mixtures were prepared containing 300 nM of each forward and reverse primers and the SYBR Green PCR Master Mix (according to Applied Biosystems procedures). The following primers were used: for hNAGLU, forward 5’-TTGCACTCATGACCCATTTCTC-3’, reverse 5’-GGTGAATCTCGTCTCCACAGACA-3’, and for hARPO forward 5’-CGAGAGTGAAAGGATGGTG-3’, reverse 5’-CTCTGGTATCAGCTGCGTGTAGT-3’. For the amplification of GM130 and GAPDH (endogenous control) cDNAs, the primers and TaqMan probes were designed by Applied Biosystems. Reaction mixtures containing the primers, the TaqMan probe and the TaqMan Universal PCR Master Mix were prepared according to Applied Biosystems procedures. Universal cycling conditions (2 min at 50°C, 10 min at 95°C, 40 cycles of 15 s at 95°C and 1 min at 60°C) were used. Each sample was analyzed in triplicate. Negative controls included omission of reverse transcriptase at the cDNA synthesis step and omission of the template at the PCR step. cDNA amounts were expressed as 2exp(Ct1-Ct2), in which Ct1 is a reference Ct measured for the amplification of the endogenous control cDNAs, and Ct2 is the Ct measured for the amplification of the examined cDNA.

Enzyme assays
To determine NAGLU enzymatic activity, cell pellets collected at different time points after tetracycline induction were suspended in water, submitted to 10 freeze thaw cycles and clarified by centrifugation. Samples were diluted to a total protein concentration (determined with a BCA protein assay kit, Pierce, Rockford, IL, USA) of 2 mg/ml, and the enzyme assay for NAGLU was carried out as previously described (57) using 4-methylumbelliferyl-N-acetyl-α-D-glucosaminide (4MU) (Merck, Nottingham, UK) as fluorogenic substrate. Hydrolysis of 1 nmol of substrate per hour per milligram of protein was defined as one catalytic unit.

Glycosaminoglycan analysis
For metabolic labeling, cells treated with tetracycline for 4, 7 or 11 days were further incubated for 3 days with 10 μCi/ml [3H]-glucosamine (Perkin Elmer, Waltham, MA, USA) in medium containing 2% FBS before harvesting, washed with DPBS, suspended in water and lysed using freeze thaw cycles. An aliquot was taken out for determination of the protein concentration using a BCA protein assay kit. Lipids were extracted by the addition of chloroform and methanol (chloroform-methanol-water 4:8:3, v/v/v). After a 10 min incubation at room temperature, extracts were recovered by centrifugation (1000g for 10 min), washed with acetone, dried and subjected to proteolysis overnight at 65°C with 1 mg/ml papain (Sigma) in 100 mM sodium acetate buffer containing 5 mM ethylene diamine tetra-acetic acid and 5 mM cysteine (pH 5.5). The incorporation of [3H]-glucosamine was measured by liquid scintillation counting, and normalized against protein concentration determined with a BCA protein assay kit.

Immunofluorescence microscopy
Cells grown in 10% FBS were processed at day 7. Live-cell staining was performed using LysoTracker® (Invitrogen), a marker of acidic vesicles, Organelle Lights™ Lysosome-RFP (Invitrogen), a baculovirus inducing the production of fluorescent LAMP1, and a lentivirus vector coding for the fluorescent lysosomal hydrolase IDUA-GFP. LysoTracker and Organelle Lights Lysosome-RFP were used according to the manufacturer’s instructions. HIV-IDUA-GFP was produced and used as previously described (58).

All other staining was performed on fixed cells. For immunocytochemistry of cytoskeletal elements, cells were fixed for 10 min with ice-cold methanol at −20°C. In other cases, cells were fixed for 15 min with warm 4% paraformaldehyde and permeabilized with saponin (0.01%). Cells were then processed as previously described (2).

Cells were imaged either with an Axioplan 2 imaging optic microscope equipped with Apotome and AxioCam TR camera controlled by the AxioVision software (Zeiss, Le Pecq, France), or with a SP5 confocal system (Leica, Downloaded from https://academic.oup.com/hmg/article-abstract/21/7/1481/2900622 by guest on 29 December 2018
Rueil-Malmaison, France). Time exposure was kept constant for all analyses in the same experiment. For quantification of immuno-labeling, fluorescent signal was binarized, keeping threshold constant for all acquisitions in the same experiment. High threshold allowed recording of specific signals produced by anti-GM130, anti-golgin97, anti-giantin, anti-pericentrin or anti-LAMP1 antibody stainings. Low threshold produced background signal on the entire cell surface, which was used to manually outline the cells, and normalize specific signal to the cell surface. For AKAP450 localization studies, a high threshold was used to restrict analysis to the most intense pixels, mostly localized at the centrosome. A lower threshold was used to bring out Golgi-localized AKAP450 pixels. Signal pixel numbers (specific signal, background signal or co-localized signal) were measured by using the AxioVision co-localization module.

Electron microscopy

Samples and ultrathin sections were prepared at day 7 as previously described (2). Analyses were performed on a JEOL 1200EXII transmission electron microscope (JEOL, Croissysur-Seine, France) equipped with an Eloise Megaview camera controlled by analysis Pro 3.1 software (Eloise, Roissy-CDG, France). Quantifications were performed using the interactive measurements module of the AxioVision software.

Western blot analysis

To obtain total protein extracts, cell monolayers were washed with DPBS at day 7 and lysed on ice for 2 min in radio-immunoprecipitation assay buffer (Sigma) containing a protease inhibitor cocktail and a phosphatase inhibitor cocktail (Roche). 1 U/μl of a Benzonase nuclease (Merck) was added to the cell lysate, followed by a 20 min incubation at 37°C. Protein samples (10 μg) were resolved by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (SDS–PAGE) using 7% Tris-acetate gels (Invitrogen). For GM130 and LAMP1 detection, chemiluminescent western blots were used, as previously described (46). For double detection of total α-tubulin, and acetylated-α-tubulin, infrared fluorescent western blots were used. Gels were blotted onto Immobilon-FL polyvinylidene difluoride membranes (Millipore), which were blocked with Odyssey blocking buffer (Li-Cor Biosciences, Lincoln, NE, USA). The primary antibodies diluted in blocking buffer containing 0.1% (v/v) Tween 20 were added to the membrane and incubated overnight at 4°C. Membranes were then washed with PBST (PBS plus 0.1% (v/v) Tween 20), and revealed with anti-mouse Ig AF680 (Invitrogen) or anti-rat Ig IRDye800 (Li-Cor Biosciences) secondary antibodies, diluted at 1:5000 in Odyssey blocking buffer containing 0.01% (v/v) SDS and 0.1% (v/v) Tween 20. The membrane was washed three times with PBST and visualized using the Odyssey imaging system (Li-Cor Biosciences).

The following antibody dilutions were used: mouse mAb IgG1 anti-LAMP1 (1:500), mouse mAb IgG1 anti-GM130 (1:200), rabbit polyclonal anti-LC3B (1:200, Novus Biologicals), mouse mAb IgG2a anti-actin (1:2000, Sigma), rabbit polyclonal anti-GAPDH (1:5000, Sigma), rat mAb IgG2a anti-α-tubulin (1:1000) and mouse mAb IgG2b anti-acetylated-α-tubulin (1:500).

Statistical analysis

Statistics were performed using the SPSS software (SPSS).

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG online.

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Conflict of Interest statement. None declared.

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