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A mutation in *SCARB2* is a modifier in Gaucher disease

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Abstract

Lysosomal integral membrane protein type 2 (LIMP-2) is responsible for proper sorting and lysosomal targeting of glucocerebrosidase, the enzyme deficient in Gaucher disease (GD). Mutations in the gene for LIMP-2, *SCARB2*, are implicated in inherited forms of myoclonic epilepsy, and myoclonic epilepsy is part of the phenotypic spectrum associated with GD. We investigated whether *SCARB2* mutations impact the Gaucher phenotype focusing on patients with myoclonic epilepsy, including a pair of siblings with GD who were discordant for myoclonic seizures. Sequencing of *SCARB2* genomic and cDNA identified a heterozygous, maternally-inherited novel mutation, c.1412A>G (p.Glu471Gly), in the brother with GD and myoclonic epilepsy, absent from his sibling and controls. Glucocerebrosidase activity, Western blots, real-time PCR, and immunofluorescence studies demonstrated markedly decreased LIMP-2 and glucocerebrosidase in cells from the sibling with (p.Glu471Gly) LIMP-2, and diminished glucocerebrosidase in lysosomes. The cells secreted highly-glycosylated enzyme and showed mis-trafficking of glucocerebrosidase. Sequencing of *SCARB2* in 13 other subjects with GD and myoclonic epilepsy and 40 controls failed to identify additional mutations. The study provides further evidence for the association of LIMP-2 and myoclonic epilepsy, explains the drastically different phenotypes encountered in the siblings, and demonstrates that LIMP-2 can serve as a modifier in Gaucher disease.

Keywords

Gaucher disease; myoclonic epilepsy; LIMP-2; *SCARB2*; glucocerebrosidase

Introduction

Understanding phenotypic heterogeneity in monogenic disorders is a major challenge in human genetics. Much of the emphasis in the era of molecular genetics has been on identifying genes and mutations. However, most genotype-phenotype studies have unequivocally demonstrated that there is a great phenotypic variation, even among individuals sharing the same genotypes. Siblings with discordant phenotypes implicate a contribution of other genetic, epigenetic and environmental factors to clinical variation among patients with the same disease.

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Gaucher disease (GD), the recessively inherited deficiency of lysosomal glucocerebrosidase (GCase, EC 3.2.1.45), encoding by *GBA* (MIM# 606463), manifests with diverse symptoms, and is commonly divided into three types, based on the absence (type 1) or rate of progression of neurological manifestations (types 2 and 3) [Beutler and Grabowski, 2001]. Type 3 GD is particularly heterogeneous. One subgroup develops progressive myoclonic epilepsy, albeit few visceral manifestations. GD, with almost 300 known mutations, was an early example of complexity in a monogenic disorder and the limitations of genotype-phenotype studies [Hruska et al., 2008, Dipple and McCabe, 2000].

Recent insights from cell biology provide new explanations for the heterogeneity seen in Mendelian disorders. The amount of functional protein can reflect how well the mutant protein is sorted, trafficked and delivered to a target organ. In fact, efforts to enhance the folding and trafficking of mutant proteins have opened new therapeutic directions. Unlike most lysosomal enzymes, GCase is not targeted to the lysosome via the mannose-6-phosphate receptor pathway, but instead, interacts with lysosomal integral membrane protein type 2 (LIMP-2) via a coiled domain within the luminal domain [Reczek et al., 2007, Blanz et al., 2010]. In cell lines from LIMP-2 deficient mice, GCase is missorted and secreted [Berkovic et al., 2008]. LIMP-2, encoded by the *SCARB2* gene (MIM# 602257), is composed of 478 amino acids, belongs to the CD36 protein superfamily, and is ubiquitously expressed. It is targeted to lysosomes and endosomes by a di-leucine motif on its C-terminal. *SCARB2* mutations were co-incidentally found to be associated with action myoclonus-renal failure (AMRF) [Berkovic et al., 2008]. Subsequently, *SCARB2* mutations were found in siblings with progressive myoclonic epilepsy and nephrotic syndrome [Balreira et al., 2008], and in a patient with myoclonic epilepsy without renal involvement. We postulated that mutations in *SCARB2* or altered LIMP-2 might explain the rare cases of neuronopathic Gaucher disease manifesting with myoclonic epilepsy.

A perplexing report in the literature described siblings with Gaucher disease with very disparate phenotypes [Eyal et al., 1991, Ron and Horowitz, 2008]. One sibling was followed closely for over a decade with progressive myoclonic epilepsy and dementia, while the second was subsequently diagnosed through family screenings, and has had few disease manifestations throughout his life, and no neurologic involvement. Both shared three *GBA* alterations, c.535G>C (p.Asp140His) and c.10936G>A (p.Glu326Lys), inherited from the mother, and c.586A>C (p.Lys157Gln) from the father [Eyal et al., 1991]. Extensive analyses, including studies of ER associated degradation (ERAD), have previously been performed on fibroblasts from both siblings, demonstrating that the one with myoclonic epilepsy had increased ERAD of GCase, as well as elevated intracellular cholesterol [Ron and Horowitz, 2008]. We evaluated whether differences in LIMP-2 in this discordant sib pair might explain their phenotypic differences. We also screened for mutations in *SCARB2* in other patients with GD, with and without myoclonic epilepsy.

Materials and Methods

Case Report

The proband had a normal childhood with good school performance. At age 13 years, in conjunction with a fever and flu-like symptoms, he experienced a generalized seizure lasting four minutes upon awakening from sleep, followed by a second seizure on awakening eleven days later. An EEG revealed bilateral slow waves and generalized spike activity which became especially marked with photic stimulation, and he was treated with diphenylhydantoin, then phenobarbital, which caused excessive drowsiness and finally primidone. The patient remained well until a sledding accident at age 14, which resulted in a ruptured spleen, requiring an emergency splenectomy. In the succeeding months, generalized grand mal seizures became increasingly frequent, and he began having

myoclonic jerks and staring spells. Multiple hospital admissions followed for almost continuous seizure activity and myoclonus, which became intractable, in spite of multiple drug regimes. Serum copper and ceruloplasmin were normal. A brain CT scan at age 17 was normal. By the age of 18 he was functioning well below his chronological age intellectually, with poor memory, poor fund of knowledge and slow mentation. He was dysarthric with slow hand movements, upper extremity myoclonus and staring spells. The EEG revealed bilateral slowing and spike activity. An electroretinogram, technetium brain scan and lumbar puncture were normal.

A work-up at age 19 revealed deficient GCase and Gaucher cells were found in the bone marrow and liver, as well as the previously removed spleen. His renal function and hearing were normal. The patient underwent a series of plasmapheresis treatments to reduce blood glucocerebroside accumulation, which decreased the frequency of generalized seizures, but the myoclonic and staring spells continued. An evaluation at age 20 revealed impaired memory, difficulty naming common objects and a poor fund of knowledge. Visual pursuit was very slow. There was asterixis of the upper extremities and very frequent myoclonic movements, more severe in the arms than legs. Muscle tone and strength were normal, and deep tendon reflexes were absent at the ankles, but preserved elsewhere. He was clumsy and dysmetric. By age 21, he became wheelchair-bound. Intravenous apomorphine dramatically reduced his myoclonus and photoconvulsive responses. He was continued on Sinemet for 10 months with improvement in physical activity. He was then switched to bromocriptine but this was discontinued at age 25 because of adverse behavioral effects and increasing unresponsiveness to the drug. Lithium was tried for six months but was also ineffective.

On examination at age 26 years, there was perseveration and interruption of speech by myoclonic jerking, and he was transiently inattentive during flurries of myoclonic seizures. There was marked impairment of horizontal saccadic eye movements, and muscle tone was mildly increased. A vitamin B₁₂ level was normal. He was treated for a urinary tract infection. Urinalyses done on prior and subsequent admissions were negative for protein. The following year a gastrostomy was performed because the patient refused to eat and was losing weight. Prior to his death at age 28, he was diagnosed with bacterial endocarditis. A full autopsy was performed demonstrating hepatomegaly, accessory splenic tissue, osteoporosis and Gaucher cells in the bone marrow and lymph nodes. Neuropathology revealed meningeal fibrosis, with mild focal gliosis of the left cingulate gyrus and anterior basal ganglia and degeneration of the lateral columns of the spinal cord, but no Gaucher cells. His kidneys were unremarkable.

Following his diagnosis, GCase levels were measured on his parents and three siblings. His brother was also deficient in GCase activity and carried the identical *GBA* genotype. A thorough examination at age 26 was normal, and a technetium liver-spleen scan showed the spleen to be at the upper limit of normal. He has remained healthy without palpable hepatosplenomegaly, bone pain, episodes of bleeding, seizures or cognitive impairment. Now 60 years of age, he has his own business and has worked in physically demanding occupations. He has not received enzyme replacement therapy.

The proband's mother, a carrier now in her 80's, also has no cognitive or neurologic impairment. Her *GBA* genotype was c.535G>C (p.Asp140His) + c.10936G>A (p.Glu326Lys)/WT.

Patient samples

Lymphocytes, lymphoblasts or fibroblasts were collected from the siblings, their mother, 13 other patients with GD and myoclonic epilepsy and 40 controls under NIH Institute Review Board approved clinical protocols.

DNA/cDNA Sequencing

RNA was isolated from fibroblasts from the siblings, patients with GD and normal controls using the Qiagen RNeasy Mini Kit (QIAGEN, Valencia, CA) and cDNA was generated using the High Capacity RNA-to-cDNA Mastermix (Applied Biosystems, Forest City, CA). *SCARB2* cDNA was synthesized using the following two primer sets: exon 1-1F: 5'-GTCTTCGACGCCTCTGCGGC-3'; exon 12-1R: 5'-CAACTCATGGGTATTGCC-3' and exon 12-8F: 5'-GGTAGCTTCATCCAATATATC-3'; exon 12-2R 5'-GTGAACCAACTGTATAAGCTAC-3', and sequenced on an Applied Biosystems 3130XL Sequencing Analyzer using the primers listed in Supp. Table S1A. Genomic sequencing of *SCARB2* was performed on genomic DNA from patients and controls using the primers in Supp. Table S1B. The Gaucher genotypes were established by sequencing *GBA*, as previously described [Stone et al., 2000]. All mutations are described using the nomenclature specified at www.hgvs.org/mutnomen. *GBA* cDNA nucleotides are numbered designating the adenine of the first ATG translation initiation codon as nucleotide 1 (GenBank reference sequence NM_000157.2). Amino acid designations are based on the primary *GBA* translation product, including the 39-residue signal peptide. For *SCARB2*, coding DNA nucleotides are numbered based on the adenine of the first ATG translation initiation codon as nucleotide 1 (GenBank reference sequence NM_005506.3). The reference sequence NP_005497.1 was used for amino acid numbering.

Plasmids, site-directed mutagenesis and transfection

A pCMV6-Neo vector expressing wild type LIMP-2 protein was purchased from Origene Inc. (Rockville, MD). The LIMP-2 mutation c.1412A>G was generated by PCR-based site-directed mutagenesis using the Quickchange mutagenesis kit according to the manufacturer's guidelines (Stratagene, La Jolla, CA). Fibroblasts from the proband's sibling (P2) were stably transfected with the mutated and wild type *SCARB2* using FuGENE 6 according to the guidelines of the manufacturer (Roche, Indianapolis, IN), and were selected with Neomycin (300ng/ml) for 3 weeks. Selected colonies were grown and utilized for enzyme assays and Western blot analyses.

Quantitative real time PCR analysis

RNA expression was quantified using the StepOnePlus Real-Time PCR System (Applied Biosystems) in a 20ul volume containing 10 ul of TaqMan Universal PCR master mix, 1 ul of *SCARB2* or *GBA* probe (with primer), 1 ul β -Actin probe and primer and 25ng cDNA. One cycle at 50°C for 2 min and one at 95°C for 10 min were followed by 40 cycles at 95°C for 15 sec and 60°C for 1 min. Three replicates of each sample were assayed.

GCase activity assay

GCase activity was assayed on fibroblasts from the siblings, mother and controls using 4-methylumbelliferyl- β -D-glucopyranoside substrate (Sigma, St. Louis, MO) as previously described [Raghavan et al., 1980]. Three replicates of each sample were performed.

SDS-PAGE and Western blotting

Fibroblasts, grown in monolayers to 90% confluence, were harvested and sonicated at 4°C in lysis buffer (60 mM KH_2PO_4 , 0.1% Triton-X and protease inhibitor, pH 5.9). After quantification with BCA (Thermo scientific, Rockford, IL) the lysate was separated by SDS-PAGE, and transferred to iBlot PVDF nitrocellulose membranes (Invitrogen, Carlsbad CA). Blots were blocked in phosphate-buffered saline (PBS) containing 0.1% Tween-20 (Sigma) and 5% fat-free milk for 1 h at room temperature. The blocked membrane was incubated in blocking buffer containing primary antibody LIMP-2, 1:1000, (Santa Cruz Biologics, Santa Cruz, CA,) and glucocerebrosidase 1:15000, (custom-made antibody, R386) overnight at 4

°C, followed by three 10 min washes, and was incubated in blocking buffer containing horseradish peroxidase (HRP)-conjugated secondary antibody 1:3000 (KPL, Gaithersburg, MD) for 1 h at room temperature. HRP immunoblots were developed using enhanced chemiluminescence (ECL Plus, GE Healthcare).

Immunofluorescence staining and confocal microscopy

Fibroblasts were grown in Lab-Tek 4 chamber slides (Fisher Scientific, Pittsburgh, PA) to 60% confluency, fixed in 3% paraformaldehyde, permeabilized with 0.1 % Triton-X for 10 min and blocked in PBS containing 0.1% saponin, 100 μ M glycine, 0.1% BSA and 2% donkey serum. They were incubated with mouse monoclonal anti-LAMP-2 (1:100)

(Developmental Studies Hybridoma bank, University of Iowa), rabbit polyclonal anti-GCase antibody R386 (1:500), or mouse monoclonal calnexin (1:100, Abcam) for 2 h at room temperature. The cells were washed, and incubated with secondary donkey anti-mouse or anti-rabbit antibodies conjugated to ALEXA-488 or ALEXA-555 (Invitrogen), washed again, and mounted in VectaShield with DAPI (Vector Laboratories, Burlingame, CA). Cells were imaged with a Zeiss LSM510 META confocal laser-scanning microscope (Carl Zeiss, Microimaging Inc, Germany) using an Argon (458, 477, 488, 514 nm) 30 mW laser, a HeNe (543 nm) 1 mW laser, and a laser diode (405 nm). Images were acquired using a Plan Apochromat 63X/1.4 oil DIC objective. All the images were taken at the same laser setting. Zeiss LSM510 META quantification software was used to analyze the images. Briefly, the co-localization coefficients of a total of 16 cells from 6 different fields were calculated. The mean and SD of these values are represented in Figure 2D. The experiment was performed three independent times.

Analysis of extra-cellular secretion

Approximately the same quantity of fibroblasts from the siblings and controls were seeded in T75 culture flasks. The next day, media was replaced with an equal volume of serum free OPTI-MEM medium. After 24 hours, the conditioned medium (CM) was passed through a 0.22 μ m filter and concentrated at 4000 rpm at 4 C for 45 min in iCON concentrators (9K pore size) (Pierce) to a volume of 250 μ L. 30 μ L of lysate from the CM was loaded on a 4–12% bis-tris acrylamide gel, blotted and probed with antibody to GCase and LIMP-2. To analyze different glycosylated forms of GCase, the medium was treated with N-Glycanase, Glyko PNGase F (GKE-5003, Prozyme, CA) for 6 h at 37°C. Treated and non-treated samples were analyzed by SDS-PAGE.

Results

Differing levels of GCase activity in the siblings reflect differences in GCase expression or trafficking

Although P1 and P2 have identical *GBA* genotypes, fibroblasts from P1 had only 15% of control residual activity, while levels were 2–3 folds higher in the sibling (P2) (Figure 1A). This discrepancy in GCase activity led us to evaluate the protein expression of GCase and its transporter LIMP-2. Western blot analyses of fibroblast cell lysates showed decreased levels of GCase in both siblings (Figure 1B, lane 3 and 5), while LIMP-2 was diminished in the proband P1 (Figure 1B, lane 3) and his mother (Figure 1B, lane 4), although not in the sibling (P2) (Figure 1B, lane 5). Next, quantitative real-time PCR (q-PCR) was performed, demonstrating similar levels of *SCARB2* and *GBA* expression in fibroblasts from the siblings and controls (Figure 1C), where less than a twofold increase or decrease was considered to be non-significant. These q-PCR results indicate that GCase and LIMP-2 expression levels are not altered.

Next, GCase trafficking to the lysosomal compartment was evaluated in fibroblasts from P1 and P2 and compared to wild type (WT) cells using immunofluorescence laser scanning confocal microscopy (Figure 2A,B,C). The co-localization coefficient of GCase (red) and the lysosomal marker LAMP-2 (green) was determined in 16 individual cells for each genotype using Zeiss LSM510 software (Figure 2D). In WT fibroblasts, (Figure 2A) clear co-localization (yellow) between GCase (red) and LAMP-2 (green) was observed, with a mean co-localization coefficient of 0.466 ± 0.072 (Figure 2D). Co-localization between the two markers was decreased in fibroblasts from P2 (Figure 2B), with a mean co-localization co-efficient of 0.247 ± 0.068 (Figure 2D), and was lowest in fibroblasts from P1 (Figure 2C), with a co-localization coefficient of 0.153 ± 0.075 (Figure 2D). This experiment was repeated three independent times, and results were reproducible. GCase staining (Figure 2, red marker) indicated less of GCase protein in P2 and P1 compared to WT, in agreement with the Western blot data (Figure 1B).

We then tested whether GCase was retained in other non-lysosomal cellular compartments since it is known that some mutant forms of GCase are retained in the ER, and will eventually undergo proteasome-mediated breakdown (Ron and Horowitz, 2005; Ron and Horowitz, 2008; Bendikov-Bar et al., 2011). Immunofluorescence studies of fibroblasts from WT, P1, and P2 showed no retention of GCase in the ER (Figure 2E,F,G).

A novel mutation in SCARB2 results in reduced LIMP-2 levels

The discrepant phenotypes and decreased amount of LIMP-2 in proband P1 prompted sequencing of *SCARB2* cDNA. One heterozygote mutation, c.1412A>G, resulting in the amino acid change, p.Glu471Gly was identified in the proband and his mother, but no mutations were detected on the second allele. The mutation was not detected in the sibling (P2), or in an additional 103 alleles amplified from controls or other patients.

Cells with the p.Glu471Gly SCARB2 mutation show extracellular excretion of GCase

In fibroblasts from P1, both GCase activity and protein were diminished compared to P2 (Figure 1A, B), although both have the same *GBA* genotype. Moreover, fibroblasts from P1 had decreased GCase in the lysosomal compartments and no retention of GCase in the ER (Figure 2C,D,G). It has been reported that in cells from LIMP-2 deficient mice, GCase is missorted and secreted into the extracellular environment [Berkovic et al., 2008]. To investigate whether GCase secretion could cause the different levels of GCase expression and activity in P1 and P2, we analyzed the GCase secretion of fibroblasts from both siblings. Conditioned medium harvested from cultured fibroblasts, showed that only the proband with myoclonic epilepsy (P1) excreted the 64 KDa pre-lysosomal high molecular weight form of GCase (Figure 3A). N-glycanase treatment confirmed this to be the glycosylated form of GCase (Figure 3B).

Next, to mimic the *SCARB2* genotype conditions of P1, fibroblasts from the sibling without myoclonic epilepsy (P2) were transfected with a pCMV6-Neo vector expressing WT or p.Glu471Gly mutant LIMP-2. Western blot (Figure 4A,B) and GCase activity (Figure 4C), performed on the stably transfected cell line, demonstrated that the introduction of mutant LIMP-2 resulted in a significant reduction in levels of endogenous GCase (Figure 4A, P1) and reduced GCase activity (Figure 4C).

SCARB2 in other patients with Gaucher disease and myoclonic epilepsy

We then evaluated whether mutations in *SCARB2* were associated with the development of myoclonic epilepsy in other patients with GD, by sequencing all coding and flanking intronic regions of *SCARB2* in 13 subjects with GD and myoclonic epilepsy, and 40 controls. No non-synonymous alternations were detected. The screening identified more

than 30 different SNPs in *SCARB2*. However none were significantly more frequent among patients with myoclonic epilepsy.

Discussion

The elucidation of the molecular mechanism whereby GCase is targeted to the lysosome provides a logical pathway to screen for candidate genetic modifiers for GD. Adequate production of LIMP-2 could be a prerequisite for proper GCase function, particularly in patients with deficient enzyme, and have an impact on phenotype. In this specific case, it appears that a mutation in *SCARB2* can convert a type 1 Gaucher phenotype to a type 3. This is particularly interesting in light of recent reports identifying homozygous and heterozygous *SCARB2* mutations in patients with myoclonic epilepsy [Balreira et al. and Berkovic et al., 2008, Dardis et al., 2009].

The recessively inherited progressive myoclonic epilepsies include Lafora disease, Unverricht-Lundborg disease, neuronal ceroid lipofuscinoses, type 1 sialidosis, action myoclonus-renal failure syndrome as well as type 3 GD. With the exception of Lafora disease, all are lysosomal disorders. Thus, a better understanding of how lysosomal proteins are processed and trafficked to the lysosome should shed light on the pathologic processes resulting in these disorders, and may lead to new therapeutic targets.

Interestingly, Glu471, the amino acid in LIMP-2 mutated in our patient, was previously discussed in the literature. Two acidic amino acid residues, Asp470 and Glu471 in the cytoplasmic tail, were shown to be crucial for the intracellular distribution of LIMP-2, playing an important role in regulating LIMP-2 movement within the endocytic pathway [Tabuchi et al., 2000]. The current report is the first *SCARB2* mutation identified in this region of LIMP-2.

Although myoclonic epilepsy has previously been reported in heterozygotes with *SCARB2* mutations [Reczek et al., 2007], in this case, based upon the normal clinical phenotype of the mother, it appears that the p.Glu471Gly mutation alone does not necessarily cause myoclonic epilepsy. However, in the proband it seems that the deficiency of enzymatic activity, accompanied by a mutation in the transporter resulting in mis-trafficking of the enzyme, contributes to the observed phenotype. However, *SCARB2* mutations were not found among the other unrelated patients with type 3 GD manifesting with myoclonic epilepsy that we were able to evaluate. The development of myoclonic epilepsy in neuronopathic GD could be due to multiple different modifiers. Other alterations in the same molecular pathway as LIMP-2 might similarly reduce the amount of active enzyme in the brain of these patients, and merits further exploration.

Studies of fibroblasts from the siblings discussed in this manuscript previously focused on endoplasmic reticulum associated degradation (ERAD) of GCase [Ron and Horowitz, 2008]. More ERAD and higher total and free cholesterol were seen in the proband, suggesting that high intracellular cholesterol levels modify the processing and maturation of mutant GCase. While lysosomal membrane proteins are important in intracellular cholesterol metabolism, the possible association between LIMP-2 deficiency and cholesterol or lipofuscin accumulation remains to be elucidated.

The identification of the *SCARB2* mutation only in the sibling with myoclonic epilepsy suggests that LIMP-2 serves as a modifier in Gaucher disease. This case clearly illustrates that all manifestations encountered in a single gene disorder may not be thoroughly explained by the primary mutation in the gene of interest. A mutation of the GCase activator, saposin-C in type 1 GD was also recently reported [Tylki-Szymańska et al., 2007]. Therefore, in Gaucher disease, abnormalities of the transporter (LIMP-2) and activator

(saposin-C) should be considered as possible disease modifiers in cases where the genotype and phenotype do not seem to match. While in this case LIMP-2 seems to be associated with for the myoclonic epilepsy that developed, other proteins involved in the trafficking or processing of GCase or LIMP-2 could likewise impact patient phenotype.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

GCase enzyme activity and expression levels in patient and control fibroblasts. **A)** Markedly low GCase enzyme activity is seen in the proband (P1) compared to the sibling (P2) and control (WT). **B)** Western blot indicates decreased endogenous protein levels of LIMP-2 and GCase in the fibroblasts from P1 (lane 3) compared to two controls (lanes 1 and 2) and P2 (lane 5). Fibroblasts from the mother (lane 4) of P1 and P2 showed diminished LIMP-2 and GCase compared to WT. Protein levels were normalized to the corresponding β -actin bands as shown in the graphs. **C)** Quantitative real-time PCR of RNA expression levels of *SCARB2* and *GBA* were not significantly different in fibroblasts from the siblings and controls.

Figure 2. Localization of GCase in patient and control fibroblasts. **A)** GCase (red) and LAMP-2 (green) stain in cells from a control (WT). **B)** The non-affected sibling P2 and **C)** the affected proband P1. **D)** Relative colocalization of GCase and LAMP-2 based on the analysis of 16 cells in 6 different fields. **E)** GCase (red) and the ER marker calnexin (green) in WT, **F)** P1, and **G)** P2 fibroblasts. Scale bar is 10μm.

Figure 3. Evaluation of extra-cellular secretion of GCase. **A)** A 64 kDa band is observed in the conditioned medium of the proband (P1), corresponding to the pre-lysosomal form of GCase. **B)** N-Glycosidase treatment of the conditioned medium demonstrates that the 64kDa band is the glycosylated form of GCase. (1) Before treatment. (2) After treatment.

Figure 4.

GCase protein expression and enzyme activity in Gaucher fibroblasts transfected with LIMP-2 (p.Glu471Gly). **A)** P2 Cells transfected with the p.Glu471Gly LIMP-2 variant show a significant reduction in the amount of GCase compared to WT LIMP-2 transfected fibroblasts. **B)** GCase protein levels were normalized to β-actin. **C)** The GCase activity is significantly reduced in p.Glu471Gly LIMP-2 transfected cells compared to cells transfected with WT LIMP-2.