Histone deacetylase inhibitors increase glucocerebrosidase activity in Gaucher disease by modulation of molecular chaperones

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Gaucher disease is caused by mutations of the *GBA* gene that encodes the lysosomal enzyme glucocerebrosidase (GCase). *GBA* mutations often result in protein misfolding and premature degradation, but usually exert less effect on catalytic activity. In this study, we identified the molecular mechanism by which histone deacetylase inhibitors increase the quantity and activity of GCase. Specifically, these inhibitors limit the deacetylation of heat shock protein 90, resulting in less recognition of the mutant peptide and GCase degradation. These findings provide insight into a possible therapeutic strategy for Gaucher disease and other genetic disorders by modifying molecular chaperone and protein degradation pathways.

Gaucher disease (GD) is an autosomal recessive lysosomal storage disorder resulting from mutations in the *GBA* gene encoding the lysosomal enzyme glucocerebrosidase (GCase), leading to accumulation of toxic amounts of glucocerebroside and subsequent organ and metabolic dysfunction. Approximately 360 unique mutations have been identified in GD, most of them missense mutations (1, 2). Our previous study revealed that these missense mutations result in a reduction of protein stability, rather than disruption of intrinsic enzymatic activity (3, 4). GCase undergoes significant posttranslational modification in the endoplasmic reticulum (ER). Nascent peptides form transient protein complexes with chaperone and cochaperone proteins, which facilitate proper folding and modification (5). Missense mutations in GCase destabilize the protein by introducing an unnatural conformation that results in altered chaperone binding, rendering the peptide vulnerable to recognition by E3 ligases (parkin and c-cbl) and proteasome-associated degradation (3, 6). Identifying key chaperone proteins that determine GCase proteostasis is potentially of great importance in targeting treatment of patients with GD.

Histone deacetylase inhibitors (HDACis) are a class of compounds first found to interfere with histone acetylation. HDACis such as valproic acid have been used to treat psychiatric/neurologic disorders, inflammatory diseases, and cancers (7–9). Along with their histone-modifying effects, HDACis translocate from the cell nucleus to the cytoplasm and are involved in posttranslational modification of nonhistone and cytoplasmic proteins (10, 11). Indeed, HDACis have been shown to remove acetyl moieties from heat shock protein (Hsp) 70, Hsp90, and tubulin (12–15). Several recent discoveries suggest that HDACis are effective in treating inherited diseases that arise from misfolding of proteins, such as GD, cystic fibrosis, Huntington disease, and type C Niemann–Pick disease (16–19). The molecular mechanism of how HDACis affect proteostasis remains unclear, however.

In the present study, we investigated key molecular chaperones that mediate GCase degradation. Using two common mutations for type I (N370S/N370S) and type II/III (L444P/L444P) GD, we discovered that misfolding of GCase results in fundamental changes in the protein expression profile of ER stress/ER-associated degradation (ERAD)-related genes as well as molecular chaperones. Among these chaperones, Hsp90β is essential for the degradation of misfolded GCase. Hsp90β recognizes misfolded GCase and guides the nascent protein through a valosin-containing protein (VCP)-associated degradation pathway (20, 21). HDACis cause hyperacetylation of the middle domain of Hsp90β, resulting in limited recognition of GCase mutants by Hsp90β and increased levels of GCase.

Results

Abnormal Degradation and ER Retention of *GBA* Mutants. In patients with GD, nascent GCase peptides bearing different pathogenic mutations acquire unnatural conformations and are not folded into the appropriate tertiary structure. We first investigated the subcellular distribution of GCase mutants in fibroblasts derived from either type I (N370S) or type II (L444P) GD. Consistent with previous findings, we confirmed a fundamental loss of GCase in patient-derived fibroblasts. In addition, GCase from patients with GD was consistently restricted to the ER, implying that GCase cannot be targeted to the correct subcellular compartment for assembly and function. In contrast to this, in normal fibroblasts GCase was successfully exported from ER, suggesting correct protein folding and translocation (Fig. 1*A*). In addition, we observed enhanced ubiquitination and binding of VCP to GCase mutants compared with WT. We also confirmed abnormal Hsp90 binding to GCase mutants in the same assay (Fig. 1*B*). ER retention and enhanced degradation resulted in a >90% loss of GCase activity in fibroblasts from patients with GD (Fig. 1*C*).

We next evaluated the dependence of mutant GCase on the presence of molecular chaperones and possibly a VCP-dependent pathway. We found that gene silencing of *STIP1* or *VCP* increased the quantity of mutant GCases, whereas inhibition on *HSPA5* reduced the quantity of GCase protein (Fig. 1*D*). We confirmed this result by measuring GCase activity in fibroblasts from patients with type I and type II GD, and found consistent changes in enzyme activity with the quantity of GCase (Fig. 1*E*). We found that Eeyarestatin I, an inhibitor targeting the VCP D1 ATPase domain (22), induced accumulation of ubiquitinated GCase mutants, but not of WT protein (Fig. 1*F*). This result is in agreement with previous findings that VCP interacts with E3 protein ligases and aids the elimination of ubiquitinated proteins (21, 23). Ubiquitination of WT GCase was not detected. Thus, we conclude that GCase mutants are recognized and efficiently processed in a noncanonical degradation pathway that involves molecular chaperones and ER-associated degradation.

Differential Expression of Molecular Chaperones and ERAD-Associated Genes from Accumulation of Misfolded GCase. To elucidate the molecular machinery related to GCase misfolding and degradation,

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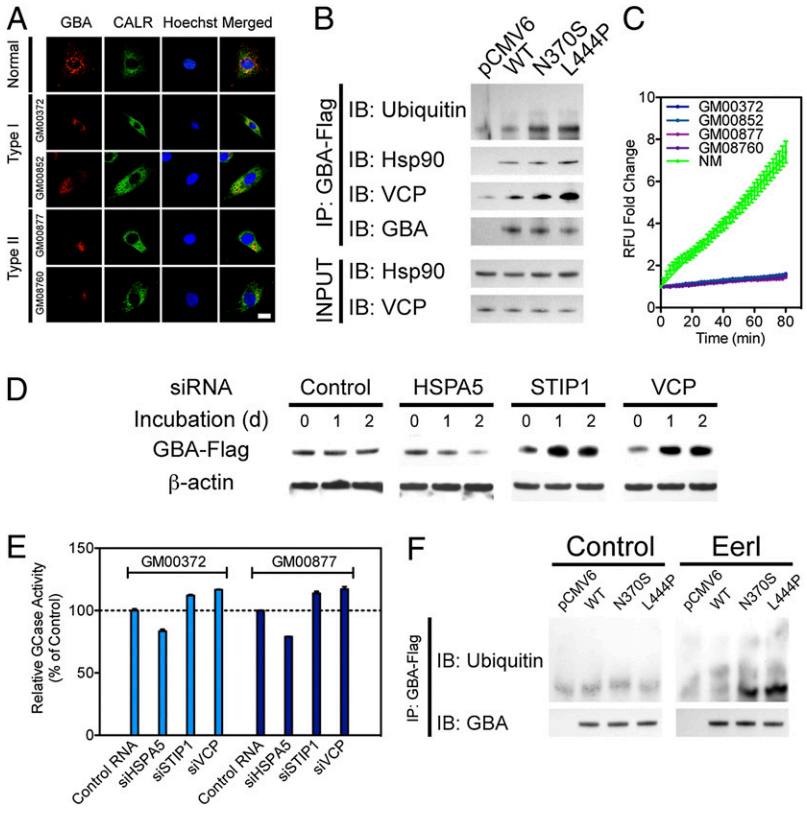

Fig. 1. Abnormal localization, protein binding, and degradation of mutant GCase. (*A*) Mutant GCase (red) was colocalized with the ER marker calreticulin (CALR, green) in fibroblasts from GD patients. (*B*) Immunoprecipitation assay of WT GCase showed increased ubiquitination of mutant GCase, with abnormal Hsp90 and VCP binding. Total cell lysate (INPUT) was used as a loading control. (*C*) GCase activity from mutant cells demonstrated 90% less activity than normal fibroblasts (NM). (*D*) RNA interference of stress-induced-phosphoprotein 1 (*STIP1*) and *VCP* increased GCase over a 2-d period. Inhibition on *HSP5A* resulted in decreased protein levels. (*E*) Gene silencing of *STIP1* or *VCP* increased GCase enzyme activity in fibroblasts derived from patients with GD. Inhibition of *HSPA5* further decreased GCase activity. (*F*) Treatment with Eeyarestatin I (EerI) prevented GCase degradation of mutated Gcases, but had no effect on WT GCase.

we performed expression array screening on protein misfolding-related genes in mRNA from HeLa cells stably expressing N370S *GBA* mutants (N370S). We used the same cell line expressing WT GBA as a baseline. We identified a global increase in chaperonin/cochaperonin gene expression in N370S cells compared with WT. These include critical protein folding machinery genes, such as *CCT4*/*CCT7*/*TCP1*, *DNAJC3*, and *HSPA5*, as well as key regulators of protein degradation, such as *SYVN1*, *DERL2*, and *VCP* (Fig. 2*A*). We confirmed the changes in fibroblasts derived from patients with type I or type II GD. Quantitative PCR confirmed up-regulation of ER stress/ERAD- related genes, as well as chaperone/cochaperone genes in patient fibroblasts (Fig. 2*B*). These findings are consistent with previous reports showing that mutant GCase degradation requires ER-associated machinery (24). The gene expression pattern was also found to correlate with disease severity.

Hsp90 Recognizes GCase Mutants and Mediates Their Degradation. Hsp90 is a key molecular chaperone that aids in protein folding, protein complex assembly, and protein degradation (25, 26). Hsp90 exhibits increased affinity to GCase mutants, suggesting that this molecular chaperone is involved in the folding and degradation of GCase mutants (4). To explore the functional relationship between Hsp90 and GCase degradation, we overexpressed *GBA* mutants in HeLa cells, combined with WT (Hsp90β-WT) or dominant-negative Hsp90β recombinant (Hsp90β-D88N). Consistent with previous findings, we identified abnormally increased ubiquitination of GCase mutants. Cotransfection of Hsp90β-WT resulted in similar trends in ubiquitination, indicating that endogenous Hsp90 is sufficient for the posttranslational modification and degradation of GCase mutants. Pharmacologic inhibition of Hsp90 through either Hsp90β-D88N or the small-molecule inhibitor 17-*N*-Allylamino-17-demethoxygeldanamycin (17-AAG) resulted in decreased ubiquitination of GCase mutants (Fig. 3*A*).

We calculated protein ubiquitination and found a similar reduction of GCase ubiquitination in N370S and L444P mutants (Fig. 3*B*). Changes in GCase degradation efficiency were confirmed through measurement of the half-lives of GCase mutants with a cycloheximide (CHX) assay. Inhibition of Hsp90 increased the life span of both GCase mutants (Fig. 3 *C* and *D*). These studies demonstrate the essential roles of Hsp90β in the degradation of GCase mutants.

Deacetylated Hsp90β Recognizes GCase Mutants and Directs Them for Degradation. In concert with other chaperones and cofactors, Hsp90 facilitates the folding and establishment of appropriate tertiary protein structures. A recent study demonstrated that the amino acid residue K294 appears to be located at the junction of the charged linker region of the Hsp90α middle domain (27). This lysine residue has been found to be modified by protein acetylases, such as HDAC6 (13, 28). Acetylation of K294 in Hsp90α may serve as a molecular switch for functional alterations of this chaperone. To investigate the relationship of Hsp90β acetylation and GCase degradation, we designed acetylation-mimicking recombinants in the identical acetylation domain in Hsp90β. The recombinants mimic the acetylated (K286Q and K286A) and the deacetylated (K286R) form of Hsp90β. An immunoprecipitation assay demonstrated that WT Hsp90β recognized both N370S and L444P GCase mutants (Fig. 4*A*). Deacetylation-mimicking Hsp90β recombinant K286R exhibited identical affinity to both GCase mutants, suggesting that Hsp90β is normally involved in mutant protein recognition and consequent degradation. In contrast, recombinants that mimic acetylated Hsp90β failed to recognize GCase mutants, consistent with the finding that acetylation in this precise domain alters Hsp90β affinity to substrate peptides.

We further confirmed that acetylation of Hsp90β abolishes ubiquitination of GCase mutants. Immunoprecipitation assays showed that ubiquitination was decreased by >50% in K286Q and K286A recombinants, but to a lesser degree in K286R

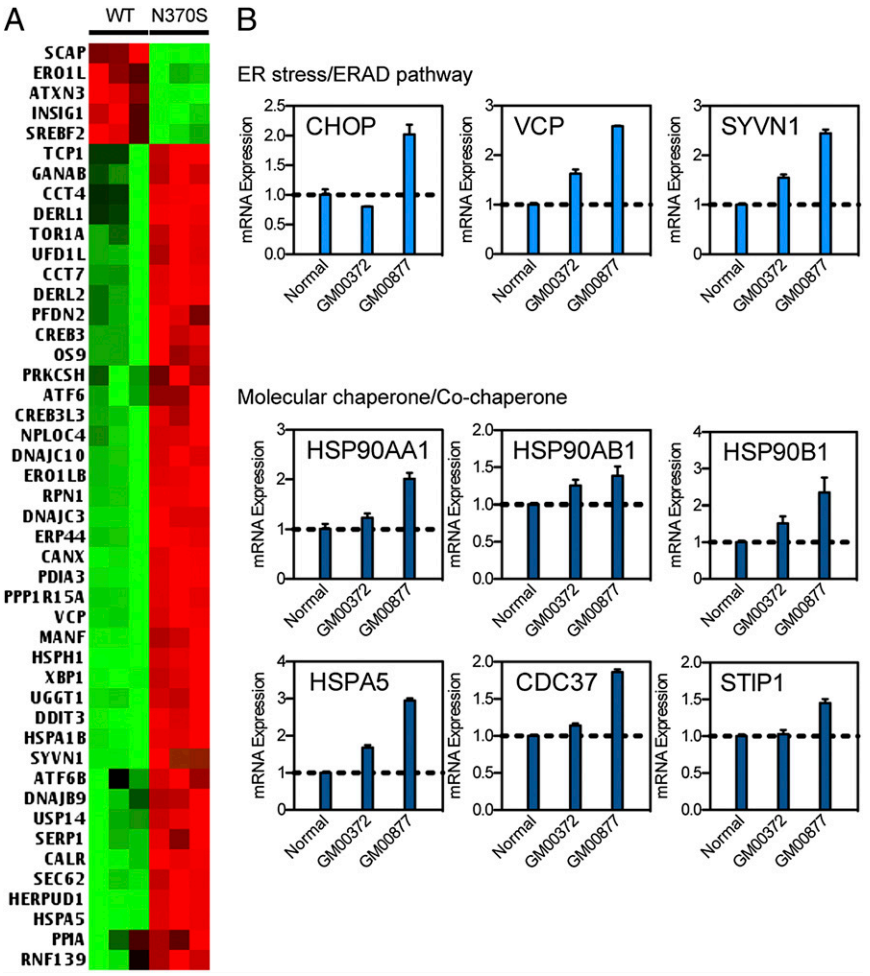

Fig. 2. Misfolded GCase directly impacts the expression levels of chaperone genes. (*A*) Gene expression profile of WT and mutant (*N370S*) in transfected HeLa cells showed key differences in relevant genes. Notably, proteins involved in folding (*CCT4*, *CCT7*, *TCP1*, *DNAJC3*, *HSPA5*) and regulation of degradation (*SYVN1*, *VCP*, and *DERL2*) were up-regulated in the mutant cells. (*B*) Quantitative PCR confirmed elevated degradation-related genes (*Upper*) and chaperone- and cochaperone-related genes (*Lower*) in fibroblasts derived from patients with GD.

analogs (Fig. 4 *B* and *C*). Finally, we measured the stability of GCase mutants by CHX assays with various Hsp90β mutants. The results indicated that acetylated Hsp90β contributes to the stabilization of mutated GCases, whereas transfection of the deacetylated

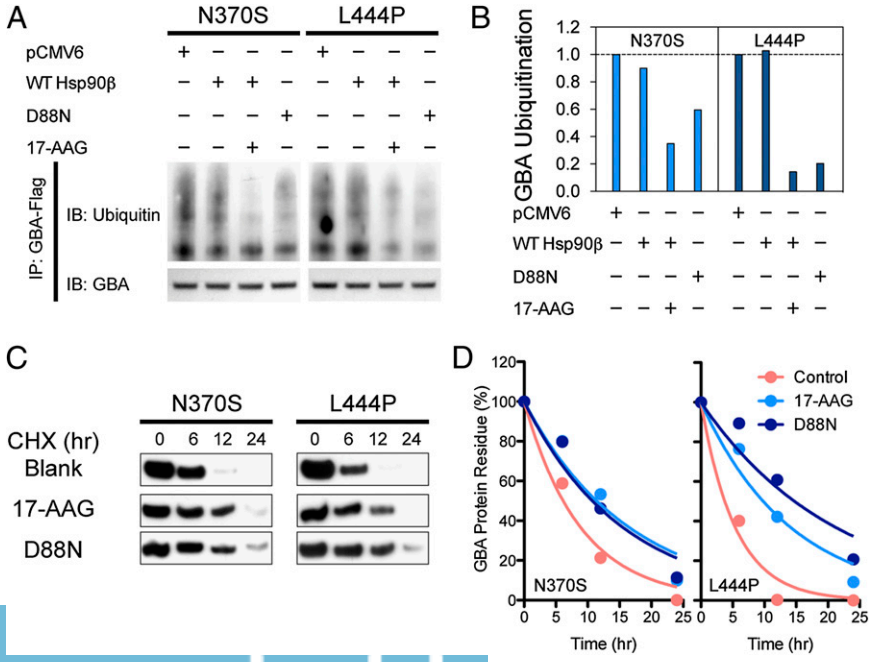

Fig. 3. Hsp90 mediates mutant GCase degradation. (*A*) Immunoprecipitation assay showed pharmacologic (17-AAG) and dominant-negative (D88N) inhibition of Hsp90 resulted in decreased ubiquitination of mutant GCase. (*B*) Quantified ubiquitination levels with these inhibitors supported Hsp90 dependence on GCase degradation. (*C*) CHX assay, which halts protein synthesis, showed an elongated protein half-life with the two inhibitors compared with nontreated cells (Blank). (*D*) Quantification of protein half-lives of GCase mutants N370S (*Left*) and L444P (*Right*).

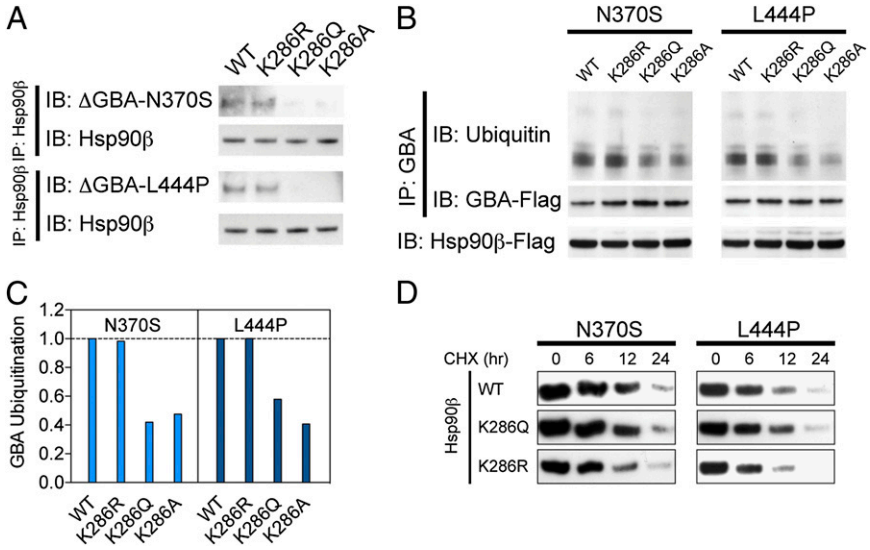

Fig. 4. Deacetylated Hsp90β recognizes GCase mutants and guides degradation. (*A*) WT and Hsp90β recombinants that mimic acetylated (K286Q and K286A) and deacetylated (K286R) forms of Hsp90β were coexpressed with mutant GCase. Immunoprecipitation assays showed that WT and deacetylated Hsp90 precipitate with mutant GCase, whereas acetylated Hsp90β does not bind to GCase. (*B*) An immunoprecipitation assay showed that decreased recognition by an acetylated form of Hsp90β results in decreased ubiquitination. (*C*) Quantification of ubiquitination of GCase mutants with transfection of different Hsp90β recombinants. (*D*) CHX assay showed that the half-life of GCase is improved in the presence of the acetylated form of Hsp90β recombinants.

form results in similar degradation efficiency with WT Hsp90β (Fig. 4 *C* and *D*).

HDACis Modulate Acetylation of Hsp90 and Increase the Quantity of Protein of GCase Mutants. Finally, we evaluated the effect of HDACis on increasing the quantity of mutant GCase proteins. HDACis such as LB205 and SAHA have been shown to potently increase GCase activity in patient-derived fibroblasts (4); however, the molecular mechanism through which HDACis stabilize GCase mutants is largely unknown. HDACis interact physically with Hsp90 and modify its acetylation (28), raising the possibility that HDACis may increase GCase half-life through the modulation of molecular chaperones by antagonizing their deacetylation. To confirm this, we measured Hsp90 acetylation in patient-derived fibroblasts treated with HDACis. We detected a global increase in lysine acetylation in Hsp90 treated with either LB205 or SAHA (Fig. 5*A*). Recognition and ubiquitination of mutated GCases were limited with WT Hsp90β transfection and SAHA treatment. Recombinant protein that mimics acetylated Hsp90β (K286R) was less affected by SAHA treatment (Fig. 5*C*). Finally, we confirmed that both LB205 and SAHA increased GBA activity in fibroblasts derived from patients with type II and type III GD (Fig. 5*B*).

Discussion

We have confirmed that pathogenic mutations of *GBA* cause protein misfolding and chaperone-dependent premature degradation via a VCP-dependent pathway. Furthermore, HDACis effectively rescue GCase mutants from degradation by increasing acetylated Hsp90β and subsequently altering its function as a molecular chaperone. Thus, HDACis increase functional GCase and may serve as a valuable therapeutic paradigm for inherited diseases (Fig. 6).

HDACis are a group of small-molecule compounds that were initially found to antagonize histone deacetylase. HDACis have since been applied in therapeutics strategies for psychiatric/neurologic disorders, inflammatory diseases, and cancers. Recent studies have shown that HDACis are effective in genetic disorders resulting from protein misfolding. HDACis were found to increase the quantity of cystic fibrosis transmembrane conductance regulator by reducing its degradation (29). Our previous study showed that HDACis can effectively rescue GCase mutants from premature degradation and thus increase GCase activity (4). The molecular mechanism of HDACis' affect on GCase stability has remained obscure, however.

Hsp90 is involved in both protein folding and degradation (25, 26). We have found that Hsp90 is essential for recognizing GCase mutants and guiding them to degradation. Inhibition of Hsp90 chaperone function via the chemical inhibitor 17-AAG or dominant

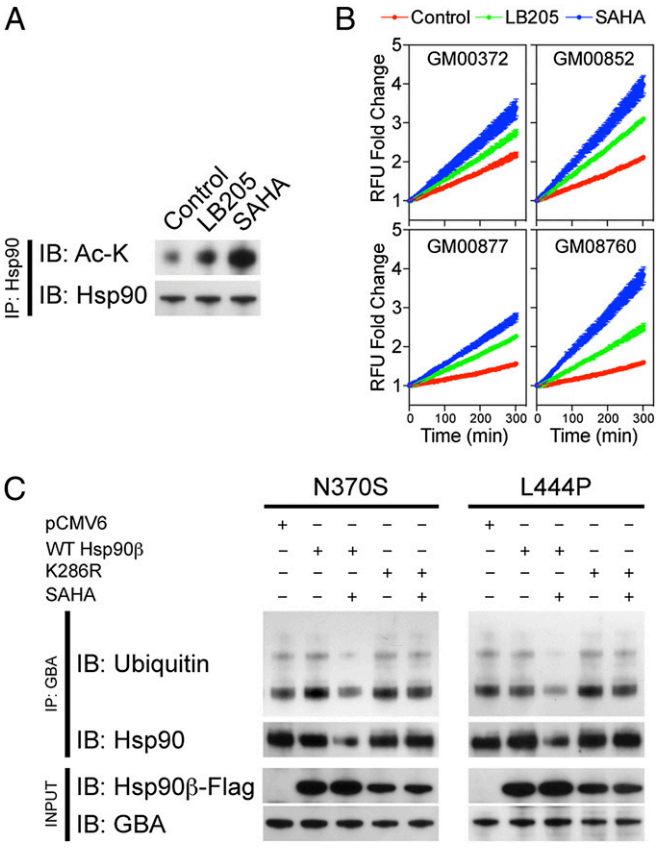

Fig. 5. HDACis increase GCase activity by reducing the deacetylation of Hsp90. (*A*) Immunoprecipitation assays showed that HDACis SAHA and LB205 result in increased acetylation of lysine in Hsp90. (*B*) HDACis increased GCase enzyme activity in fibroblasts derived from patients with GD. (*C*) SAHA treatment of WT Hsp90 resulted in decreased recognition and ubiquitination of GCase mutants. The Hsp90β deacetylated form of recombinant K286R was unaffected by treatment with SAHA.

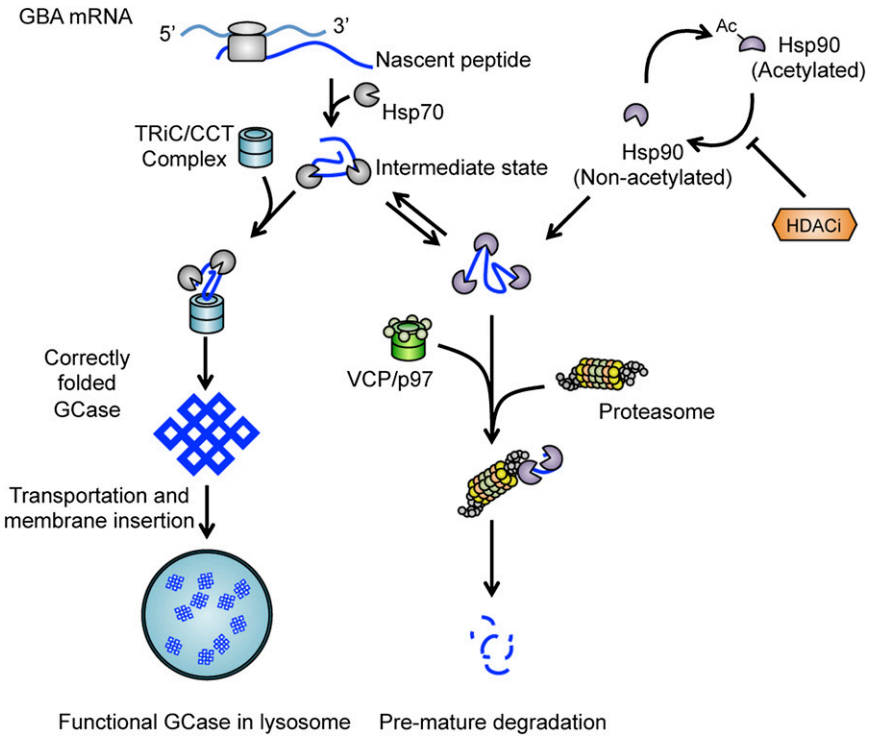

Fig. 6. Hsp90 acetylation regulates degradation of GCase. GCase nascent peptides are recognized efficiently by molecular chaperone/cochaperone system (Hsp70 and TRiC/CCT complex) and folded to the proper tertiary structure. Correctly folded GCases are functional after being transported into lysosome. However, mutant GCases are recognized by Hsp90 and retained in the ER compartment. Mutant peptides are efficiently degraded through the VCP/p97 and proteasome pathways. HDACis accumulate the acetylated form of Hsp90 and thus abolish Hsp90 chaperone function, limit Hsp90 affinity to GCase mutants, and increase the likelihood of correct folding of GCase mutants.

negative recombinants not only reduces the affinity to GCase mutants, but also extends protein half-life. A recent study suggested that Hsp90 chaperone function could be modulated by taking advantage of reversible acetylation (30, 31), which raises the possibility of modulating proteostasis of GCase mutants by affecting the acetylation of Hsp90. Acetylation of a particular amino acid residue (e.g., K294 in Hsp90a, K286 in Hsp90b) fundamentally changes chaperonin function (27). Indeed, it has been shown that inhibition of histone deacetylase results in hyperacetylation of Hsp90 and subsequent alteration of its complex structure and protein assembly (32).

We have demonstrated that HDACi treatment modulates K286 acetylation in Hsp90β, thereby reducing its affinity to mutant GCase peptides, decreasing ubiquitination, and elongating the life span of protein. Our findings reveal a unique molecular mechanism in which HDACis stabilize misfolded proteins by affecting the acetylation status of key molecular chaperones and their affinity to client proteins, leading to an increase in functional GCase levels.

Finally, ERAD designates a cellular pathway that targets misfolded proteins to the ER compartment and subsequent degradation through a protein-degrading complex (33). The ERAD pathway is likely involved in the degradation of GCase mutants and thus contributes to the pathogenesis of GD (34). Our results show that GCase mutants exhibit abnormal affinity to VCP, a key protein regulator in the ERAD process. The molecular details of this compartmentalized degradation are not entirely clear, however.

Our findings demonstrate that molecular chaperones may be a valuable therapeutic option for GD, especially for the neuronopathic phenotypes. Small-molecule compounds such as HDACis result in hyperacetylation of chaperones such as Hsp90, limiting their affinity to misfolded protein and increasing the quantity of GCase and overall catalytic activity of GCase mutants. In general, pharmacologic targeting of the chaperone system may be useful in disorders caused by misfolded proteins, such as other lysosomal storage disorders, cystic fibrosis, neurofibromatosis type 2, SDHB-associated neuroendocrine tumors, and neurofibromatosis type II (18, 29, 35, 36). More specific molecular targeting, as well as in vivo analysis, would be significant in further elucidating the therapeutic effects of HDACis.

Materials and Methods

Cell Culture. Fibroblasts from patients with GD were maintained as described previously (4). Cells from patients with type I GD (GM00372 and GM00852) and those with type II GD (GM00877 and GM08760) were obtained from Coriell. HeLa cells were purchased from American Type Culture Collection. Cells were incubated in Eagle's minimum essential medium (Invitrogen) supplemented with 10% FBS.

Immunofluorescence Analysis. Patient-derived fibroblasts were fixed in Histochoice (Mandel) and labeled with primary antibodies against GBA (Abcam) and CALR (Thermo Scientific) overnight. The specimens were then labeled with secondary antibodies with Alexa Fluor 488 or Alexa Fluor 594 conjugation (Invitrogen). Cell nuclei were counterstained with Hoechst 33342 (Invitrogen). Specimens were visualized through a Zeiss LSM 510 confocal microscope.

DNA Cloning and Site-Directed Mutagenesis. WT, N370S, and L444P *GBA* recombinants were prepared as described previously (4). Human *HSP90AB1* gene was incorporated into the pCMV6-entry vector (Origene). Mutagenesis of the K286 site was performed using the QuikChange Lightning Site-Directed Mutagenesis Kit (Agilent). The nucleotide sequence of the *HSP90AB1* gene was verified by analyzing the entire coding regions through Sanger sequencing. The *HSP90AB1* dominant negative vector (Hsp90β-D88N) was a gift from William Sessa (Department of Pharmacology and Vascular Biology and Therapeutics Program, Boyer Center for Molecular Medicine, Yale University School of Medicine, New Haven, CT) (37).

Immunoprecipitation. Immunoprecipitation was performed as described previously (19). In brief, cell pellets were lysed in Nonidet P-40 lysis buffer supplemented with Halt proteasome inhibitor (Thermo Scientific). Total cell lysate was precipitated using the DynaBeads Protein G Immunoprecipitation Kit (Invitrogen) and antibodies against FLAG tag (Origene), GBA (Sigma-Aldrich), or Hsp90 (Abcam). Precipitated protein was eluted and resolved through Western blot analysis.

Western Blot Analysis. Cell pellets were harvested and lysed in RIPA lysis buffer with Halt proteasome inhibitor mixture (Thermo Scientific). Protein was quantified using the Bio-Rad Protein Assay Kit. Samples were separated on NuPAGE Bis-Tris 4–15% (vol/vol) gel (Invitrogen) and transferred to PVDF

membranes (Millipore). The membranes were probed with primary antibody and detected through an HRP-conjugated species-specific secondary antibody and an ECL kit (Pierce). The intensity of the image was determined by densitometric analysis using ImageJ software. The primary antibodies used in this study include GCase (Sigma-Aldrich and Abcam), CALR (Thermo Scientific), ubiquitin (Abcam), Hsp90 (Cell Signaling Technology and Abcam), VCP (Cell Signaling Technology and Abcam), DDK (Origene), and acetyl-lysine (Millipore).

Assay of GCase Activity. The fluorometric GCase enzyme activity assay was performed as described previously (38). In brief, 1 million cells were pelleted and flash-frozen on dry ice. Cells were lysed in a buffer containing 50 mM citric acid/potassium phosphate (pH 6.0), 0.2% Triton X-100, and 1% (wt/vol) sodium taurocholate (Sigma-Aldrich) for protein extraction. The 30 μg of protein was incubated with GC assay buffer containing 100 mM potassium phosphate (pH 6.0), 15 mM 4-methylumbelliferylglucopyranoside (Sigma-Aldrich), 0.15% Triton X-100, 0.125% (wt/vol) sodium taurocholate (Sigma-Aldrich), and 0.1% (wt/vol) BSA for 5 h. Fluorescence was measured with a BioTek plate reader at an excitation/emission setting of 355/460 nm. GCase activity was normalized compared with normal fibroblast control.

RNA Interference. RNA interference was carried out with Qiagen FlexiTube siRNA HSPA5 (SI02780554), STIP1 (SI03019471), and VCP (SI03019730). For this, 100 pmol siRNA oligo was transfected into HeLa cells or patient-derived fibroblasts using RNAiMAX reagent (Invitrogen). Expression of GBA-Flag and GCase activity were subsequently investigated.

Quantitative PCR. Total RNA was extracted from cell pellets using the RNeasy Mini Kit (Qiagen). Genomic DNA was removed through in-column DNase I digestion (Qiagen). RNA was reverse-transcribed into cDNA using SuperScript III First-Strand Synthesis SuperMix (Invitrogen). The cDNA products were analyzed on an Eco Real-Time PCR System (Illumina). The following primer sets were used: CHOP (QT00082278), VCP (QT00089712), SYVN1 (QT01669983), HSP90AA1 (QT00246967), HSP90AB1 (QT01039864), HSP90B1 (QT00046963), HSPA5 (QT00096404), CDC37 (QT01667078), and STIP1 (QT00054873). RT^2 Profiler PCR Unfolded Protein Response PCR Array (PAHS-089Z, Qiagen) was used according to the manufacturer's protocol.

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