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Cytosolic glucosylceramide regulates endolysosomal function in Niemann-Pick type C disease

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ABSTRACT

Niemann-Pick type C disease (NPCD) is a neurodegenerative disease associated with increases in cellular cholesterol and glycolipids and most commonly caused by defective NPC1, a late endosomal protein. Using ratiometric probes we find that NPCD cells show increased endolysosomal pH. In addition U18666A, an inhibitor of NPC1, was found to increase endolysosomal pH, and the number, size and heterogeneity of endolysosomal vesicles. NPCD fibroblasts and cells treated with U18666A also show disrupted targeting of fluorescent lipid BODIPY-LacCer to high pH vesicles. Inhibiting non-lysosomal glucocerebrosidase (GBA2) reversed increases in endolysosomal pH and restored disrupted BODIPY-LacCer trafficking in NPCD fibroblasts. GBA2 KO cells also show decreased endolysosomal pH. NPCD fibroblasts also show increased expression of a key subunit of the lysosomal proton pump vATPase on GBA2 inhibition. The results are consistent with a model where both endolysosomal pH and Golgi targeting of BODIPY-LacCer are dependent on adequate levels of cytosolic-facing GlcCer, which are reduced in NPC disease.

Niemann-Pick type C disease (NPCD) is a devastating neurodegenerative condition most commonly due to mutations in NPC1 (Puri et al., 1999; Sugimoto et al., 2001) a protein of late endosomes and lysosomes (Higgins et al., 1999). (Due to difficulties in precisely distinguishing these two sets of organelles the term endolysosome will be used here to include both.) Mutations in NPC1 are associated with impaired endocytic transport via decreased endolysosomal calcium release (Shen et al., 2012; Lloyd-Evans et al., 2008). In turn, endocytosis and luminal calcium are dependent on correct endolysosomal acidification (Shen et al., 2012) and have been found to be controlled by glycolipids in neurons (Shen et al., 2014), melanocytes (van der Poel et al., 2011), plant vacuoles (Yamaguchi and Kasamo, 2001) and *C. elegans* (Zhu et al., 2013). It is increasingly apparent that aberrant lysosomal GlcCer in Gaucher disease is associated with elevated endolysosomal pH (Sillence, 2013; De La Mata et al., 2017; Magalhaes et al., 2015; Bourdenx et al., 2016). Glycolipids, vital for mammals (Yamashita et al., 1999), are also implicated in membrane trafficking (Sillence, 2013; Sillence et al., 2002). Similarly in yeast the NPC1 homologue ncr1 regulates both vacuolar pH (Brett et al., 2011) and glycolipid transport (Malathi et al., 2004). An overview of sphingolipid metabolism highlighting the connection with endocytosis is offered in Fig. S1.

Does NPC1 affect endolysosomal pH? Conflicting evidence has been found both in disease fibroblasts (Lloyd-Evans et al., 2008; Brett et al., 2011; Bach et al., 1999; Tharkeshwar et al., 2017; Chakraborty et al., 2017) and in cells treated with U18666A, a putative inhibitor of NPC1 (Lloyd-Evans et al., 2008; Lafourcade et al., 2008; Lu et al., 2015; Shoemaker et al., 2013). If endocytic traffic is delayed then probes which permeate all acidic organelles will show increased pH values even though fully mature lysosomes still acidify correctly. We used such a general probe to measure endolysosomal pH in NPCD cells and cells treated with U18666A and found the pH higher than normal. Endocytosis was also disrupted.

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To address these issues we also examined glycolipid transport. NPCD cells and culture models were found to have decreased non-vesicular glucosylceramide (GlcCer) transport. In contrast, inhibiting non-lysosomal glucocerebrosidase (GBA2) decreased endolysosomal pH in normal cells, reversed increases in endolysosomal pH, increased ATP6V0a1 expression and restored disrupted BODIPY-LacCer trafficking in NPCD fibroblasts. The results are consistent with a model where both endolysosomal pH and Golgi targeting of BODIPY-LacCer are dependent on adequate levels of GlcCer on the cytosolic face of membranes; NPC disease reduces this lipid subpopulation.

1. Results

1.1. U18666A increases the size and pH of the endolysosomal compartment

An NPC disease phenotype can be induced by a variety of cationic amphiphiles (which also inhibit filoviral fusion) (Shoemaker et al., 2013; Ng et al., 2014; Johansen et al., 2013). Previously it has been reported that the cationic amphiphile U18666A increases the pH of endolysosomes as measured by fluorescence ratio imaging (Lafourcade et al., 2008). Since acidification is a critical step in endosomal maturation the endolysosomal pH of U18666A-treated RAW macrophages was measured using acridine orange as a pH sensor. Acridine orange accumulates within endolysosomes due to protonation and changes colour from green to red. Fig. 1A,D show that U18666A treatment decreases the red/green ratio of punctates labelled with acridine orange (control 2.2 ± 0.2 to 1.0 ± 0.1 U18666A treated), consistent with an alkalinising effect on endolysosomes. U18666A treatment also increased the size (Fig. 1E) and red/green heterogeneity (Fig. 1A) of acridine labelled punctates, similar to what has been reported before (Funk and Krise, 2012; te Vruchte et al., 2014; Xu et al., 2012). In order to control for changes in lysosome size experiments were performed with NH_4Cl which led to similar decreases in red/green ratio alone or in the presence of U18666A (Fig. 1D).

1.2. Niemann-Pick C cells show increased endolysosomal pH

Due to potential pitfalls of acridine orange staining such as unequal loading and effects of cholesterol (Wang et al., 2006) further experiments were performed using LysoSensor yellow/blue (Diwu et al., 1999) as a ratiometric probe which changes colour in a concentration-independent manner. Using this probe we obtained a pH value for the total endolysosomal compartment of RAW cells of 5.4 ± 0.1; treatment with 5 μM U18666A for 2.25 h increased this to 6.4 ± 0.3 (Fig. 2A,B).

Control fibroblasts gave a value of 4.5 ± 0.1 in line with previously reported values (Otomo et al., 2011; Coffey et al., 2014) while in NPC patient fibroblasts this increased to 5.4 ± 0.3; patient lymphoblasts showed similar increases in total endolysosomal pH (Fig. 2A). (For correlation curves see Fig. S2.) This increase in NPCD endolysosomal pH is in agreement with previous studies (Tharkeshwar et al., 2017; Chakraborty et al., 2017) and with reports that GlcCer accumulation leads to delayed endolysosomal acidification (Tharkeshwar et al., 2017; Chakraborty et al., 2017; Lafourcade et al., 2008). It is not inconsistent with contrary observations of mature NPCD lysosomes.

We were also interested in the kinetics of pH increase resulting from U18666A treatment. In RAW cells endolysosomal pH increased over 2 h on U18666A treatment (Fig. 2B), though the rise was evident after 45 min and mostly complete by 2 h. Thus the pH increase with this

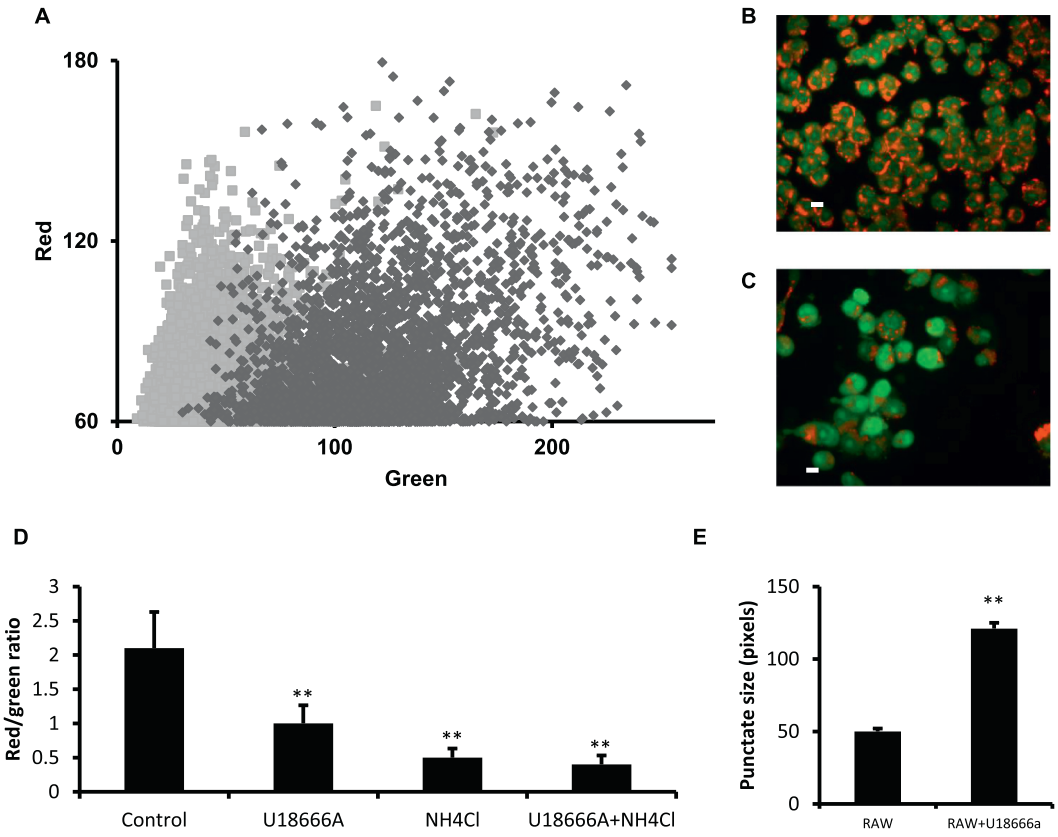

Fig. 1. The size and pH of the endolysosomal compartment increases in RAW cells treated with U18666A A) Red/Green scattergraph of acridine labelled punctates; untreated (■); U18666A (♦). B, C) Representative images of untreated (B) RAW cells and cells treated with U18666A (C). Scale bars represent 10 μm. D) Quantitation of red/green ratio in RAW cells in the presence of U18666A, and incubations with cells treated with 10 mM NH_4Cl. E) Quantitation of punctate size in the presence of U18666A. (** $p < .001$ $n = 5–10$, *t*-test). Results are presented as mean ± SD (D) and mean ± SEM (E). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

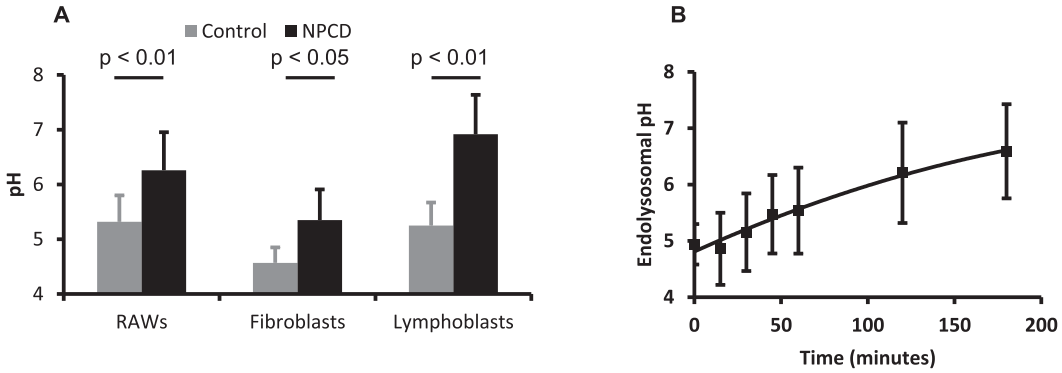

Fig. 2. NPC cell culture models show increased lysosomal pH measured using LysoSensor yellow-blue. Cells were labelled with 5 μM lysosensor yellow/blue in 1 ml RPMI or DMEM (10%FCS) for 5 min at 37 °C. Excess dye was removed by cold PBS. The emission ratio at 451/520 was measured at Ex 320/360 nm. RAW cells were treated with 5 μM U18666A for 2.25 h unless otherwise stated; A) Quantitation of lysosomal pH in RAW cells and fibroblasts; n = 4–11, significance measured by t-test and ANOVA) B) time course of increased pH in RAW cells treated with U18666A. Significant difference (t-test, $p < .05$) at 180 min but not before. All results are presented as mean ± SD. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

agent precedes the accumulation of both cholesterol (Lloyd-Evans et al., 2008) and GlcCer (Fig. 6D).

1.3. BODIPY-LacCer traffics to a high pH compartment in U18666A-treated RAW macrophages and NPC fibroblasts

BODIPY-LacCer can be used as a marker of endocytosis. This fluorescent lipid traffics from late endosomes to the Golgi via a pathway dependent on NPC1, rab 7/9 and TRPML1 (Pryor et al., 2006; Choudhury et al., 2002); correct endolysosomal acidification is also important for the successful completion of endocytosis (Baravalle et al., 2005; Bayer et al., 1998; Clague et al., 1994; van Weert et al., 1995). In many glycolipid storage diseases such transport is disrupted and instead the fluorescent lipid co-localises with late endosomes and lysosomes (Puri et al., 1999; Chen et al., 1999). Given the effects of U18666A on endolysosomal pH we next studied evidence for BODIPY-LacCer endocytic trafficking to high pH compartments. RAW cells were pulse-labelled with BODIPY-LacCer and, in the presence of U18666A, the fluorescent marker incompletely co-localised with LysoTracker red (Fig. 3A, Pearson's co-localisation coefficient 0.51 ± 0.01). Further experiments in NPC human fibroblasts were conducted and revealed a similar effect (Fig. 3B, Pearson's co-localisation coefficient 0.17 ± 0.02). This result contrasts with other storage diseases where co-localisation of BODIPY-LacCer and a variety of late endosomal and lysosomal markers (Puri et al., 1999; Chen et al., 1999) including LysoTracker red (Sillence et al., 2002) occurs. In NPC cell culture models it appears that BODIPY LacCer sorting is diverted to a high pH compartment (pH > 6.5), consistent with increased endolysosomal pH.

1.4. Inhibition of GBA2 decreases endolysosomal pH and reverses altered BODIPY-LacCer targeting in NPC fibroblasts

Previous studies have used brefeldin A to merge the ER and Golgi thus co-localising ceramide and GlcCer synthase and increasing the amount of GlcCer on the cytosolic face of membranes ('cytosolic GlcCer') (Warnock et al., 1994). (In our hands this tactic increased GlcCer levels by about 50% in CHO cells (Fig. 6D), similar findings were obtained in fibroblasts (data not shown).) In fibroblasts, we discovered that endolysosomal pH was lowered under these conditions in both control and disease cells (4.5 ± 0.1 to 4.2 ± 0.1 for control cells, 5.4 ± 0.3 to 4.6 ± 0.1 for NPCD cells, Fig. 4A). The converse is also true: high endolysosomal pH is linked to low levels of GlcCer in melanocytes (7) and mouse macrophages (10). Consequently we increased cytosolic GlcCer by inhibiting its hydrolase GBA2 an enzyme which is upregulated in a mouse model of NPCD (Marques et al., 2015). Initial experiments with 6 μM NB-DGJ and 1 μM NB-DNJ lowered endolysosomal pH (data not shown). (Although these imino-sugars have been developed as a GlcCer synthase inhibitors they are more potent GBA2 inhibitors (Ridley et al., 2013); NB-DNJ (miglustat) is approved for the treatment of NPCD.) We then switched attention to AMP-DNJ (Overkleeft et al., 1998), a version of NB-DNJ but an even more potent inhibitor of GBA2, which has shown promise in a recent in vivo study of a mouse model of NPCD (40). Following precedent (Dekker et al., 2011; Marques et al., 2016) the use of 20 nM AMP-DNJ indeed corrected the endolysosomal pH defect by inhibition of GBA2. Thus the endolysosomal pH of NPC cells reduced from 5.4 ± 0.3 to 4.5 ± 0.17 (Fig. 4B). When normal fibroblasts were subjected to the same treatment the endolysosomal pH also reduced to 4.1 ± 0.08 (Fig. 4B). To exclude the possibility of off-target pharmacology we also measured endolysosomal pH in a near-haploid chronic myelogenous leukaemia cell line (HAP1 cells) Consistent with our previous results, CRISPR genetic knockout of GBA2 led to a decrease in endolysosomal pH (Fig. S3). AMP-DNJ treatment increases total GlcCer between 1 and 20 nM in NPC fibroblasts (Fig. S4).

Previous experiments in Gaucher disease have suggested that not only endolysosomal pH but also BODIPY-LacCer trafficking are dependent on cytosolic GlcCer levels (Sillence et al., 2002). Thus inhibiting GlcCer synthase by high concentrations of NB-DNJ and NB-DGJ (100 μM) gave aberrant trafficking which was repaired by specifically replenishing cytosolic GlcCer by adding GlcSph (Sillence et al., 2002). In order to test if this finding could be replicated in NPCD cells we initially treated NPC fibroblasts with imino-sugars at concentrations where they would be expected to inhibit GBA2 selectively. Fig. 4C,D show reversal of disrupted BODIPY-LacCer targeting using both inhibitors. Representative images from fluorescence microscopy are shown in Fig. 4E-F; BODIPY-TR-ceramide is used as a Golgi marker in these images which therefore show how Golgi targeting can be assessed. Use of NB-DGJ at a concentration expected to inhibit GlcCer synthase (> 12 μM) did not rectify trafficking (Fig. 4C). The results are consistent with a model where both endolysosomal pH and Golgi targeting of BODIPY-LacCer are dependent on cytosolic GlcCer which is disrupted in NPC disease.

1.5. vATPase a subunit expression is increased on GBA2 inhibition

Our findings above suggest that cytosolic GlcCer is necessary for activation of the endolysosomal proton pump vATPase, as previously argued (van der Poel et al., 2011; Zhu et al., 2015). If this lipid pool is deficient in NPC disease then we may expect altered expression of vATPase subunits. Expression of subunit a (ATP6V0a1) was examined by PCR and western blotting and was similar in both healthy and NPC cells (Fig. 5A-C). In contrast, GBA2 inhibition with AMP-DNJ

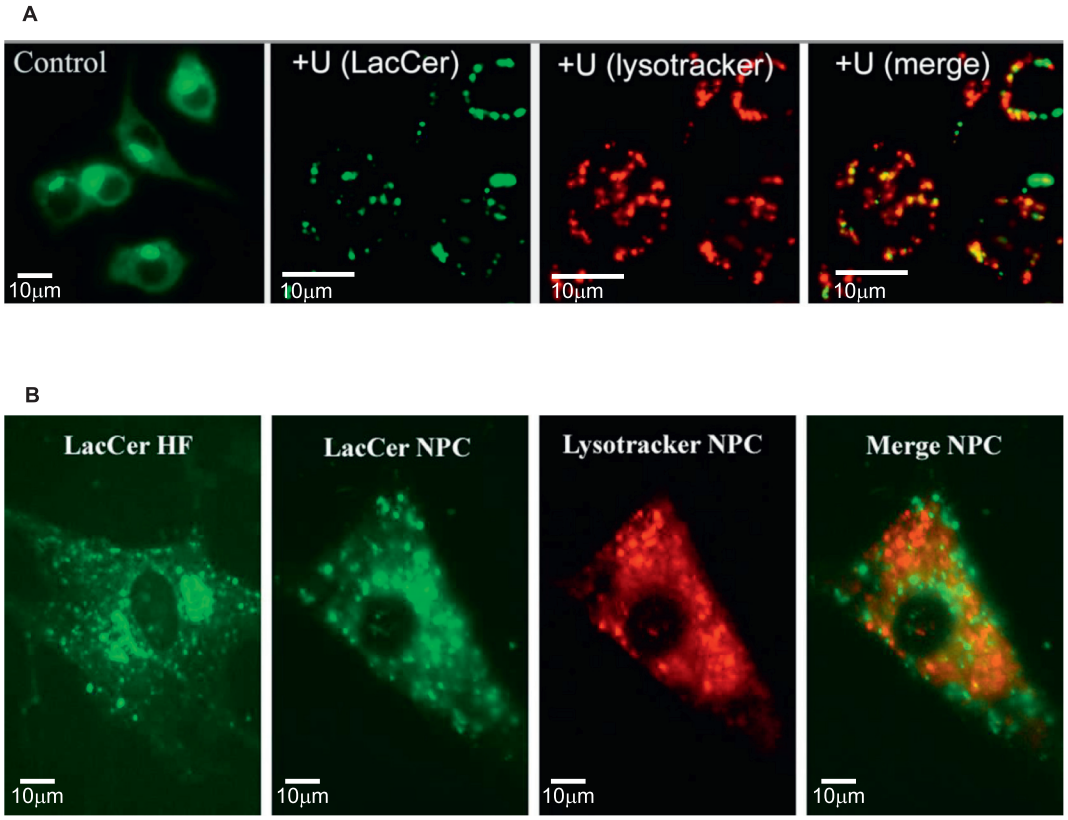

Fig. 3. U18666A gives BODIPY-LacCer trafficking that is disrupted and targets a punctate compartment that partially co-localises with LysoTracker red. Representative images of RAW macrophages (A) and healthy and NPC fibroblasts (B) showing the endocytic sorting of BODIPY-LacCer. (Pearson's coefficient RAW 0.51 ± 0.01 n = 5; NPCFs 0.17 ± 0.02 $n = 9$). Cells were pulsed for 45 mins with 15 μM BODIPY-LacCer; RAWs were chased for 60 min, fibroblasts were chased for 90 min. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

significantly increased ATP6V0a1 expression at the protein level (Fig. 5A-C) in both cell types. A similar increase was observed with mRNA, though this was not significant. These experiments are consistent with our observations of endolysosomal pH (Fig. 4A,B). (A dose of 10 nM AMP-DNJ had given optimal pH reductions in a variety of other cell types (data not shown) so this concentration was used in the blotting experiments.)

Using the recently determined structure (Mazhab-Jafari et al., 2016) of the yeast homologue (PDB: 5TJ5) as a template we built a model (Arnold et al., 2006; Biasini et al., 2014) of part of the membrane resident portion of human vATPase (3 of the ten c subunits and the a subunit) and validated it using QMEANBrane (Studer et al., 2014) which indicated acceptable quality (local score 0.50–0.79 for relevant regions, Fig. S5). Using molecular docking software ROSIE (Lyskov et al., 2013; Lyskov and Gray, 2008; Combs et al., 2013) which allows some flexibility of both sidechains and backbone we found a number of binding poses where the GlcCer headgroup uses hydrogen−bonds to bridge the a and c subunits. An example is shown in Fig. 5D: the headgroup is positioned where the membrane border is expected to be with the tails oriented correctly, (see also the QMEANBrane output in Fig. S5.) This binding is in agreement with a previous report of cross-linking of photoactivatable pacGlcCer with the vATPase c subunit (van der Poel, 2010).

The vATPase a subunit is highly conserved across eukaryotes. However the predicted critical residue for GlcCer binding only showed a high level of conservation from mammals to GlcCer containing yeast (Fig. 5E). In most non-GlcCer expressing yeast this residue was replaced by a glycine which would not be able to support GlcCer binding (Fig. 5C, S6). This is highly suggestive that this important residue correlates with GlcCer. (The presence of Asp rather than Asn in most GlcCer containing yeasts is not significant as this ionised residue is still able to perform the H−bond acceptor function (Gorbitz and Etter, 1992) of Asn depicted in Fig. 5D.)

1.6. The impact of NPC1 inhibition on transport of GlcCer to the cell surface

To probe further this putative insufficiency in cytosolic GlcCer we measured cell surface transport which would be likely reduced in NPCD. To this end glycolipid transfer protein (GLTP) was used to extract radiolabelled GlcCer from the cell surface following a previous procedure (Halter et al., 2007). GlcCer is transported by both vesicular and non-vesicular mechanisms (Halter et al., 2007; D'Angelo et al., 2013, 2007) and cytosolic GlcCer levels are limited by translocation ('flipping') in the ER and a post-Golgi compartment (Halter et al., 2007). Over 2 h inhibition of vesicular transport by brefeldin A (BFA) partially inhibited transport of GlcCer to the cell surface by 30–40% while U18666A inhibited GlcCer transport by ~60% even in the presence of BFA (Fig. 6A). Thus U18666A rapidly inhibits non-vesicular GlcCer transport. Broadly similar observations were made in NPC fibroblasts (Fig. 6C). However, inhibition of GlcCer transport did not result in significant increases in GlcCer synthesis and only small increases in levels of this lipid occurred in the timeframe of the experiment (Fig. 6D). At longer time points significant increases in GlcCer labelling were detected probably due to the eventual storage of lysosomal GlcCer (Fig. 6D).

In order to understand the selectivity of cytosolic glycolipid transport we next studied traffic of galactosylceramide (GalCer) to the cell surface. The results were initially similar to those obtained with GlcCer: BFA inhibited transport of GalCer to the cell surface by 30–40% consistent with significant non-vesicular transport from the ER/Golgi to the cell surface; U18666A also inhibited GalCer transport by ~30% in the absence of BFA. However, in contrast to GlcCer, GalCer transport was

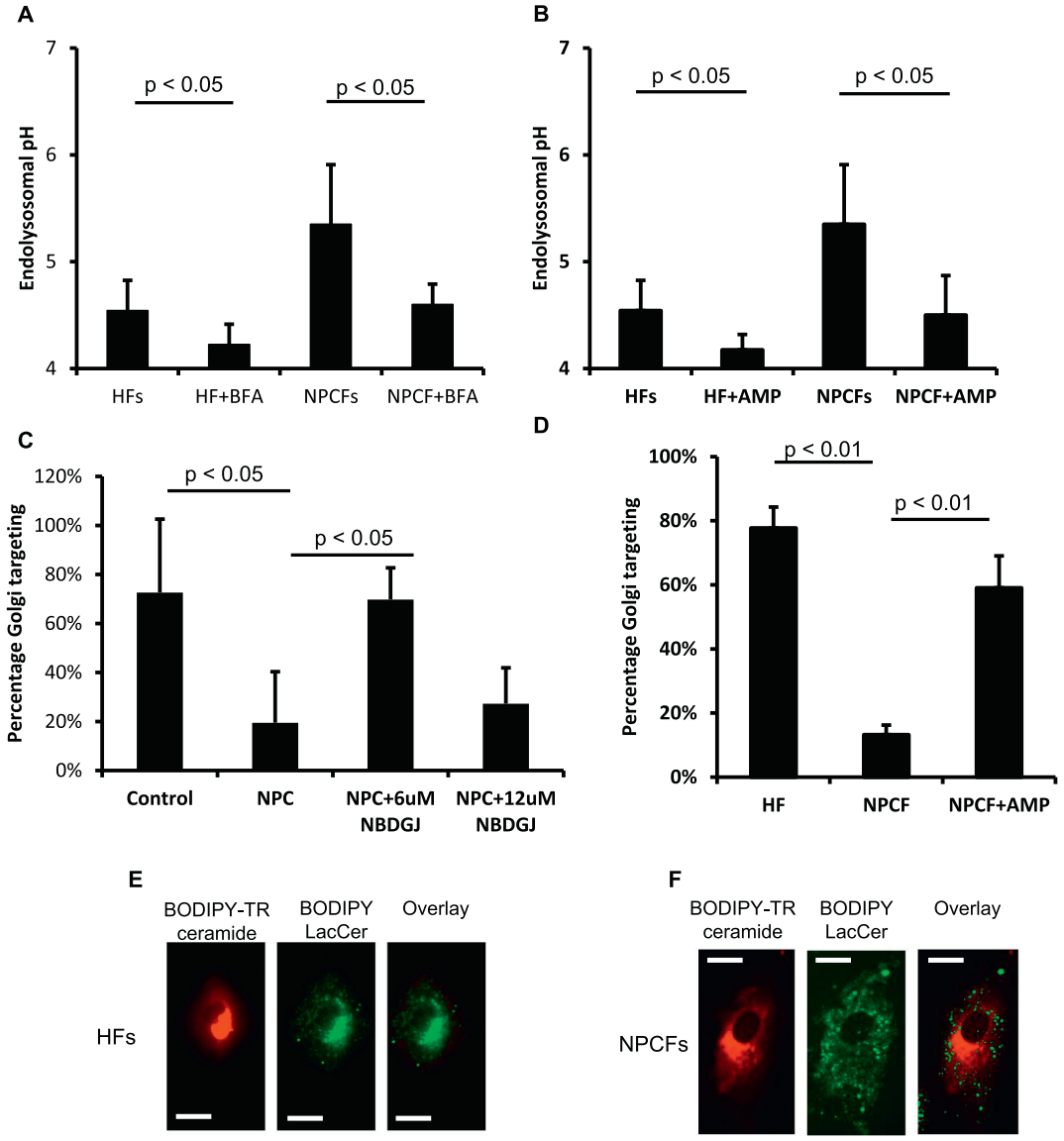

Fig. 4. Repaired endolysosomal pH and Golgi targeting on GBA2 inhibition. A) Treatment with brefeldin A reduces endolysosomal pH in control and disease fibroblasts. B) Endolysosomal pH with and without treatment with AMP-DNJ. Results in A and B are mean ± SD. C) Percentage Golgi targeting in fibroblasts assessed by blind scoring. Percentages are means ± SD from at least 40 cells in at least 2 independent experiments; D) Percentage Golgi targeting in fibroblasts with and without treatment with AMP-DNJ as assessed by blind scoring. Percentages are means ± SD from at least 90 cells in at least 2 independent experiments. *E*-F) Representative images of control (E) and disease (F) cells after pulse-chase treatment with BODIPY-LacCer and BODIPY-TR-ceramide. Cells were pulsed for 45 min with 15 μM BODIPY-LacCer with 5 μM BODIPY-TR-ceramide added for the last 30 min. Cells were chased for 90 min. Scale bars represent 10 μm. Significance measured by t-test and ANOVA.

not inhibited by U18666A in the presence of BFA (Fig. 6B) suggesting that U18666A inhibits vesicular but not non-vesicular GalCer transport. Control experiments showed that BFA was effective in inhibiting vesicular transport as it completely inhibited movement of GM3; U18666A also reduced surface GM3 consistent with inhibition of vesicular transport (Fig. S7).

2. Discussion

We discovered defects in NPC cell culture models (increased endolysosomal pH and aberrant endocytic trafficking) consistent with a reduction in the cytosolic pool of GlcCer. Accordingly increasing cytosolic GlcCer by specifically inhibiting non-lysosomal glucocerebrosidase (GBA2) corrected both errors and increased ATP6VOa1 expression. This work consequently suggests GBA2 and vATPase as new therapeutic targets in NPCD. GlcCer has previously been suggested as a potential modulator of vATPase (van der Poel et al., 2011). However previous studies have exclusively referred to melanocytes which are a specialised cell type with a particularly low Golgi and lysosomal pH needed for correct sorting to melanosomes. The generality of previous findings or the application to human disease has not been previously addressed. The current study raises the question of how cytosolic GlcCer can be integrated with current understanding of NPC1 function. We wish to present two possible interpretations of these results.

The first interpretation relies on the classical role of NPC1 as a cholesterol export protein. Soluble partner protein NPC2 transfers the lipid to NPC1 from where it can transfer to the ER, via a partner protein. Since NPC2 can transfer cholesterol to membranes in the absence of NPC1 (Cheruku et al., 2006; Infante et al., 2008), and cholesterol is generally believed to flip between membrane leaflets with ease (Steck et al., 2002) dysfunctional NPC1 may reduce lysosome-ER trafficking and enrich the cytosolic leaflet of the endolysosome membrane in

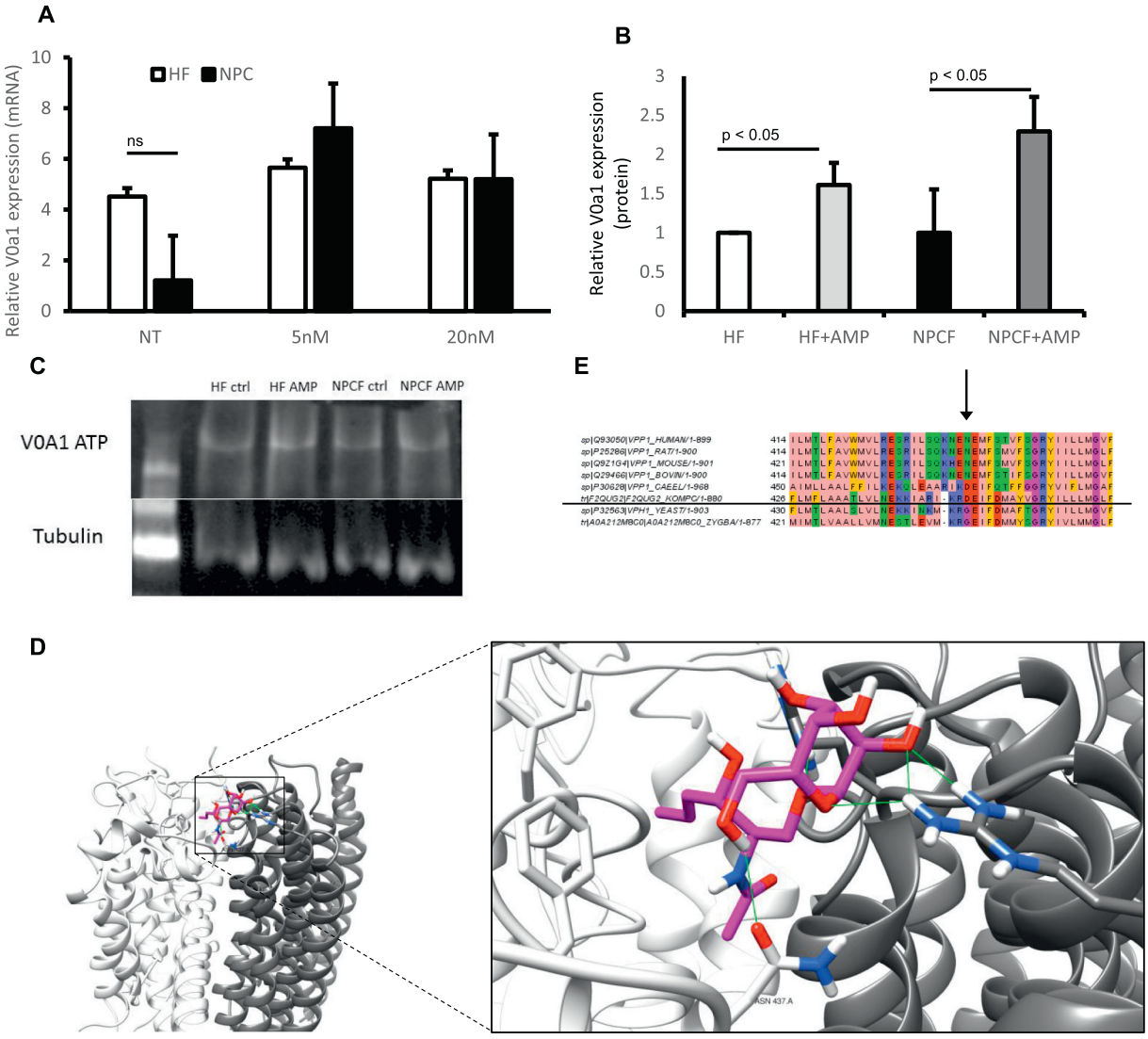

Fig. 5. GlcCer increases expression of vATPase a subunit; potentially critical role of Asn437 A) In fibroblasts ATP6V0a1 mRNA is decreased in NPCD, but increased by AMP-DNJ. Results are presented as mean ± SD relative to TBP, significance measured by t-test and ANOVA. B,C) ATP6V0a1 protein expression is increased by AMP-DNJ in both control and disease fibroblasts consistent with the endolysosomal pH decreases seen with this compound. Results are normalised to untreated healthy cells ($n = 3$). D) GlcCer headgroup bridges the vATPase a and c subunits; a subunit reside Asn437 accepts an H-bond (a subunit shown in white, c subunits in grey, GlcCer in magenta, hydrogen bonds as green lines). E) Multi-sequence alignment relevant section of the vATPase a subunit from species containing GlcCer (above the line) and in species lacking GlcCer (below the line). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

cholesterol. Within that leaflet cholesterol can be glycosylated by GBA2 using GlcCer, likely the major sphingolipid on the lysosomal surface, as a glucose donor. Thus NPC1 dysfunction will decrease GlcCer on the lysosomal surface in order to increase GlcChol (Marques et al., 2016). Constant GlcCer levels during our experiments with U18666A (Fig. 6D) and modest elevations of GBA2 in NPCD mice (Marques et al., 2015) perhaps make this explanation less likely. However we cannot rule out increased degradation of a minor cytosolic GlcCer pool due to an increase in cholesterol levels.

Secondly, since inhibiting NPC1 function reduces cell surface transport (Fig. 6A,C) NPC1 may act as a GlcCer flippase (Halter et al., 2007) alone or in concert with another protein eg TMEM97 (Bartz et al., 2009) or LAMP2 (Li and Pfeffer, 2016). The region of NPC1 traditionally known as the sterol sensing domain (SSD) has significant sequence and structural similarity with transporters from the Resistance-Nodulation-Cell Division family (Li et al., 2016; Davies et al., 2000) and can potentially bind mycolic acids (Fineran et al., 2016). It may therefore be large enough to accommodate other lipids, including GlcCer. The question remains of the precise role of U18666A in reducing transport of GlcCer, though not GalCer. Whilst this study did not address this, it could inhibit either non-vesicular transport or flipping. It has been reported on spectroscopic and crystallographic grounds that GalCer and GlcCer may share the same transfer proteins GLTP and FAPP2 (Malinina et al., 2006; Samygina et al., 2013), in which case U18666A specifically inhibits GlcCer flipping.

By whatever mechanism cytosolic GlcCer levels are controlled, this lipid may bind to and activate the vATPase (van der Poel et al., 2011; Sillence, 2013; Lafourcade et al., 2008) ensuring correct acidification, leading in turn to a functional endocytic pathway (Baravalle et al., 2005; Bayer et al., 1998; Clague et al., 1994; van Weert et al., 1995). Whilst our findings point to this hypothesis further experiments will be necessary to confirm it, including investigations of whether other GlcCer binding proteins are involved, eg Galectin-3 which forms endocytic carrier structures in a GSL-dependent manner (Lakshminarayan et al., 2014). In NPC disease, levels of cytosolic GlcCer are reduced leading to impaired acidification and disrupted BODIPY-LacCer

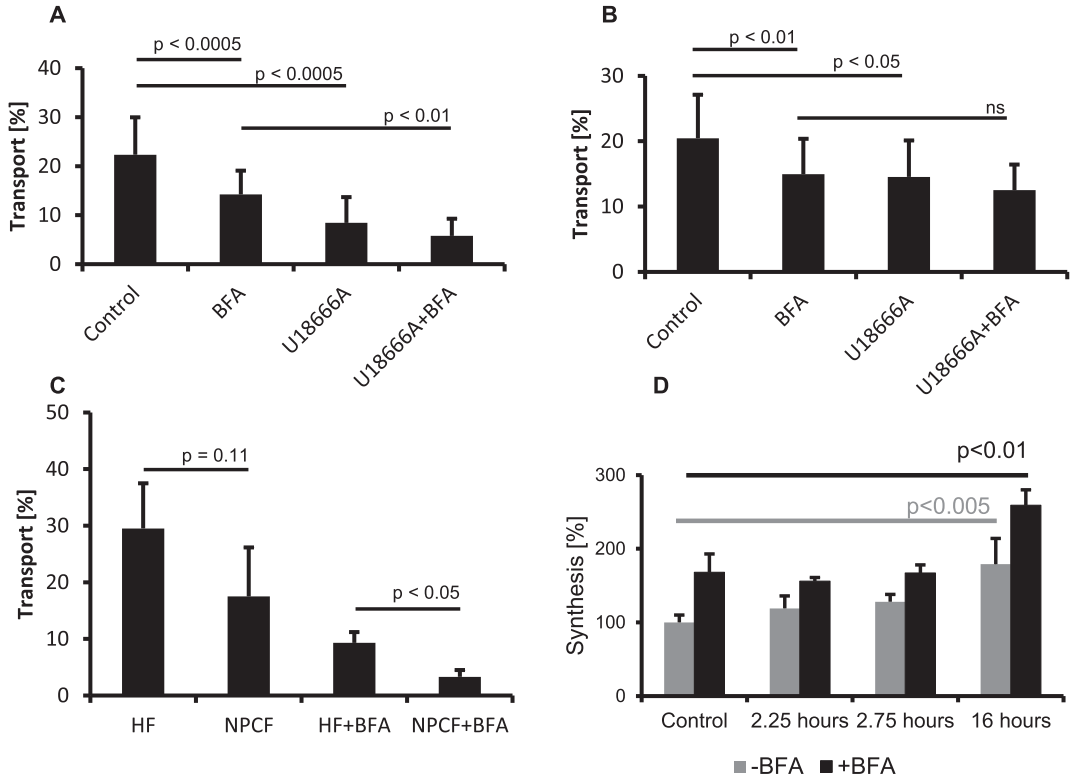

Fig. 6. Transport of newly synthesised GlcCer to the cell surface is reduced in NPCD cell culture models. The effect of 1 μg/ml of brefeldin A (BFA) and 5 μM U18666A on transport of GlcCer (A) and GalCer (B) by means of the GLTP assay in CHO cells expressed as % transport to the cell surface. C) Surface transport of GlcCer in NPC fibroblasts. D) U18666A significantly increased amounts of GlcCer at 16 h in either the presence or absence of BFA; no increases were observed at earlier time points. All samples were pre-incubated ± BFA for 30 mins. Data shown as mean ± SD, significance measured by t-test.

transport to a near-neutral compartment. The deficit of GlcCer at the cytosolic face of endolysosomes can be repaired by the addition of a GBA2 inhibitor. Reduced GlcCer levels have previously been associated with disrupted endocytic trafficking (Sillence, 2013; Sillence et al., 2002). Our putative understanding is summarised in Fig. S8.

Other interpretations are less likely. The selectivity of U18666A inhibition of GlcCer transport could be explained by inducing GlcCer storage. However: 1) the presence of Brefeldin A in these experiments blocks vesicular transport and so newly synthesised cytoplasmic GlcCer wouldn't be expected to be stored; 2) U18666A-does not induce changes in GlcCer in the timeframe of the experiment (Fig. 6D). Decreased cell surface GlcCer could also be explained by lower endosomal recycling. However this explanation cannot adequately account for the selectivity we observe for GlcCer over GalCer (Fig. 6A, B).

Strong support for our interpretation would be derived from directly measured changes in levels of cytosolic GlcCer and these changes correlating with lysosomal function. Unfortunately such a direct measurement is not currently possible. The best available surrogate is to measure GlcCer at the cellular level. In healthy cells treatment with AMP-DNJ does not result in changes to whole cell GlcCer (Fig. S4) despite this compound being a potent GBA2 inhibitor (IC_{50} ~ 1 nM (Ridley et al., 2013)). We suggest that this is because AMP-DNJ acts only on a minor pool of GlcCer and so gives changes which are undetectable at the cellular level. In contrast, in NPCD cells GlcCer levels are elevated by ~100% (Fig. S4) and doses of 1–20 nM AMP-DNJ lead to further increased GlcCer (up to ~400%). We believe that when our data is taken together, especially that concerning the use of 1 μM NB-DNJ, 6 μM NB-DGJ and brefeldin A (cytoplasmic GlcCer increases in ER/Golgi), increased GlcCer is the best explanation. Overall 5–10 nM AMP-DNJ would be recommended based on experiments in a variety of cell types (data not shown).

Other pathways involving lipids and regulating endolysosomal pH also operate. 1) sorting of vATPase via FAPP2-mediated recycling of non-vATPase containing tubules (Cao et al., 2009) and endocytic maturation involves the sorting and activation of the vATPase (Lafourcade et al., 2008; Kane, 2006; Trombetta et al., 2003). 2) GlcCer binding to the mTORC1/ragulator complex which is a key regulator of vATPase (Zhu et al., 2013). Evidence for this possibility also includes the observation the GlcCer precursors palmitate, serine and glucose, as well as GlcCer itself, are all implicated in the regulation of mTORC1 (Bar-Peled et al., 2012; Zoncu et al., 2011). The relationship between NPC1 and mTOR requires further elucidation as cholesterol accumulation has variously been reported to result in mTORC1 inhibition (Xu et al., 2010), mTORC1 activation (Castellano et al., 2017) and no difference in mTORC1 status (Pacheco et al., 2007). 3) association between NPC1 and components of the vATPase (Castellano et al., 2017; Macías-Vidal et al., 2016). 4) the potential involvement of GlcSph, although this is less likely here as GBA2 inhibition does not correct GlcSph elevation (Marques et al., 2015; Mistry et al., 2014; Hamler et al., 2017). 5) the possibility that NPC1 exports sphingosine (Lloyd-Evans et al., 2008; Höglinger et al., 2015, 2017), reported as an endogenous inhibitor of GBA2 (Schonauer et al., 2017); recent molecular modelling work from our laboratory supports this (Wheeler et al., 2019). 6) accumulating lipids permeabilise the lysosome (Amritraj et al., 2013; Chung et al., 2016; Gabande-Rodriguez et al., 2014; Kosicek et al., 2018) which would be expected to lead to proton leak.

It is commonly thought that increasing pH will inevitably result in dysfunction of lysosomal hydrolases. In fact approximately half of such enzymes, not least most of the cathepsin family, have pH optima of 5 or above (Xiong and Zhu, 2016). Thus the pH increase reported here will not in itself lead to widespread failure of lysosomal catabolism. Consistently, vATPase inhibition does not increase lysosomal amino acids (Abu-Remaileh et al., 2017). Instead lysosomal hydrolases with low pH optima and high substrate flux, such as lysosomal acid lipase, will be

affected as previously reported (van der Poel et al., 2011; Sillence, 2013; Magalhaes et al., 2015; Soyombo et al., 2006).

It is important to consider the details of the measurement of endolysosomal pH. As noted, various results have been reported and it's possible that some of the confusion regarding pH in NPCD cells is due to different techniques measuring different compartments. We do not consider our data to be in direct conflict with some previous reports of no increase in *lysosomal* pH in NPCD cells as measured by other probes. We subscribe to the popular view that lysosomal storage diseases are a 'traffic jam' (Liscum, 2000; Simons and Gruenberg, 2000). Thus results with our probe, which reports a single value for the combination of endosomal and lysosomal pH, represent delayed endolysosomal acidification, and do not preclude mature lysosomes fully acidifying. This explains why our results are different to those using other probes, and also to those gained using LysoTracker red (Shoemaker et al., 2013) as fluorescence from this probe (pKa 7.5) is not sensitive to pH changes below about 6.5 (Duvvuri et al., 2004). Differing reported pH values are thus a reflection of differing experimental techniques measuring different compartments.

It is not known why different pools of GlcCer have opposite effects on endolysosomal pH. GlcCer storage within the lysosome increases endolysosomal pH (Sillence, 2013; De La Mata et al., 2017; Magalhaes et al., 2015; Bourdenx et al., 2016; Chakraborty et al., 2017) whereas increasing *cytosolic* GlcCer decreases pH (current study). Possibly different pools of GlcCer bind different vATPase domains with opposite effects. It is also possible that overexpression of GBA2, which occurs both in Gaucher and NPC disease, increases the degradation of cytosolic GlcCer. New techniques that are able to measure different pools of GlcCer will be needed to address this issue.

In summary we have found defects in NPCD cells consistent with a reduced cytosolic pool of GlcCer and demonstrated the repair of these defects by inhibiting cytosolic GlcCer breakdown. This led in turn to further evidence that GlcCer may bind vATPase a subunit which may show decreased expression and could explain increased endolysosomal pH in GlcCer synthase knockout cells. Our findings are consistent with studies in mouse models of NPCD (Nietupski et al., 2012) as well as other LSDs (Lee et al., 2015; Folts et al., 2016) where miglustat was found to be disease-modifying despite increased levels of brain GlcCer, which is inconsistent with synthase inhibition. We think it is significant that recent reports point to a key role for lysosomal acidification in LSDs and neurodegenerative diseases more generally (Bourdenx et al., 2016; Ashe et al., 2011; Boudewyn et al., 2017). GBA2 and vATPase are therefore potential drug targets for NPCD and other diseases of lysosomal dysfunction.

3. Experimental procedures

Materials were obtained from Sigma unless otherwise indicated. Tissue culture media and supplements were from Gibco. Foetal Calf Serum (FCS) was from PAA laboratories. LysoSensor yellow/blue, BODIPY-LacCer and LysoTracker red were from Invitrogen. U18666A was from Affinity Research Chemicals (Exeter, UK). Brefeldin A was from Cayman Chemical. AMP-DNJ was prepared as previously described (Overkleeft et al., 1998).

3.1. Cell culture

Normal and storing patient lymphoblasts and fibroblasts (GM03124 (mutation P237S), GM03299, GM03123 (mutation P237S), GM05399 and GM00380) were obtained from the Coriell Institute, Human Genetic Mutant Cell Repository (New Jersey, USA). RAW 264.1 mouse macrophages were obtained from the ECACC (Porton Down, UK). HAP1 cells (wt and GBA2 KO) were obtained from Horizon (Cambridge, UK). Cells were maintained in RPMI (lymphoblasts and RAWs), DMEM (fibroblasts, CHOs) or IMDM (HAP1s) supplemented with 10 mM glutamine, 50 U/ml penicillin/streptomycin and 10% FCS. U18666A was at a final concentration of 5 μM, dissolved in ethanol at 1000× concentration and stored at −20 °C. AMP-DNJ (AMP-DNJ) was added at 1–20 nM and was dissolved in DMSO at 500× concentration and stored at −20 °C.

3.2. Fluorescence microscopy

Cells were placed on glass coverslips and left to adhere overnight. Cells were labelled by pulsing with BODIPY-C_5-LacCer in medium containing 1% serum for 30–60 min, removing cell surface fluorescence by washing 3 times for 1–5 min and chasing for 60–90 min (15). Fluorescent cells were observed using Leica or EVOS fluorescence microscopes. BODIPY was excited at 450–490 nm and viewed at > 520 nm. BODIPY-TR and LysoTracker were excited at 530–540 nm and viewed at > 560 nm. Pearson's coefficients were calculated using the Image J JACoP plugin.

Golgi targeting can be assessed by co-localisation with a Golgi marker, eg BODIPY-TR ceramide (see Fig. 4).

3.3. Endolysosomal pH measurement

Experiments with acridine orange were performed as previously described (Bach et al., 1999). Typically 10–15 Images were collected from 3 separate experiments and the red green ratio of the (200–500) punctates were calculated using Image J. Endolysosomal pH was also determined using LysoSensor yellow/blue (Diwu et al., 1999; Lin et al., 2001) using a previously published method (Fraldi et al., 2010). GLTP surface assay was as described by (Halter et al., 2007).

3.4. Lipid analysis

Lipids were extracted and applied to TLC plates, which, when used to separate GalCer from GlcCer, had been dipped in 2.5% wt/vol boric acid in MeOH and dried. Lipids were generally separated by 2D TLC using either $CHCl_3$/MeOH/25% vol/vol NH_4OH/water (65:35:4:4 vol/vol) or $CHCl_3$/MeOH/0.2% aqueous $CaCl_2$ (55:45:10 vol/vol) for the first dimension $CHCl_3$/MeOH/acetone/HOAc/water (50:20:10:10:5 vol/vol) for the second dimension. Radiolabelled spots were detected by exposure of phosphorimaging screens and read-out on a Personal FX phosphorimager. TLC plates with fluorescent lipids were directly developed using a phosphorimager (STORM 860; Molecular Dynamics). Spots were identified by comparison with standards and quantified using Quantity One software (Bio-Rad Laboratories).

GlcCer quantitation was performed as previously described (Mirzaian et al., 2017)

3.5. Molecular modelling

A model for the a and c subunit of vATPase where no experimental structure is available was constructed using SwissModel using 5TJ5 (Mazhab-Jafari et al., 2016) (67% identical, downloaded from the PDB) as a template (Arnold et al., 2006; Biasini et al., 2014).

Docking of GlcCer headgroup was conducted using ROSIE (rosie.rosettacommons.org) (Lyskov et al., 2013; Lyskov and Gray, 2008; Combs et al., 2013). All settings were defaults; NZ of Lys54 (subunit c) was used as the starting position. ROSIE output was visualised using UCSF Chimera (Pettersen et al., 2004).

3.6. PCR and Blotting

All cultured cells were prepared immediately before RNA extraction. Total RNA was extracted using RNeasy mini kit (Qiagen) and final RNA pellets were re-suspended in PCR grade water. The RNA purity was crudely assessed using a Nanodrop (ThermoFisher) which gave a 260/280 nm ratio of 1.9–2.1 for all samples. cDNA was synthesised from 1 μg of RNA using SensiFast cDNA Synthesis Kit (BioLine, London,

UK). Quantitative RT-PCR was performed using SensiFast SYBR® Hi-Rox kit assays in a StepOne instrument. The thermal profile was 2 min at 95 °C, 40 cycles of 5 s at 95 °C, and 30 s at 58 °C. Non-template controls were included for all samples. Results were expressed as expression relative to TATA box binding protein (TBP) $2^{-\Delta ct}$ (mean ± SD). Primers: human ATP6V0A1 (NM_001130020.1),. TBP was used as a housekeeping gene.Healthy and disease fibroblast were cultured until they reach an 80% confluence. At that point, the proteins were extracted using RIPA buffer. The protein concentration was assessed using DC Protein essay kit (Bio-Rad). An equal amount of protein was separated on polyacrylamide gel electrophoresis and transferred onto polyvinylidene fluoride membranes (BioRad). The membrane were incubated with 5% skimmed milk solution in TBST (Tris-buffered saline, 0.1% Tween 20) for 1 h and then treated with primary antibodies overnight at 4 °C. On the follow day, the membranes were washed 3 times with TBST and then treated with secondary antibody at room temperature for 1 h. The primary antibodies used were an Anti-ATP6V0a1 (Abcam 1: 500) and anti-Tubulin (Sigma 1:200). The second antibody were HRP-labelled anti-rabbit (Sigma-1:5000) and anti-mouse (Sigma-1:2000). The blots were developed using an ECL reagent (Thermofisher) and the results were normalised to Tubulin.

3.7. Sequence alignment

This was performed in Clustal Omega (Sievers et al., 2011) and results visualised in Jalview (Waterhouse et al., 2009).

3.8. Statistical analysis

Results are presented as mean ± SEM in the text and mean ± SD in the figures unless otherwise stated. Statistical significance was assessed using Student's one- or two-tailed *t*-tests and two-way ANOVA calculated using Microsoft Excel.

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Conflict of interest

The authors declare they have no conflicts of interest with the contents of this article

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.nbd.2019.03.005.

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