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UPR activation and CHOP mediated induction of *GBA1* transcription in Gaucher disease

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ABSTRACT

Chronic presence of mutant, misfolded proteins in the endoplasmic reticulum (ER) initiates ER stress and induces the Unfolded Protein Response (UPR).
In Gaucher disease (GD), resulting from mutations in the *GBA1* gene, encoding lysosomal acid β-glucocerebrosidase (GCase), a certain fraction of the mutant variants is retained in the ER and activates the UPR. We have previously shown UPR activation in GD derived fibroblasts, in fibroblasts that derived from carriers of GD mutations and in *Drosophila* models of carriers of GD mutations.
In the present work we extended our studies to include a large collection of fibroblasts, EBV-transformed B-cells and white blood cells (WBCs) that derived from GD patients.
The results showed UPR activation in all tested cells. They also indicated that transcription of the *GBA1* gene is upregulated through activation of the UPR-induced CHOP transcription factor. Transcription of the *MAN2B* gene, encoding alpha-mannosidase and of the *ACP* gene, encoding acid phosphatase was also elevated presumably through CHOP activation.
Our results highlight the existence of chronic stress in GD derived cells due to the presence of ER-retained mutant GCase, which leads to upregulation of *GBA1* expression.
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1. Introduction

Gaucher disease (GD), an autosomal recessive genetic disorder, results mainly from mutations in the *GBA1* gene (OMIM #606463), encoding acid β-glucocerebrosidase (GCase; EC 3.2.1.45) [1]. Mutations in the saposin C domain of the *prosaposin* gene (*PSAP*) also result in GD [2–4]. Presence of mutant enzyme leads to decreased lysosomal activity and accumulation of the substrate glucosylceramide (glucocerebroside) [1]. More so, a certain fraction of the mutant enzyme is retained in the ER and undergoes ER associated degradation (ERAD) [5]. The chronic presence of mutant enzyme in the ER leads to ER stress and upregulates the Unfolded Protein Response (UPR), a cascade of signaling pathways aiming at reaching homeostasis in the cells. If not achieved, the cells undergo apoptotic death. The ER membrane harbors three ER stress sensors: the type 1 transmembrane protein kinase endoribonuclease (IRE1), the type 1 protein kinase (PERK), and the activating transcription factor 6 (ATF6). These three UPR transducers are constitutively expressed in metazoan cells, and are maintained in an inactive state through interaction with the ER chaperone BiP (Immunoglobulin heavy chain-binding protein) [6,7]. ER-accumulated unfolded proteins bind and sequester BiP, thus promoting its dissociation from PERK, IRE1 and ATF6. Dissociation of BiP from the three stress sensors allows their modification and activation, which results in a response to the accumulation of misfolded proteins [7–9]. Thus, IRE1 undergoes dimerization and phosphorylation, and participates in a cytoplasmic complex, which splices the transcription factor X-box binding protein 1 (Xbp1). Upon its splicing the *Xbp1* mRNA (*Xbp1s*) is translated into a protein that translocates into the nucleus and activates UPR related genes [10–13]. PERK is a kinase that undergoes dimerization and autophosphorylation, and mediates phosphorylation of the eukaryotic translation initiation factor 2α (eIF2α) [8]. Phosphorylated eIF2α attenuates general protein translation in the cells [7,8,12,14,15]. Modified PERK also initiates translation of ATF4, which activates transcription of UPR related genes, like the CAAT/Enhancer binding protein (C/EBP) homologous protein (CHOP), which is a proapoptotic bZIP transcription factor [16,17]. CHOP is essential for cell cycle arrest as part of the apoptotic response to chronic ER stress [6,14,18]. ATF6 shuttles to the Golgi, where it is sequentially cleaved by proteases. Its cleaved N-terminal cytosolic

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fragment serves as a transcription factor of UPR upregulated genes [7, 11,12,14,19].

Activation of UPR in GD derived cells has already been noted in fibroblast lines that originated from GD patients, homozygous for the N370S or the L444P mutations [20,21]. We documented it in several fibroblast lines that derived from GD patients and carriers of GD mutations by assaying the increase in *BiP* and *CHOP* mRNAs and proteins, the increase in *Xbp1* splicing and phosphorylation of eIF2α [22]. Accumulation of glucosylceramide per se, induced by conduritol-β-epoxide (CBE), did not result in UPR [22]. Likewise, in the absence of mutant GCase there was no UPR [23], underscoring the importance of mutant GCase in the activation of UPR.

GD patients and carriers of GD mutations have a higher propensity to develop Parkinson disease (PD) than the non-GD population [24]. We and others have shown that the presence of mutant GCase activates UPR and leads to development of parkinsonian signs or neurodegeneration in *Drosophila melanogaster* [22,25,26].

It has already been documented that *GBA1* mRNA levels are increased in GD derived cells, the nature of which was unknown [27]. Previous studies indicated that a 630 bp promoter fragment of the human *GBA1* gene was sufficient to confer the same tissue specificity as the entire gene. This fragment contains 365 bp upstream the transcription start site and 265 bp of the first exon [27, 28]. Using Electrophoretic Mobility Shift Assays (EMSAs) and Chloramphenicol Acetyl Transferase (CAT) assays our lab showed that OCTA binding protein (OBP), activator protein 1 (AP-1), polyoma enhancer activator 3 (PEA3) and a CAAT enhancer binding protein (C/EBP) participate in regulating the *GBA1* gene [29].

In the present study we document activation of UPR in GD derived cultured fibroblasts, in cultured lymphoblasts and in white blood cells (WBCs) from GD patients. We also show that the increase in *GBA1* transcription in GD-derived cells is mediated by the UPR-activated CHOP protein.

2. Materials and methods

2.1. Cell lines

Human primary skin fibroblasts and EBV-transformed B-cells derived from GD patients and carriers were from the "Cell Line and DNA Biobank from Patients Affected by Genetic Diseases" (G. Gaslini Institute). Skin fibroblasts (cultured fibroblasts) were grown in DMEM supplemented with 20% FBS (Biological Industries, Beit Haemek, Israel). Human EBV transformed B-cells (cultured lymphoblasts) were grown in RPMI supplemented with 10–20% FBS (Biological Industries, Beit Haemek, Israel). HEK293 (Human epithelial embryonic kidney) cells were grown in DMEM supplemented with 10% FBS. All cells were grown at 37 °C in the presence of 5% CO_2.

2.2. Antibodies

The following primary antibodies were used in this study: rabbit polyclonal anti-phospho-eIF2α (Ser51) antibodies, rabbit polyclonal anti-eIF2α antibodies (from cell signaling Technology, Beverly, MA, USA), Rabbit polyclonal anti-ERK antibodies (Santa Cruz Biotechnology, CA, USA) and mouse monoclonal anti-myc antibody (Cell Signaling Technology, Beverly, MA, USA).

Secondary antibodies used were: horseradish peroxidase-conjugated goat anti-mouse antibodies and horseradish peroxidase-conjugated goat anti-rabbit antibodies (both from Jackson Immuno Research Laboratories, West Grove, PA, USA).

2.3. Chemicals

Leupeptin, phenylmethylsulfonyl fluoride (PMSF), aprotinin and phosphatase inhibitor cocktails 2 and 3 were from Sigma-Aldrich (Rehovot, Israel). KAPA SYBR FAST UNIVERSAL qPCR kit was from KAPA Biosystems Inc. (Wilmington, MA, USA). CBE and thapsigargin were from Sigma-Aldrich (Rehovot, Israel).

2.4. Plasmids

pGL3-N: A 630 bp normal human *GBA1* promoter fragment, prepared by cleavage of an existing clone [24] with the restriction enzyme *Sac*I, was introduced into the *Sac*I restriction site of the mammalian vector pGL3 (Promega Corporation, Madison, WI, USA), harboring the luciferase gene.

pGL3-M: The above mentioned vector, harboring a 630 bp human *GBA1* promoter fragment, mutated at its CHOP binding site (CCAAT → CTGGC).

pGL2: A mammalian vector expressing the renilla gene coupled to the CMV promoter (Promega Corporation, Madison, WI, USA).

pcDNA4-myc-His-CHOP: CHOP sequence was amplified from a human cDNA library, prepared from a normal fibroblast line (F0908, kindly provided by Prof. Eli Sprecher, Department of Dermatology, Tel Aviv Sourasky Medical Center, Tel Aviv, Israel), using the primers: CHOP cDNA-F: TTAGCG**GAATTC**ATGGCAACT and CHOP cDNA-R: TCCAAT**CTCGAG**ATTGCTTGG. The 1028 bp amplified CHOP cDNA fragment, which contained *Eco*RI and *Xho*I restriction sites on its 5′ and 3′ ends, respectively (appears in bold), was cloned between the *Eco*RI and *Xho*I restriction sites of pcDNA4-myc-His-plasmid (Invitrogen Life Technologies Co., Carlsbad, CA, USA).

2.5. Separation of WBCs from whole blood

Pellets of WBCs were isolated by applying whole blood samples on polysucrose sodium metrizoate in UNI-SEP lymphocyte separation tubes (Novamed, Jerusalem, Israel) and processed according to the manufacturer's instructions.

2.6. Patients' genotypes

Molecular characterization of the samples used in the present study was performed at the Gaslini Institute (Genoa, Italy) on fibroblasts and EBV transformed B-cells and at Shaare Tzedek Hospital (Jerusalem, Israel) on patients' derived blood samples.

Since the traditional amino-acid residue numbering, which excludes the first 39 amino acids of the leader sequence (GenBank accession *GBA1* no. NP_000148.2) is regularly used in GD literature and not the

Table 1
Primers used in this study. The table contains the sequence of all the primers used in this work. RT = real time, R = reverse, F = forward.

Primer	Primer sequence
Human-GAPDH-RT-F	5′-CTCCTCCTGTTCGACAGTCA-3′
Human-GAPDH-RT-R	5′-GTTGACTCCGACCTTCACCT-3′
Human-CHOP-RT-F	5′-AGCGACAGAGCCAAAATCAG-3′
Human-CHOP-RT-R	5′-TCTGCTTTCAGGTGTGGTGA-3′
Human-BiP-RT-F	5′-CATCAAGTTCTTGCCGTTCA-3′
Human-BiP-RT-R	5′-ATGTCTTTGTTTGCCCACCT-3′
Human-ATF4-RT-F	5′-GTTCTCCAGCGACAAGGCTA-3′
Human-ATF4-RT-R	5′-ATCCTGCTTGCTGTTGTTGG-3′
Human-GBA1-RT-F	5′-AGGCAGTGTCGTGGGCAT-3′
Human-GBA1-RT-R	5′-ACCAAGGGCAGGAAAGGT-3′
Human-MAN2B-RT-F	5′-GATCATTGGACAAGCCAGAC-3′
Human-MAN2B-RT-R	5′-CGTCTGCCCTATTTACCCAT-3′
Human-ACP-RT-F	5′-AACCTAAACCAGCAGCCATC-3′
Human-ACP-RT-R	5′-AGCACATCAAGATCATGGGA-3′
Human-GAPDH-F	5′-CCATCAATGACCCCTTCATTGACC-3′
Human-GAPDH-R	5′-CTCAYGGYYCACACCCATGAC-3′
Human-Xbp1s-F	5′-TCTGCTGAGTCCGCAGCAG-3′
Human-Xbp1s-R	5′-GAAAAGGGAGGCTGGTAAGGAAC-3′

recommended nomenclature (http://www.hgvs.org/mutnomen), we used it in the present study.

2.7. RNA extraction and Reverse Transcription (RT)

Total RNA was isolated from cell pellets using TRIZOL reagent (Life-technologies Co. Carlsbad, CA, USA) according to the manufacturer's instructions. One microgram of RNA was reverse transcribed with M-MLV reverse transcriptase (Promega Corporation, CA, USA), using oligo-dT primer in a total volume of 25 μl, at 42 °C for 60 min. Reactions were stopped by incubation at 70 °C for 15 min.

2.8. PCR

Two microliters of the cDNA mixture were amplified by PCR in Red Load Taq Master ×5 buffer (Larova, Germany), carried out for 35 cycles by denaturing at 94 °C for 30 s, annealing at 52 °C for 30 s, and extending at 72 °C for 30 s. PCR reactions were carried out in an Eppendorff Master-cycler EP Gradient S (Eppendorf, Hamburg,

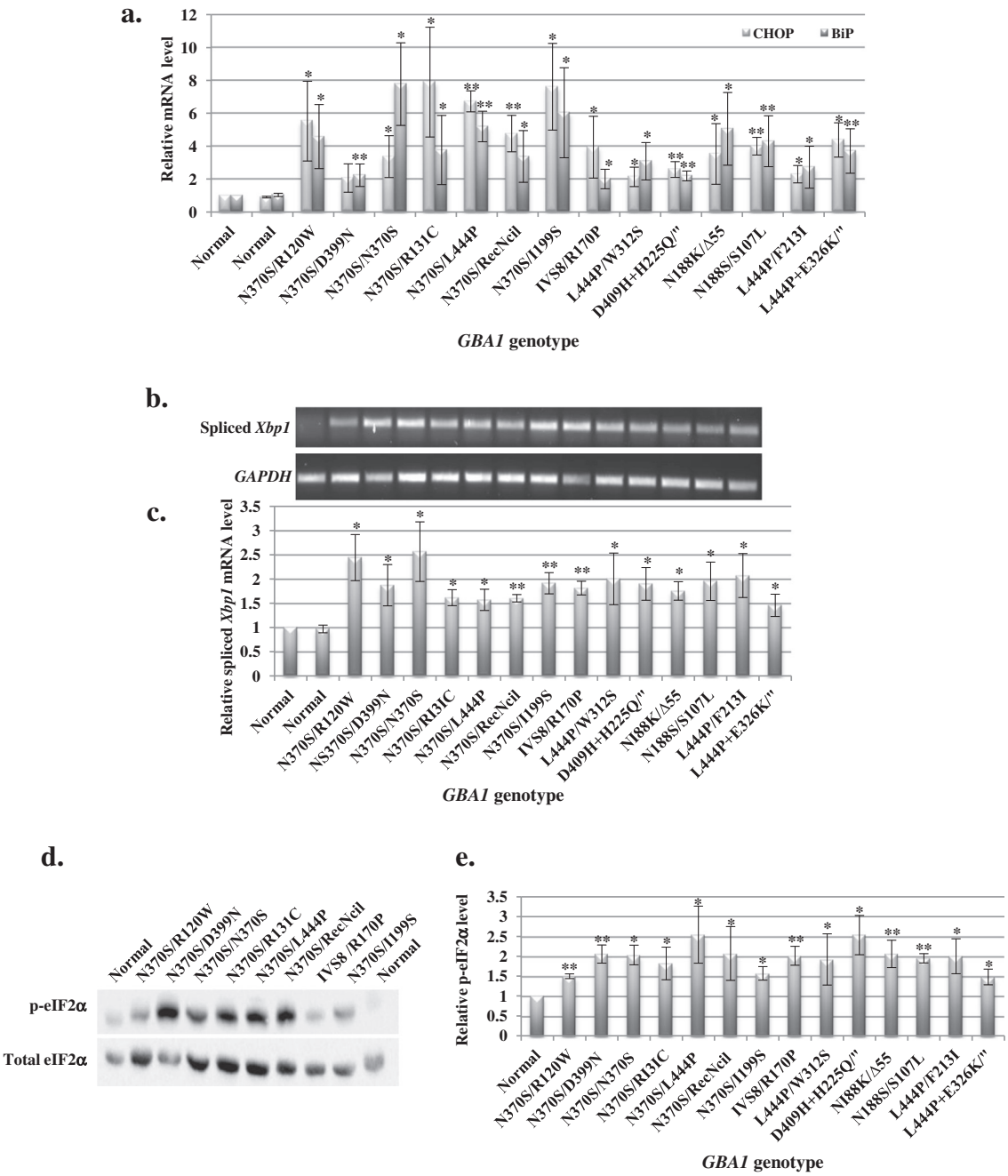

Fig. 1. UPR activation in GD cultured fibroblasts. a. Quantitative RT-PCR of *BiP* and *CHOP* mRNAs. b. RT-PCR of spliced *Xbp1* mRNA isolated from 14 cultured fibroblasts. Shown are results obtained for 13 samples. c. Quantification of results (for 14 cultured fibroblasts) as presented in b. The columns in the graph appear beneath the corresponding gel lanes. The results are the mean ± SEM of five independent experiments. d. Protein lysates were prepared from 14 GD cultured fibroblasts and subjected to western blotting, which were interacted with anti p-eIF2α antibodies and as a loading control, with anti eIF2α antibodies. Shown are results from 8 out of 14 tested samples. e. Quantification of results as shown in d. The blots were quantified by dividing the amount of p-eIF2α by that of total-eIF2α in the same lane. Values obtained for normal cell line was considered 1. The results are the mean ± SEM of three independent experiments. Significance: * <0.05; ** <0.01.

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Germany). RT-PCR products were separated through 1% agarose gels and visualized with 0.1% ethidium bromide. Sequence of the primers used is listed in Table 1.

2.9. Detection of Xbp1 mRNA splicing

Human spliced *Xbp1* was amplified from cDNA using the primers: Human Xbp1s-F and Human Xbp1s-R (Table 1). These primers allow amplification of the spliced *Xbp1* cDNA only. *GAPDH* (Glyceraldehyde 3-phosphate dehydrogenase) was used as a normalizing control (amplified with primers: Human-GAPDH-F and Human-GAPDH-R, Table 1).

2.10. Quantitative real time (qRT) PCR

PCR amplification was performed using the KAPA SYBR FAST UNIVERSAL qPCR kit in a Rotor-Gene 6000 thermal cycler (Qiagen, Hilden, Germany). The reaction was executed in 10 μl containing 1.5 μl of cDNA (diluted 1:64), 10ρmol of each primer and 5 μl SYBR mix. Thermal cycling conditions were: 95 °C (10 min), and 40 cycles of 95 °C (10 s), 60 °C (20 s) and 72 °C (20 s). Each sample was loaded in triplicate. The quantitative analysis was performed using the Rotor-Gene 6000 Series software (Qiagen, Hilden, Germany) and relative gene expression was determined by the Ct value. The results were quantified by dividing the value obtained for each sample by that obtained for *GAPDH* (as a normalizing gene). Values obtained for the cDNA that derived from the normal cell line was considered 1. Sequence of the primers used appears in Table 1.

2.11. SDS-PAGE and western blotting

Cell monolayers or cell pellets were washed three times with ice-cold phosphate-buffered saline (PBS) and lysed at 4 °C in lysis buffer (20 mM TRIS HCL pH, 7.5, 100 mM NaCl, 1 mM $MgCl_2$, 5 mM EDTA and 0.5% NP-40) containing 10 μg/ml aprotinin, 0.1 mM PMSF, 10 μg/ml leupeptin and 1% phosphatase inhibitor cocktails 2 and 3. Lysates were incubated on ice for 30 min and centrifuged at 10,000 g for 15 min at 4 °C. The lysates were electrophoresed through 10% SDS-PAGE, after which the corresponding blots were interacted with antibodies. The blots were scanned using Image Scan scanner (Amersham Pharmacia Biotech, Buckinghamshire, England), and the intensity of each band was measured by the Image Master 1DPrime densitometer (Amersham Pharmacia Biotech, Buckinghamshire, England) and GelQuant (BiochemLabSolutions, California).

2.12. Transfections and reporter assays

HEK293 cells were transfected using calcium phosphate solutions. A mixture of DNA in 250 μl of 250 mM $CaCl_2$ was dropped into a tube containing HBSX2 solution (50 mM Hepes, 280 mM NaCl, 1.5 mM Na_2HPO_4, pH 7.09) and incubated for 20 min at RT. The mixture was then added dropwise to subconfluent cells. Forty-eight hours later, cell lysates were prepared and luciferase activity was tested using the Dual-Luciferase Reporter Assay System according to the manufacturer's instructions (Promega Corporation, Madison, WI, USA). Samples were analyzed in a Veritas GloMax-96 Microplate Luminometer (Promega Corporation, Madison, WI, USA).

2.13. Statistical analysis

All the results were statistically analyzed using the student *t*-test.

3. Results

3.1. Activation of UPR in GD derived cells

Certain fraction of mutant GCase is retained in the ER and undergoes ERAD [5]. The chronic presence of mutant GCase in the ER leads to ER stress, which activates the UPR. We have previously shown activation of UPR in GD derived fibroblasts as well as in fibroblasts that derived from carriers of *GBA1* mutations [22]. In the present work we extended our previous studies to show that in different patients, some with mutations distinct from those we described before, there is UPR activation. For UPR activation in GD derived fibroblasts, mRNA levels of *BiP* and *CHOP*, mRNA levels of spliced *Xbp1* (*Xbp1s*) and protein levels of phosphorylated translation initiation factor eIF2α were tested. The results (Fig. 1, Table 2) showed that UPR is activated in all tested GD derived fibroblasts. The same experiments were carried out in two other sample types, EBV-transformed B-cells and WBCs, derived directly from patients. The results, presented in Fig. 2 and Table 3, showed elevation in *BiP* and *CHOP* mRNA levels as well as elevation in *Xbp1* splicing (tested by a set of primers that amplify only the spliced form of *Xbp1* mRNA, designated *Xbp1s*). No significant elevation in phosphorylation of eIF2α was observed, likely due to EBV transformation. These results strongly indicated activation of UPR in cultured lymphoblasts. In WBCs elevation in mRNA levels of *BiP*, *CHOP* and *ATF4* was tested (Fig. 3, Table 4). Due to sample size constrains we were unable to test phosphorylation of eIF2α using western blotting. Therefore, instead of phosphorylation of eIF2α, the increase in transcription of *ATF4* in the PERK arm of UPR was tested, using quantitative RT-PCR [12]. Our results indicated activation of UPR in WBCs.

Table 2
UPR activation in cultured fibroblasts from GD patients. Values represent the mean ± SEM of three (for eIF2a) or five (qRT-PCR for *BiP* and *CHOP* mRNAs, *Xbp1* mRNA splicing) independent experiments. Significance: * <0.05; ** <0.01. N.T.—Not Tested. §All *GBA1* mutations are described according to the traditional amino-acid residue numbering, which excludes the first 39 amino acids of the leader sequence (*GBA1* GenBank accession NM_000157.3; NP_000148.2).

Genotype§	GD type	Relative *CHOP* mRNA	Relative *BiP* mRNA	Relative *Xbp1* mRNA splicing	Relative p-eIF2α
Normal	–	1	1	1	1
Normal	–	0.9 ± 0.12	1.04 ± 0.1	0.97 ± 0.08	N.T.
[N370S] + [R120W]	1	5.5 ± 2.4*	4.6 ± 1.9*	2.4 ± 0.47*	1.5 ± 0.05**
[NS370S] + [D399N]	1	2.1 ± 0.8	2.2 ± 0.7**	1.9 ± 0.42*	2.1 ± 0.23**
[N370S] + [N370S]	1	3.4 ± 1.3*	7.8 ± 2.5*	2.6 ± 0.61*	2 ± 0.25*
[N370S] + [RI3IC]	1	7.9 ± 3.3*	3.8 ± 2.1*	1.6 ± 0.16*	1.8 ± 0.41*
[N370S + [L444P]	1	6.7 ± 0.6**	5.2 ± 0.9**	1.6 ± 0.22*	2.5 ± 0.72*
[N370S] + [RecNcil]	1	4.8 ± 1.1**	1.6 ± 0.2*	1.6 ± 0.08**	2.1 ± 0.68*
[N370S] + [I199S]	1	7.6 ± 2.6*	6 ± 2.7*	1.9 ± 0.21**	1.6 ± 0.17*
[IVS8(-11delC)(-14T>A)] + [R170P]	1	3.9 ± 1.8*	2 ± 0.6*	1.8 ± 0.14**	2 ± 0.24**
[L444P] + [W312S]	1	2.1 ± 0.6*	2.7 ± 1.2*	2 ± 0.53*	1.9 ± 0.64*
[D409H;H225Q] + [D409H;H225Q]	2	2.6 ± 0.5**	2.2 ± 0.3**	1.9 ± 0.34**	2.5 ± 0.5*
[NI88K] + [Δ55]	2	3.5 ± 1.8*	5.1 ± 2.2*	1.8 ± 0.19*	2.1 ± 0.34**
[N188S] + [S107L]	3	4.0 ± 0.5**	4.3 ± 1.5**	2 ± 0.4*	2 ± 0.12**
[L444P] + [F213I]	3	2.3 ± 0.5*	2.7 ± 1.3*	2 ± 0.45*	2 ± 0.44*
[L444P;E326K] + [L444P;E326K]	3	4.4 ± 1*	3.7 ± 1.3**	1.5 ± 0.23*	1.5 ± 0.21*

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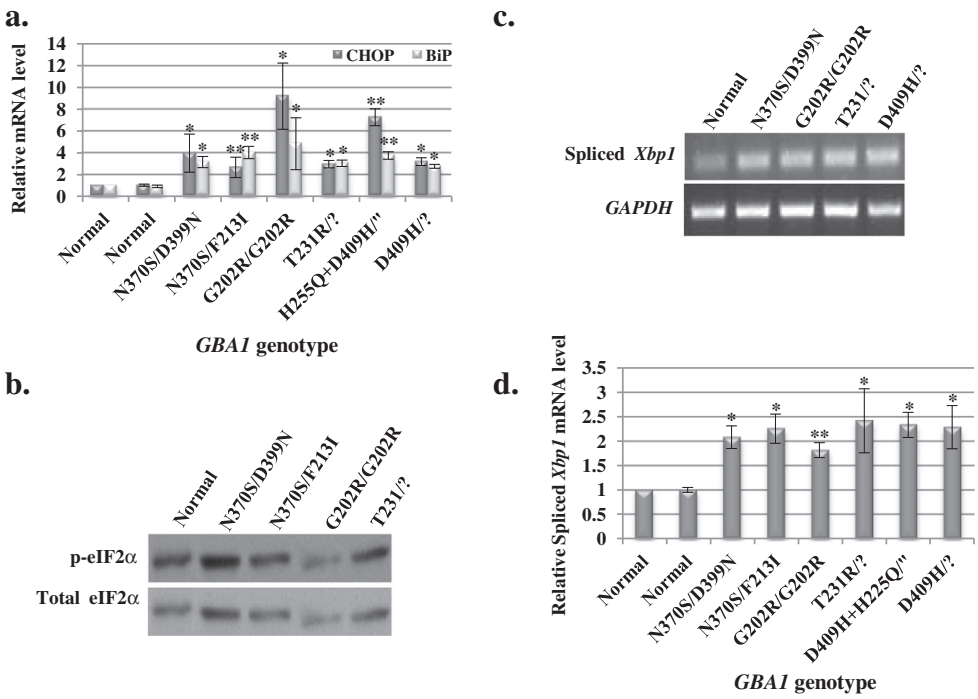

Fig. 2. UPR activation in GD EBV Transformed B-cells. a. Quantitative RT-PCR of *BiP* and *CHOP* mRNA. b. Protein lysates were prepared from different GD derived cultured B-cells and subjected to western blotting, which were interacted with anti p-eIF2α antibodies and as a loading control, with anti eIF2α antibodies. c. RT-PCR of spliced *Xbp1* mRNA. Shown are 4 out of 6 tested samples. *GAPDH* was used as a normalizing control. d. Quantification of results as shown in c. Results are the mean ± SEM of five independent experiments. Significance: * <0.05; ** <0.01.

To summarize, our results strongly suggested UPR activation in three different cell types that derived from GD patients (skin fibroblasts, EBV-transformed B cells and white WBCs) and highlight its generality in GD.

3.2. Upregulation of GBA1 transcription due to UPR activation

We have shown in the past that *GBA1* mRNA levels are elevated in GD derived cell lines [27]. We wrote then that: "comparison of the GCase mRNA levels in Gaucher-versus non-Gaucher-derived cells revealed that in Gaucher cells this RNA is always more abundant than in the corresponding non-Gaucher counterparts, suggesting the involvement of a feed-back mechanism sensitive to the levels of actual enzymatic activity" [27]. In the present study, we revisited this topic and confirmed the data by performing quantitative RT-PCR on RNA that derived from GD derived fibroblasts, from fibroblasts that originated from carriers of GD mutations, (Fig. 4a), from EBV transformed B-cells (Fig. 4b) and from WBCs that derived from GD patients (Fig. 4c). The results showed that *GBA1* mRNA level was elevated in all tested cells, that derived from GD patients or from carriers of GD mutations, in comparison to non GD cells.

Accumulation of glucosylceramide per se, induced by CBE, a non-competitive inhibitor of all cellular GCases, did not result in UPR activation. We tested whether this accumulation has an effect on *GBA1* transcription. Treatment of normal skin fibroblasts for 10 days with 200 mM CBE, did not induce elevation in *GBA1* mRNA levels (Fig. 4a), strongly suggesting that UPR activation, induced by presence of mutant GCase and not by glucosylceramide accumulation, leads to upregulation of *GBA1* transcription.

Based on our results, showing that *GBA1* transcription is upregulated in GD derived cells, which also present activation of UPR, we tested whether UPR modulates *GBA1* expression. To do so, we treated cells with thapsigargin. Thapsigargin is a non-competitive inhibitor of the sarco/endoplasmic reticulum Ca^{+2} ATPase (SERCA), which causes a decrease in ER calcium level. This decrease in ER calcium level decelerates activity of calcium-dependent ER chaperones and increases the amount of unfolded proteins in the ER [30]. Thapsigargin also inhibits fusion of autophagosomes with lysosomes, which also induces stress on the ER and activates the UPR [31]. Two hours of treatment with thapsigargin (Fig. 5a) significantly increased transcription of the *GBA1* gene in both normal and GD derived fibroblasts, strongly suggesting that UPR activation leads to elevation in *GBA1* transcription.

Table 3
UPR activation in EBV transformed B-Cells from GD patients. Values represent the mean ± SEM of five independent experiments. Significance: * <0.05; ** <0.01. N.T.—Not Tested. §All *GBA1* mutations are described according to the traditional amino-acid residue numbering, which excludes the first 39 amino acids of the leader sequence (*GBA1* GenBank accession nos. NM_000157.3; NP_000148.2).

Genotype§	GD type	Relative *CHOP* mRNA	Relative *BiP* mRNA	Relative *Xbp1* mRNA splicing	Relative p-eIF2α
Normal	–	1	1	1	1
Normal	–	1.03 ± 0.1	0.92 ± 0.09	1.1 ± 0.11	N.T.
[N370S] + [D399N]	1	3.97 ± 1.75*	3.15 ± 0.51*	2.08 ± 0.23*	0.98 ± 0.12
[N370S] + [F213I]	1	2.66 ± 0.94*	4.02 ± 0.57**	2.25 ± 0.29*	0.93 ± 0.18
[G202R] + [G202R]	2	9.2 ± 3.04*	4.83 ± 2.38*	1.81 ± 0.15**	1.04 ± 0.15
[T231R] + [?]	2	2.95 ± 0.35*	3.03 ± 0.3*	2.42 ± 0.6*	1.05 ± 0.06
[D409H;H225Q] + [D409H;H225Q]	3	7.26 ± 0.77**	3.75 ± 0.34**	2.33 ± 0.26*	0.9 ± 0.15
[D409H] + [?]	3	2.2 ± 0.35*	1.76 ± 0.18*	2.28 ± 0.44*	1.1 ± 0.14

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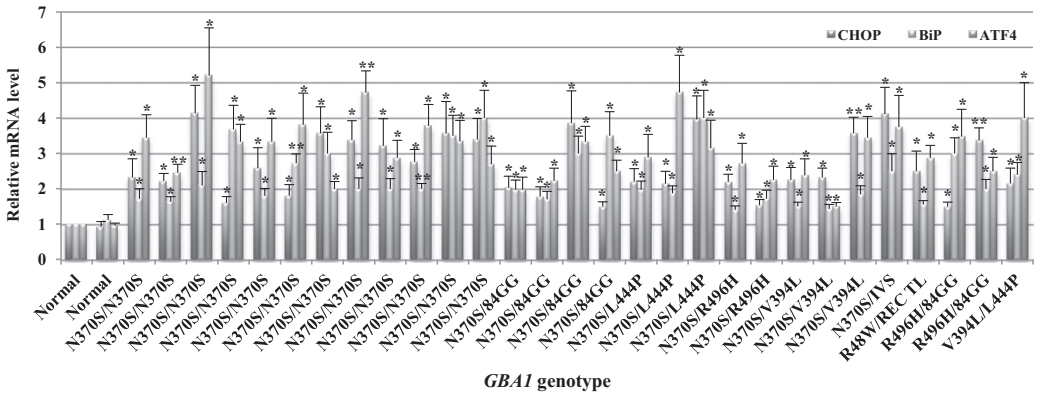

Fig. 3. UPR activation in GD derived WBCs. Quantitative RT-PCR of *BiP*, *CHOP* and *ATF4* mRNA levels. The results are the mean ± SEM of five independent experiments. Significance: * <0.05; ** <0.01.

3.3. CHOP activates the GBA1 promoter

The transcription factor CHOP is upregulated as part of UPR activation [8,15]. CHOP is a nuclear protein of the C/EBP family of transcription factors. This family was first shown to bind the "CCAAT" DNA element and to regulate a variety of genes involved in a broad range of physiological processes, including immune functions as well as cell differentiation and proliferation [32].

The *GBA1* promoter contains a "CCAAT" site, which binds C/EBP, as we have shown in the past, using EMSA and DNAse footprinting assays [29]. Since CHOP is a member of the C/EBP family [32], we decided to test whether activation of *GBA1* transcription in GD-derived cells results from UPR-mediated increase in CHOP levels. To do this, we tested the direct effect of CHOP on the 630 bp normal *GBA1* promoter fragment as well as its effect on a promoter fragment mutated in its "CCAAT" binding site (CCAAT → CTGGC, Fig. 5b). We used both promoter fragments in the past to show binding of C/EBP to the "CAAT" consensus site [28,29]. As evident from the results (Fig. 5c), CHOP increased luciferase activity directed by the normal *GBA1* promoter but not by a *GBA1* promoter mutated in its CHOP binding site. CHOP expression was confirmed by western blotting (Fig. 5d). Our results strongly suggest that UPR-activated CHOP upregulates *GBA1* transcription.

Several publications reported elevated activities of lysosomal enzymes in serum from GD patients. These included α-mannosidase and acid phosphatase [33,34]. We wondered whether this elevation reflects activation of the corresponding genes. Our results, presented in Supplementary Fig.1, indicated that there is an elevation in the *MAN2B* and *ACP* mRNA levels in all GD tested fibroblast lines as well as in WBCs. This elevation may have resulted from UPR-mediated CHOP activation.

Table 4
UPR activation in WBCs from GD patients. Values represent the mean ± SEM of five independent experiments. Significance: * <0.05; ** <0.01. §All *GBA1* mutations are described according to the traditional amino-acid residue numbering, which excludes the first 39 amino acids of the leader sequence (*GBA1* GenBank accession NM_000157.3; NP_000148.2).

Genotype§	GD type	Relative *CHOP* mRNA	Relative *BiP* mRNA	Relative *ATF4* mRNA
Normal	–	1	1	1
Normal	–	0.93 ± 0.14	1.12 ± 0.15	0.97 ± 0.06
[N370S] + [N370S]	1	2.32 ± 0.53*	1.72 ± 0.28*	3.45 ± 0.64*
[N370S] + [N370S]	1	2.22 ± 0.21*	1.64 ± 0.14*	2.46 ± 0.24**
[N370S] + [N370S]	1	4.14 ± 0.78*	2.1 ± 0.39*	5.2 ± 1.35*
[N370S] + [N370S]	1	1.6 ± 0.18*	3.68 ± 0.7*	3.33 ± 0.5*
[N370S] + [N370S]	1	2.58 ± 0.6*	1.8 ± 0.21*	3.33 ± 0.66*
[N370S] + [N370S]	1	1.81 ± 0.31*	2.73 ± 0.26**	3.81 ± 0.9*
[N370S] + [N370S]	1	3.57 ± 0.75*	3 ± 0.6*	2 ± 0.21*
[N370S] + [N370S]	1	3.38 ± 0.54*	2 ± 0.31*	4.72 ± 0.61**
[N370S] + [N370S]	1	3.22 ± 0.76*	2 ± 0.29*	2.88 ± 0.5*
[N370S] + [N370S]	1	2.76 ± 0.35*	2 ± 0.15**	3.78 ± 0.6*
[N370S] + [N370S]	1	3.57 ± 0.9*	3.5 ± 0.58*	3.34 ± 0.58*
[N370S] + [N370S]	1	3.4 ± 0.6*	4 ± 0.8*	2.7 ± 0.5*
[N370S] + [84GG]	1	2.03 ± 0.33*	1.97 ± 0.27*	1.98 ± 0.35*
[N370S] + [84GG]	1	1.78 ± 0.25*	1.71 ± 0.22*	2.23 ± 0.35*
[N370S] + [84GG]	1	3.86 ± 0.9*	3 ± 0.5*	3.33 ± 0.43*
[N370S] + [84GG]	1	1.5 ± 0.13*	3.5 ± 0.68*	2.5 ± 0.3*
[N370S] + [L444P]	1	2.2 ± 0.37*	1.98 ± 0.24*	2.89 ± 0.65*
[N370S] + [L444P]	1	2.14 ± 0.35*	1.88 ± 0.2*	4.73 ± 1.05*
[N370S] + [L444P]	1	3.97 ± 0.66*	4 ± 0.78*	3.15 ± 0.8*
[N370S] + [R496H]	1	2.2 ± 0.21*	1.41 ± 0.1*	2.72 ± 0.56*
[N370S] + [R496H]	1	1.54 ± 0.15*	1.72 ± 0.24*	2.25 ± 0.4*
[N370S] + [V394L]	1	2.25 ± 0.35*	1.51 ± 0.11*	2.39 ± 0.46*
[N370S] + [V394L]	1	2.33 ± 0.26*	1.43 ± 0.12*	1.5 ± 0.11*
[N370S] + [V394L]	1	3.58 ± 0.44**	1.85 ± 0.23*	3.45 ± 0.6*
[N370S] + [IVS2 + 1]	1	4.11 ± 0.76*	2.5 ± 0.5*	3.75 ± 0.9*
[R48W] + [RecTL]	1	2.5 ± 0.57*	1.56 ± 0.1*	2.88 ± 0.35*
[R496H] + [84GG]	1	1.5 ± 0.12*	3 ± 0.44*	3.48 ± 0.77*
[R496H] + [84GG]	1	3.37 ± 0.5**	2 ± 0.26*	2.5 ± 0.4*
[V394 L] + [L444P]	1	2.16 ± 0.43*	2.4 ± 0.34*	4 ± 1*

4. Discussion

In the present study we confirm UPR activation in fibroblasts, EBV-transformed B-cells and fresh WBCs derived from GD patients with different genotypes. We also show UPR-modulated activation of *GBA1* mRNA expression through the transcription factor CHOP.

Several previous publications documented UPR activation in GD derived skin fibroblasts [35–37]. Our lab has shown in the past that UPR is activated in fibroblast cells that derived from GD patients and from carriers of GD mutations by evaluating increase in mRNA and protein levels of *BiP* and *CHOP*, splicing of *Xbp1* and phosphorylation of eIF2α [22]. In the present work additional fibroblast lines, that derived from untested GD patients, some with genotypes not previously described, were studied for the activation of UPR. In addition, we examined EBV-transformed B-cells that derived from GD patients. UPR activation was confirmed in both cell types (Figs. 1 and 2, Tables 2 and 3). However, in EBV-transformed B-cells we could not detect a significant elevation in the level of phosphorylated eIF2α (Fig. 2b, Table 3). We assume that this is the consequence of the viral transformation and immortalization of these cells, giving them replication ability by altering different gene expression pathways [38]. Given the positive results for UPR activation in EBV-transformed GD derived B-cells, further analysis was performed on WBCs, taken directly from GD patients, in order to validate activation of UPR in these cells. *BiP*, *CHOP* and *ATF4* mRNA levels were elevated in all evaluated WBCs, highlighting UPR activation in GD patients.

We have previously shown that *GBA1* mRNA level increases in GD derived cells in comparison to non GD cells [27]. In the present study we confirmed this elevation in *GBA1* mRNA level in GD derived fibroblasts, EBV transformed B-cells and WBCs taken directly from patients

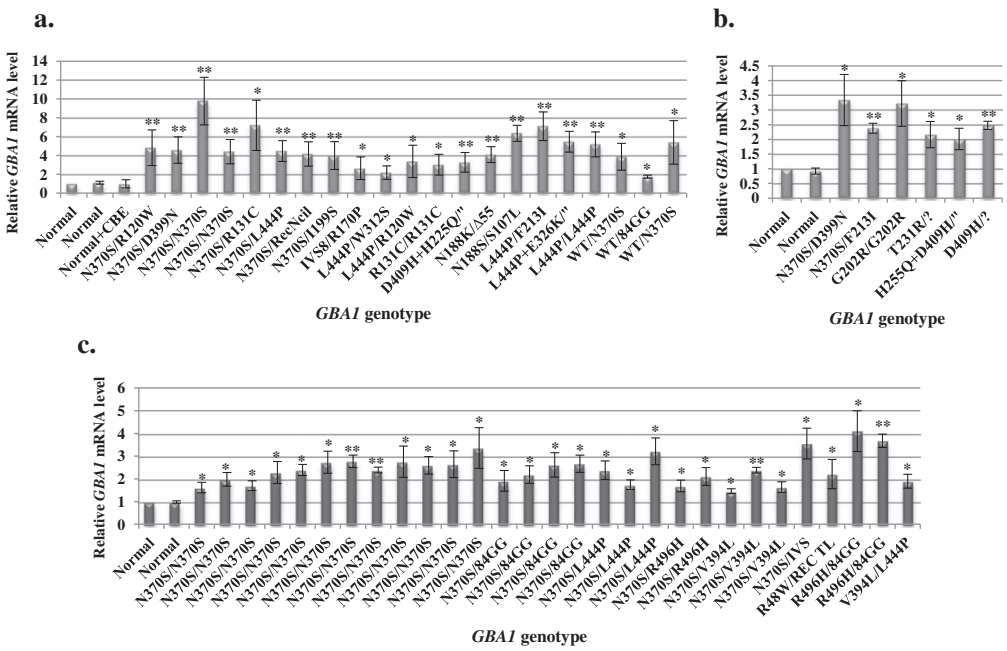

Fig. 4. Upregulation of *GBA1* transcription in GD derived cells. Quantitative RT-PCR of *GBA1* mRNA isolated from skin fibroblasts (a), EBV transformed B-cells (b) and WBCs (c). The results are the mean ± SEM of five independent experiments. Significance: * <0.05; ** <0.01.

(Fig. 4). Since UPR is activated in GD derived cells, we wondered whether the *GBA1* gene promoter contains elements that respond to this UPR activation. Thapsigargin, a known ER stressor, elevated *GBA1* transcription in both normal and GD derived cells, confirming that *GBA1* transcription is induced under activated UPR conditions (Fig. 5a).

We have previously shown, using CAT assays, that the *GBA1* promoter has an active CCAAT binding site [29]. CHOP, also known as GADD153 (growth arrest and DNA damage inducible protein) is a nuclear protein [39,40] of the CCAAT-enhancer binding protein (C/EBP) family of transcription factors, originally characterized by their sequence specific binding to CCAAT motifs in DNA [32]. *CHOP* transcription is activated by several UPR-induced pathways [8,17]. It down regulates *bcl2* and induces transcription of *GADD53* and *ERO1α*, which facilitate the dephosphorylation of eIF2α (promoting protein synthesis) and encodes an ER oxidase, respectively, thus inducing cell death [41,42]. The possible direct effect of CHOP on transcription of the *GBA1* promoter was tested using the dual luciferase assay. The results showed that the normal but not the *GBA1* promoter mutated in its CCAAT (CHOP) binding site, is activated by CHOP (Fig. 5c).

The *GBA1* promoter was evaluated in other studies [43,44]. Sidransky et al. sequenced the *GBA1* promoter region to uncover conserved sequences between species and regulatory elements within this region. Several possible transcription factor families were identified [44].

In contrast to our results, McNeill et al. showed decreased *GBA1* mRNA level in several fibroblast samples that derived from GD patients, using an Affymetrix chip array and quantitative RT-PCR. An increase in *GBA1* mRNA levels was observed following treatment of these cells with the pharmacological chaperone ambroxol [45].

Interestingly, our results also showed activation of the *MAN2B* and *ACP* genes. This activation could have also resulted from UPR-mediated CHOP activation. There is a CHOP binding site within the *MANB* gene promoter, encoding lysosomal acid α-mannosidase, as well as a CCAAT motif within the *ACP* gene promoter, encoding lysosomal acid phosphatase (prediction tool: SABiosciences' Text Mining Application). However, further studies are needed to confirm this assumption.

To summarize, our results indicated activation of UPR in different cell types derived from GD patients, highlighting the generality of this process in this disease. They also showed that the UPR-regulated CHOP transcription factor induces transcription of the *GBA1* gene. Elevated transcription of two other lysosomal enzymes, α-mannosidase and acid phosphatase, was noted as well.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bcmd.2016.10.025.

Authors' contribution

HB designed and performed experiments and wrote the manuscript. GM designed and performed experiments. GC Assisted in preparing WBCs from blood samples. MF contributed fibroblast cells and EBV transformed B-cells. AZ designed experiments and contributed blood samples from GD patients. MH designed the experiments and wrote the manuscript. All contributors edited the manuscript.

Disclosures

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Ethics approval and consent to participate

All experiments, involving blood samples from GD patients, were approved by the Shaare-Tzedek Helsinki Committee [98/13(102/12)]. All samples obtained from the Gaslini Institute had written informed consent using a form approved by the Local Ethics Committee. All the experiments described were approved by the Tel Aviv University Ethical

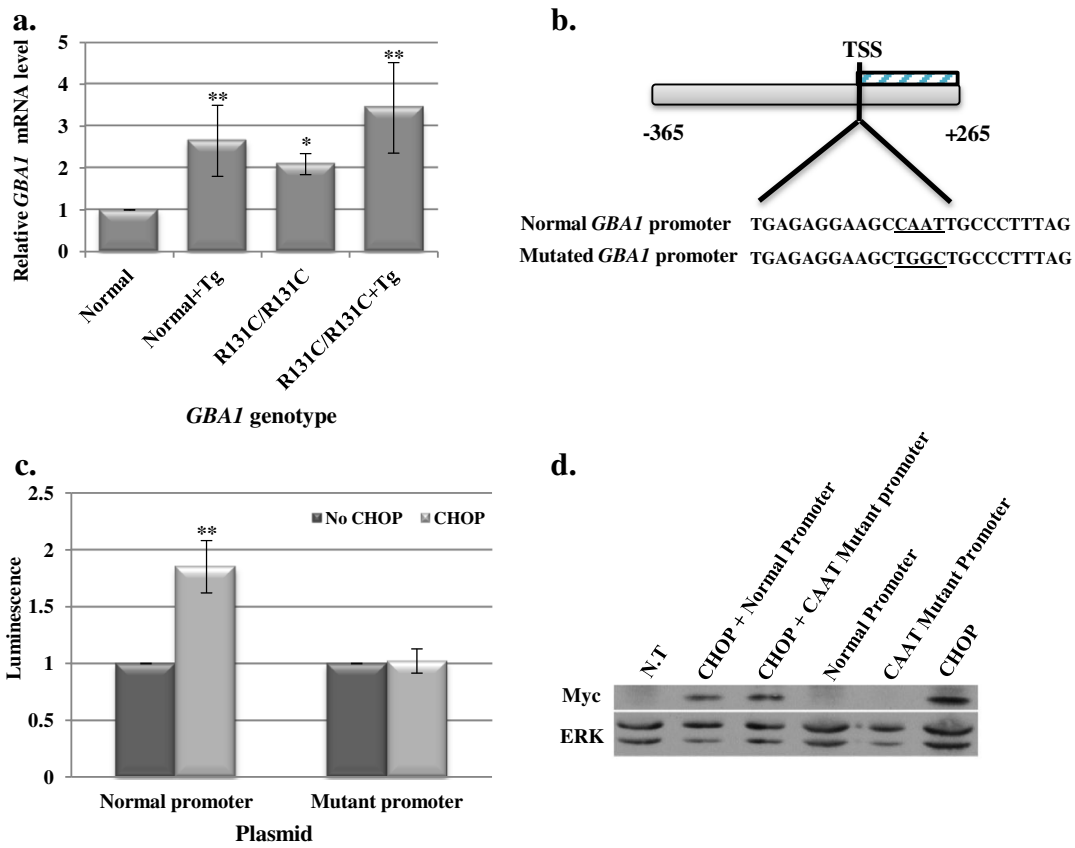

Fig. 5. GBA1 transcription is regulated by the UPR-induced transcription factor CHOP. a. Quantitative RT-PCR of *GBA1* mRNA isolated from normal or a GD derived fibroblast line, grown with or without 150 mM thapsigargin (Tg). The results are the mean ± SEM of five independent experiments and the values obtained for non-treated normal line were considered as 1. Significance: * <0.05; ** <0.01. b. Illustration of the 630 bp *GBA1* promoter fragment used in this study, which contains 365 bp upstream the transcription start site and 265 bp of the first exon. Shown beneath are the sequences at the site of mutation at the CHOP binding site, mutated from CCAAT to CTGGC. c. HEK293 cells were co-transfected with 2.4 μg of a firefly expressing plasmid, coupled to the normal (Normal Promoter) or a CCAAT mutated *GBA1* promoter fragment (CAAT mutant Promoter) with 0.6 μg of a renilla luciferase expressing plasmid as transfection control, with or without 1 μg of myc-His-CHOP expressing plasmid. Forty-eight hours later, cell lysates were prepared and luciferase activity was monitored as explained in Materials and methods. Luciferase expression is represented as firefly luciferase normalized to renilla luciferase. The results obtained for cells not transfected with CHOP were considered as 1. The results represent the mean ± SEM of 4 independent experiments. Significance: * <0.05; ** <0.01. d. Western blot of protein lysates, obtained 48h after transfection, validating the expression of the myc-His tagged CHOP. The blots were interacted with anti-myc antibody, to follow transfection levels, and with anti ERK antibodies as a loading control. N.T.—Not transfected.

Committee. All patients or patient guardians gave informed consent to the study.

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