Development of an *in vivo* plant-based screen for identifying pharmacological chaperones for treatment of human lysosomal storage diseases

by

Gholamreza Babajani

M.Sc., Simon Fraser University, 2008
B.Sc., Shahid Chamran University, 2001

Thesis Submitted in Partial Fulfillment of the
Requirements for the Degree of
Doctor of Philosophy

In the
Department of Biological Sciences
Faculty of Science

SIMON FRASER UNIVERSITY

Summer 2014

Approval

Name: **Gholamreza Babajani**

Degree: **Doctor of Philosophy (Biology)**

Title: ***Development of an in vivo plant-based screen for identifying pharmacological chaperones for treatment of human lysosomal storage diseases***

Examining Committee: **Chair:** Carl Lowenberger
Professor

Allison Kermode
Senior Supervisor
Professor

Lorne Clarke
Supervisor
Professor
Department of Medical Genetics
The University of British Columbia

Gordon Rintoul
Supervisor
Associate Professor

Don Mahuran
Internal External Examiner
Professor
Department of Laboratory and
Pathobiology Medicine
University of Toronto

Francis Choy
External Examiner
Professor
Department of Biology
University of Victoria

Date Defended: July 8, 2014

Partial Copyright Licence

Abstract

Small-molecule- enzyme enhancement therapy has emerged as an attractive approach for the treatment of lysosomal storage diseases (LSDs), a broad group of genetic diseases caused by mutations in genes encoding lysosomal enzymes or proteins required for lysosomal function. Missense mutant lysosomal enzymes normally subjected to rapid disposal by ER-associated degradation (ERAD) can be stabilized by small molecule chaperones that increase residual enzyme activity largely by increasing the transport and maturation of the mutant enzyme. Mucopolysaccharidosis I (MPS I) and Gaucher disease were my research targets – two LSDs caused by a deficiency of α-L-iduronidase (IDUA) and β-glucocerebrosidase (GCase), respectively. My goals were two-fold: (1) To determine the proteostasis of a severely defective mutant lysosomal enzyme in plant cells. (2) To develop a plant-cell-based screening system to identify putative LSD therapeutics. For the former goal, post-ER trafficking of the severely malfolded L444P GCase protein, and some aspects of cellular homeostasis, were restored to different degrees by ERAD inhibitors and proteostasis regulators, which increased the steady-state levels of the mutant protein inside the plant cells and rescued a proportion of protein from proteolysis. For goal 2, I developed a plant-cell-screening tool for identifying putative small molecule therapeutics based on selecting for library molecules capable of enhancing the post-ER transport of missense mutant lysosomal enzymes. Since the recombinant variants were equipped with a signal peptide, and the expression cells - transgenic tobacco BY2 cells - possess no lysosomes, the assay was based on increased lysosomal enzyme activity in the secretion media. I first established the proof-of-principle for the assay (i.e. its selectivity and specificity) based on recombinant N370S GCase, and two characterized chaperones - ambroxol and N-(n-nonyl) deoxynojirimycin. Two IDUA mutant proteins that underlie MPS I disease (P533R- and R383H- IDUA), formed the basis of the plant-cell-based assay that was used to screen a library of 1,040 Food and Drug Administration-approved drugs. Downstream validation of the hits identified in the primary screening by secretion and heat denaturation assays resulted in the identification of a potential candidate molecule ('X-372') for P533R IDUA. Further development of this molecule may yield a therapeutic for MPS I disease.

Keywords: Lysosomal storage diseases; missense mutations; pharmacological chaperones; tobacco BY2 cells; high-throughput screening (HTS); small molecule libraries

Dedication

I would like to dedicate this thesis to my parents who have always supported and encouraged me to achieve success in my higher education.

Acknowledgements

I am grateful for the invaluable assistance I received from many amazing people during my PhD research. First, I would like to express my deepest and sincere gratitude to my senior supervisor Dr. Allison Kermode for giving me the opportunity to study under her supervision. I would like to thank Allison for her mentorship, knowledge, endless encouragement and full support in all aspects, which helped me to overcome many hurdles along the way. The training I received under Allison over the duration of my PhD has been a very rewarding experience. Thank you very much Allison for everything.

I gratefully acknowledge Dr. Lorne Clarke, part of my supervisory committee, for his insights, valuable guidance and encouragement during my PhD. I thank Dr. Gordon Rintoul, also a member of my supervisory committee, for his great advice, support and encouragement during my research. I especially acknowledge Dr. Don Mahuran for providing me the opportunity to visit his lab at SickKids hospital and for his insights, experimental ideas and guidance on different aspects of this work. I received valuable advice from Dr. Mahuran during my visit as well as after my return to SFU. I further thank Dr. Mahuran for accepting the role of being my public examiner. I thank Dr. Francis Choy for accepting to be the external examiner of my defense. I would also like to convey my gratitude to Dr. Carl Lowenberger for being the chair of my defense.

The screening work presented here was conducted in collaboration with the Centre for Drug Research and Development (CDRD). I wish to thank Dr. Tom Pfeifer for graciously allowing me to conduct the screening project at CDRD. I specially thank Dr. Sheetal Raithatha for his tremendous help and effort with conducting the drug screening and data analysis. Without his expertise, my screening project could never have been realized.

The support of all lab members is greatly appreciated. Many thanks to Dr. Xu He, Dr. Ying Zeng, Dr. Tiehan Zhao, Dr. Kerstin Müller, Dr. Kristoffer Palma and Dr. Stephanie McInnis for all of their generous assistance and providing fruitful discussions. My special thanks go to Dr. Xu He. I obtained valuable assistance and advice from her during these years. Many thanks to Ying as well for all of his support and assistance. Thanks to

Kerstin from whom I learned qPCR. I gratefully thank Dr. Joost Lücker for his generous assistance with editing chapters 3 and 4. Many thanks to Ryan Thomas, Dhani Kalidasan and Owen Pierce for their assistance during my research. I wish to thank the newest member of our lab, Dr. Lydia Cheng, for her assistance with protein purification. Thanks to all the past and current members of the Kermode Lab.

I would like to thank Dr. Mahuran's lab for their great hospitality during my visit at SickKids. I specially thank Dr. Michael Tropak for fruitful discussions and guidance. Thanks to Dr. Brigitte Rigat, Ms. Mana Chow, Ms. Amy Leung, Ms. Sayuri Yonekawa, Ilona Tkachyova and Daphne Benedict for their assistance during my visit.

I thank Dr. Davidson and his lab especially Dr. Krzysztof Lubieniecki for providing access to the equipment needed to conduct qPCR.

I am thankful to Dr. Graham Hallson for his generous help with revising the introduction of my thesis and providing me with helpful comments.

I would like to thank our graduate program assistant Marlene for all of her help and support during my degree.

I would like to gratefully thank Dr. Mala Fernando for her support during my journey at SFU. Great thanks to Leslie Dodd, Alex Fraser, Dave Carmean and David Qu for their endless technical support.

Funding of this research was provided by the National sciences and Engineering Research Council of Canada (NSERC) Strategic, Michael Smith Foundation for Health Research and the Canadian MPS Society grants. I would also like to thank the department of Biological Sciences for providing graduate fellowships and the dean of Graduate Studies for the president's research stipend.

Words fail me to express my appreciation to my beloved parents. I take this opportunity to express my profound gratitude to my Mom and Dad. I am deeply and forever indebted to my parents for their love, support and encouragement throughout my entire life. I dedicate this thesis to my mother and father. I am also very grateful to my sisters and my brother for their love and support as well.

Finally, I would like to thank everybody who took part in the successful completion of this thesis, and express my sincere apologies if I have missed to acknowledge their names. Thank you all.

Table of Contents

List of Tables

List of Figures

List of Supplementary Figures and Tables

Abbreviations

Aβ, amyloid-β peptide

ABX, Ambroxol

Alpha (α)-Gal A, α-galactosidase A

AD, Alzheimer's disease

APP, amyloid precursor protein

ATF6, activating transcription factor 6

BBB, blood-brain barrier

BiP, binding protein

BY2, bright yellow 2

CaMV, cauliflower mosaic virus

CHO, Chinese hamster ovary

CD-MPR, cation-dependent M6P receptor

CNS, central nervous system

CNX, calnexin

COP II, coat protein II

CP, citrate phosphate

CRT, calreticulin

DGJ, 1-deoxy-galactonojirimycin

DMSO, dimethylsulfoxide

Doα10, degradation of α factor

ECL, enhanced chemiluminescence

ECM, extracellular matrixes

EDEM, ER degradation enhancing -mannosidase-like protein

EerI, Eeryastatin I

EET, enzyme enhancement therapy

eIF2, eukaryotic initiation factor 2

EndoH, endoglycosidase H

ER, endoplasmic reticulum

ERAD, endoplasmic reticulum-associated degradation

ERGIC, ER-Golgi intermediate compartment

ERSE, ER stress response elements

ERT, enzyme replacement therapy

ES5, Endosidin 5

FDA, Food and Drug Administration

GAG, glycosaminoglycans

GADD34, (growth arrest and DNA damage–inducible 34

GAPDH, glyceraldehyde-3-phosphate dehydrogenase

GCase, glucocerebrosidase

GD, Gaucher disease

Glc, glucose

GPX2, glutathione peroxidase 2

HDAC6, histone deacetylase 6

hPreP, pre-sequence protease

HSF1, heat shock transcription factor 1

HSP40, heat shock protein 40

HSP70, heat shock protein 70

HSR, heat-shock response

HTS, high-throughput screening

IDUA, α-L-iduronidase

IFG, isofagomine

IRE1, inositol-requiring protein 1

Kif, kifunensine

LACTA, library of active compounds in Arabidopsis

LIMP-2, lysosomal integral membrane protein type 2

LSD, lysosomal storage diseases

M6P, mannose 6-phosphate

MPR, M6P receptors

MPS I, mucopolysaccharidosis I

MS, Murashige and Skoog

MUGP, 4-methylumbelliferyl-β-D-glucopyranoside

MUI, 4-methylumbelliferyl α-L-idopyranosiduronic acid

NB-DNJ, N-butyldeoxynojirimycin

NBD, nucleotide-binding domain

NINDS, National Institute of Neurological Disorders and Stroke

NN-DNJ, N-(n-nonyl)deoxynojirimycin

NPC, Niemann-Pick type C

NTC, no-template-controls

OOP, organellar oligopeptidase

OST, oligosaccharyl transferase

PC, pharmacological chaperones

PD, Parkinson disease

PDI, protein-disulfide isomerases

PERK, protein kinase RNA-like endoplasmic reticulum kinase

PM, plasma membrane

PR, proteostasis regulators

RIDD, IRE1-dependent decay

ROS, reactive oxygen species

RT-PCR, reverse transcription-polymerase chain reaction

S1P, site 1 protease

S2P, site 2 protease

SBD, substrate-binding domain

SOD1, Superoxide dismutase 1

SRP, signal recognition particle

SRT, substrate reduction therapy

TGN, trans-Golgi network

TP, transit peptides

TSP, total soluble protein

UPR, unfolded protein response

UPRE, unfolded protein response elements

XBP-1, x-box binding protein 1

Chapter 1.

Introduction

1.1. Lysosomal storage diseases: Mucopolysaccharidosis I and Gaucher disease

Lysosomes (de Duve et al. 1955), which contain hydrolytic enzymes responsible for degradation of macromolecules, are the primary catabolic components of mammalian cells and are involved in maintaining cellular homeostasis (de Duve 2005; Saftig and Klumperman 2009). The majority of lysosomal enzymes are soluble glycoproteins localized in the lysosomal lumen and are activated by the highly acidic pH (between 4.5 and 5.0) of the lysosome (Journet et al. 2002; Mindell 2012).

Lysosomal storage diseases (LSDs) are a group of over 70 genetic disorders that are mostly inherited in an autosomal recessive manner (Aerts et al. 2011). Although LSDs are individually considered rare, as a group they occur in approximately 1 in 5,000 live births (Meikle et al. 2004; Menéndez-Sainz 2012). Most LSDs are caused by mutations in genes encoding lysosomal enzymes that are involved in the degradation of macromolecules. Abnormal accumulation of the substrates of the defective enzymes within the lysosome results in excess cellular storage of these materials and eventually leads to cell damage and organ dysfunction (Lieberman et al. 2012; Boustany 2013). LSDs are typically progressive, multisystem disorders; pathology may involve connective and ocular tissues, organomegaly and the central nervous system (Platt et al. 2012). Since the discovery of Pompe's disease in 1963 (Hers 1963), numerous defects of integral and lysosome-associated membrane proteins which lead to different types of LSDs have been described (Boustany 2013).

Mucopolysaccharidosis I (MPS I) is one of the most common disorders of the group of 11 mucopolysaccharidoses and is inherited in an autosomal recessive manner with an incidence of approximately 1 in 100,000 live births (Neufeld and Muenzer 2001). MPS I is caused by a deficiency or absence of activity of α-L-iduronidase (IDUA), a soluble lysosomal enzyme involved in the breakdown of the glycosaminoglycans (GAGs), heparin sulfate and dermatan sulfate (Neufeld and Muenzer 2001). Glycosaminoglycans are complex polysaccharides that are typically covalently attached to core proteins and are found in all connective tissues, extracellular matrixes (ECMs) and cell membranes (Scott 2003). In addition to playing major roles as structural molecules in connective tissues, cartilage and the cytoskeleton, GAGs also function in cell adhesion and migration, regulation of proliferation and cell-cell communication (Rapraeger et al. 1986; Bernfield et al. 1999; Martin et al. 2013). The progressive accumulation of undegraded GAGs in MPS I patients, results in widespread cellular, tissue and organ dysfunction. The most common clinical symptoms of the disease include a facial coarsening, corneal clouding, hearing loss, hydrocephaly, cardiac pathology, respiratory problems, hepatosplenomegaly, limited joint mobility and stiffness, and in severe cases MPS I patients have progressive CNS disease manifesting as neurodegeneration (Collins et al. 1990; Leighton et al. 2001; Mohan et al. 2002; Shinhar et al. 2004; Fuller et al. 2005; reviewed in Clarke 2011). The primary accumulation of GAGs can also disturb cellular homeostasis by interfering with complex molecular pathways and these secondary effects are important effectors in disease symptoms and progression (reviewed in Clarke 2011). Based on the pathology of the disease, MPS I patients are classified in three clinical phenotypes: Hurler, Hurler-Scheie and Scheie for the severe, intermediate and attenuated forms of the disease, respectively (Roubicek et al. 1985).

Gaucher disease is the most common LSD caused by a deficiency of the lysosomal hydrolase acid β-glucosidase (glucocerebrosidase, GCase) and is inherited in an autosomal recessive manner. Glucocerebrosidase is a membrane-associated lysosomal enzyme involved in hydrolyzing the β-glucosidic linkage of glucosylceramide (glucocerebroside). It is therefore not surprising that the biochemical hallmark of Gaucher disease is the accumulation of large quantities of glucocerebroside in the lysosomes of macrophages and other cells of the reticuloendothelial system (Beutler and

Grabowski 1995; Beutler and Grabowski 2001; Tropak et al. 2008). In a few instances, mutations leading to a lack of saposin C, a co-hydrolase that is required in addition to glucocerebrosidase for the breakdown of glucocerebroside, also leads to the accumulation of glucocerebroside (Brady et al. 1993; Tamargo et al. 2012). Macrophages engorged with glucosylceramide-laden lysosomes are known as Gaucher cells. In Gaucher patients these cells displace the normal cells particularly in bone marrow and visceral organs. This leads to the development of the diverse manifestations of Gaucher disease including progressive skeletal abnormalities and haematological symptoms. The estimated prevalence of Gaucher disease in the general population is 1 in 100,000. However, in ethnic groups such as Ashkenazi Jewish populations the prevalence might be as high as 1:450 (Beutler and Grabowski 2001). Despite the heterogeneity and phenotypic spectrum of Gaucher disease, three subtypes have been distinguished based on the presence or absence of neurological involvement. Type 1, the most common form of the disease, is associated with chronic non-neuronopathic symptoms. Type 1 patients present variable clinical manifestation including hepatosplenomegaly, anemia, and skeletal deformities. Type 2 (acute infantile) and Type 3 (juvenile or early adult onset) Gaucher disease can be further sub-classified into two categories based on the extent of CNS dysfunction (acute neuronopathic and subacute neuronopathic) (Brady et al. 1993).

1.2. Common mutations underlying MPS I and Gaucher disease

1.2.1. Mutations in the α-L-Iduronidase (*IDUA*) gene that cause MPS I

The α-L-iduronidase (*IDUA*) gene is located on chromosome 4p and consists of 14 exons spanning approximately 19 kb. The coding sequence of human *IDUA* encodes the 653-amino acid α-L-iduronidase (IDUA) protein (Scott et al. 1992). Since cloning of the *IDUA* gene (Scott et al. 1992), mutation analyses have elucidated a molecular basis for many of MPS I phenotypes; over 100 mutations cause various forms of MPS I (Terlato and Cox 2003). Some of the most common mutations are introduced here. The premature stop codon mutations Q70X and W402X are the two most frequent mutations

of the *IDUA* gene found in European patients and account for up to 70% of MPS I disease alleles in some populations (Bunge et al. 1995; Gort et al. 1998; Matte et al. 2003; Hein et al 2004). These null mutations result in an absence of detectable IDUA protein and are associated with a very severe clinical presentation. Interestingly, patients who carry mutations that preserve residual enzyme in amounts as low as 0.1 to 1% of normal levels can present much reduced clinical symptoms (Moskowitz et al. 1993).

The P533R mutation is a relatively frequent missense mutation in the *IDUA* gene and is associated with intermediate to severe MPS I phenotypes. This mutation is commonly detected in Brazilian, Tunisian, Italian and Moroccan MPS I patients (Gatti et al. 1997; Alif et al. 1999; Matte et al. 2003; Laradi et al. 2005; Scott et al. 1992; reviewed in Bie et al. 2013). Recent in-depth protein crystallization studies revealed that P533 is ~25 Å away from the catalytic Glu299 residue (Bie et al. 2013). The P533R mutation causes serious structural perturbations in the IDUA protein and may impair catalysis and the ability of IDUA to bind its natural substrates (Bie et al. 2013). The P533R expressed in CHO-K1 cells does not appear to undergo normal processing and was not secreted into the cell culture medium (Matte et al. 2003). These data suggest that P533R mutation interferes with normal folding, processing and trafficking of IDUA protein (Matte et al. 2003; Bie et al. 2013).

Another IDUA missense mutation is R383H; this mutation is common among Brazilian MPS I patients, who generally present a very mild clinical phenotype. The encoded product possesses only 7% of normal IDUA activity (Bunge et al. 1998; Matte et al. 2003).

1.2.2. Mutations in the *GBA* gene that cause Gaucher disease

The *GBA* gene that encodes the GCase enzyme is located on chromosome 1q and contains 11 exons and 10 introns spanning 7.6 kb of sequence (Ginns et al., 1984; Horowitz et al., 1989; Sorge et al., 1985). Almost 300 disease-causing mutations have been identified in the *GBA* gene. These include 203 missense mutations, 18 nonsense mutations, 36 small insertions or deletions that lead to either frameshifts or in-frame alterations, 14 splice-junction mutations and 13 complex alleles carrying two or more

mutations (Hruska 2008; reviewed in Song et al. 2013). Among the 203 missense mutations identified, the L444P and N370S substitutions are the most prevalent mutant alleles encountered in most Gaucher disease populations (Hruska 2008; reviewed in Song et al. 2013). Over 70% of the Ashkenazi Jewish Gaucher disease population carries the N370S substitution. The L444P mutation happens at a much higher frequency (37.5%) in the non-Jewish Gaucher disease population (Yu et al. 2007). The frequency of N370S and L444P mutants among non-Jewish Gaucher disease populations is 30 and 38%, respectively (Koprivica et al. 2000). The N370S GCase variant preserves almost 10% of wild-type activity and is associated with non-neuropathic forms of the disease (Grace et al. 1994). The L444P mutation leads to a complete loss of GCase activity and is associated with severe neuronopathic symptoms in homozygous patients (Grabowski 1997). Crystal structural analyses of GCase reveal that the N370S mutation is located at a helical domain near, but not within, the active site. By contrast, other studies have found that the L444P mutation is in a separate immunoglobulin-like domain of the protein, far from the domain containing the active site (Dvir et al. 2003).

1.3. IDUA and GCase are transported to lysosomes along the secretory pathway

1.3.1. Intracellular trafficking of IDUA

The soluble lysosomal enzyme IDUA contains a 25 amino acid N-terminal signal peptide that enables its cotranslational translocation into the lumen of the endoplasmic reticulum (ER) (Scott et al. 1991; Kakkis et al. 1994). The signal peptide is cleaved within the ER lumen during synthesis of the nascent IDUA protein. Another co-translational process that IDUA is subject to is N-glycosylation. Here preformed oligosaccharides, $Glc_3Man_9GlcNac_2$, are transferred to certain asparagine residues within the sequence motif Asn-X-Ser/Thr (where X can be any amino acid except Pro or Asp) (Braulke and Bonifacino 2009). The IDUA protein contains six Asn-X-Ser/Thr recognition sites for the addition of N-linked oligosaccharides (Brooks et al. 2001). These include N110, N190, N336, N372, N415, and N451 (Zhao et al. 1997). The N-glycans of IDUA then undergoe oligosaccharide trimming, so that by the time the protein leaves the ER, its N-glycans are in a high mannose form ($Man_{8-9}GlcNAc_2$) (Kakkis et al.

1994). The soluble lysosomal protein then leaves the ER by vesicular transport passing through the ER-Golgi intermediate compartment (Braulke and Bonifacino 2009). In the Golgi apparatus, the oligosaccharide chains of lysosomal enzymes either undergo further modification to form complex or hybrid structures, or certain high-mannose oligosaccharides on the protein become modified to form the mannose 6-phosphate (M6P) marker that mediates their transport to lysosomes. The formation of M6P occurs in two steps: in the first reaction, an N-acetylglucosamine-1-phospho-transferase transfers N-acetylglucosamine phosphate to one or more mannose residues on the lysosomal protein (Little et al., 1986, 1987). The N-acetylglucosamine residue is then removed by a N-acetylglucosamine-1-phosphodiester in the second reaction to generate the M6P tag (Reitman and Kornfeld, 1981a,b). The exposed M6P of IDUA can be recognized by a cation-dependent M6P receptor (CD-MPR) in late Golgi compartments (Ghosh et al. 2003). The sorting signal in the cytosolic tails of the M6P receptors (MPRs) (EESEERDDHLL) interacts with clathrin adaptors to form MPR-hydrolase complexes within clathrin-coated areas of the *trans*-Golgi network (TGN) (Le Borgne and Hoflack 1997; Ghosh et al. 2003; Ghosh and Kornfeld 2004; Braulke and Bonifacino 2009 and references therein). The MPR-ligand complexes leave the TGN in clathrin-coated transport intermediates, which eventually fuse with endosomes where the MPRs deliver their bound ligands (Mellman 1996; Waguri et al. 2003; van Meel and Klumperman 2008; reviewed in Braulke and Bonifacino 2009). The acidic pH of endosomes (< 5.5) dissociates hydrolases from MPRs and the released enzymes remain in the lumen during endosomal maturation and ultimate fusion with lysosomes (Kornfeld and Mellman 1989; Braulke and Bonifacino 2009). The MPR recycles back to TGN to engage in further rounds of protein transport.

1.3.2. Intracellular transport of GCase

The GCase mRNA encodes a 60-kDa mature protein which is cotranslationally glycosylated at four of the five potential N-glycosylation consensus sequences including Asn 19, 59, 146, and 270. The Asn 462 glycosylation site remains unoccupied (Berg-Fussman et al. 1993; Maegawa et al. 2009). The N-glycosylation of Asn 19 is essential for developing a catalytically active GCase protein (Berg-Fussman et al. 1993). Unlike soluble lysosomal proteins, GCase becomes membrane associated and does not traffic

to the lysosome via the MPR system (Leonova and Grabowski 2000; reviewed in Grabowski 2012). Instead the lysosomal integral membrane protein type 2 (LIMP-2) is used as a sorting receptor for GCase (Reczek et al. 2007; Blanz et al. 2010). LIMP-2 is a heavily N-glycosylated type III transmembrane protein (Fujita et al., 1991) belonging to the CD36 family of scavenger receptors and is one of the most abundant lysosomal membrane proteins (Febbraio et al., 2001). A putative coiled-coil motif spanning from amino acids 152-167 within the luminal domain of LIMP-2 is important for GCase binding; the near neutrality of the pH in the ER allows GCase to interact with LIMP-2 (Blanz et al. 2010; Zachos et al 2012). Once bound to LIMP-2 in the ER, GCase traffics through the Golgi complex to the lysosome where the dissociation of LIMP-2 and the GCase occurs as a result of the acidic pH of the lysosome. A single histidine residue at position 171 in the luminal domain of LIMP-2 acts as the critical pH sensor and regulates the dissociation of the receptor-ligand complex in late endosomal/lysosomal compartments (Zachos et al 2012). After transport, GCase becomes membrane associated in the lysosome (Grabowski 2012).

1.4. Mutant variant IDUA and GCase can be subject to degradation due to ER protein quality control mechanisms

The missense mutations underlying MPS I and GD encode mutant proteins prone to misfolding. While often catalytically competent, the mutant enzymes are unable to efficiently pass the quality control mechanisms of the endoplasmic reticulum (ER) known as endoplasmic reticulum-associated degradation pathway (ERAD), resulting in reduced lysosomal trafficking and substrate accumulation. Such mechanisms ensure the degradation of defective proteins before being targeted to lysosomes. Thus, lysosomal enzymes need to become correctly folded in order to escape degradation by the ERAD (Yu et al. 2007; Fan 2008; Boyd et al. 2013). Due to the importance of the ER protein quality control mechanisms in cellular homeostasis, a general discussion surrounding these processes is warranted.

1.5. The endomembrane system and protein quality control in eukaryotes

1.5.1. Thermodynamics of protein folding

Protein folding is the process by which a polypeptide folds into its specific three-dimensional structure. Principles of protein folding have been studied intensely, yet the identification of the molecular mechanisms involved in protein folding is one of the most fundamental open questions in biochemistry (Herczenik and Gebbink 2008). The number of conformations a polypeptide chain can adopt is very large, and thus folding processes are highly complex and heterogeneous phenomena. Folding reactions rely on cooperation of multiple weak and noncovalent interactions among which hydrophobic forces are the most critical forces causing the linear protein chain to fold into a compact structure (Kim et al., 2013). The folding kinetics is described by a funnel-shaped energy landscape model in which polypeptide chains explore a free energy gradient as they progress toward the native structure (Bryngelson et al. 1995; Dobson and Ellis 1998; Tsai et al. 1999; Kim et al. 2013). According to this model the energy of the different conformations decreases as the polypeptides move towards native conformations. Non-native conformations of a protein possess the highest energy levels (reviewed in Mogk et al. 2002). As the proteins become ordered and secondary structural elements form at certain positions of the protein chain, they shift the energy profile to a minimum (native conformation) (Herczenik and Gebbink 2008). In this phase, proteins are now folded into their native conformations with a unique set of α-helical and β-sheet motifs (Herczenik and Gebbink 2008).

1.5.2. Protein folding in the ER: general aspects, role of N-glycosylation, exit from the ER

The endoplasmic reticulum (ER) is the gateway for proteins to enter the secretory pathway (Chen et al. 2005). The presence of a 16-30 amino acid signal peptide facilitates the co-translational translocation of nascent secretory proteins across the ER membrane. The signal peptide typically is located at the N-terminus of a protein and consists of a continuous stretch of hydrophobic residues (6 to 20) with one or more basic residues (von Heijne 1990). The hydrophobic region of signal peptides is critical for

recognition by both the signal recognition particle (SRP) and the membrane-embedded translocation machinery (Hatsuzawa et al. 1997). The signal peptide interacts with the SRP which in turn results in elongation arrest of the nascent chain (Walter and Johnson 1994). The SRP-ribosome nascent chain complex is targeted to the ER membrane through an interaction with the SRP receptor (Janda et al. 2010; reviewed in Braakman and Bulleid 2011). Translation then resumes and the nascent chain is translocated across the membrane and into the ER lumen via the Sec61 translocon complex (reviewed in Deng et al. 2013). Upon entry into the ER, protein folding starts with assistance from chaperones and enzymes essential for the folding process. Chaperones are defined as proteins that transiently interact with other proteins to aid them in the folding or assembly process without being part of their final structure (Hartl 1996; Hartl and Hayer-Hartl 2009). The ER lumen is a crowded environment in which the concentration of resident and nascent proteins may be 100 g L^{-1} (Kleizen and Braakman 2004). In such a crowded environment, protein folding intermediates can interact non-specifically through their hydrophobic interactions and form protein aggregates. The exposure of hydrophobic regions at the surface while the protein is in an unfolded state can make the protein susceptible to aggregation (Howell 2013). Chaperones partly function to prevent protein aggregation.

The ER chaperones involved in the protein folding process include the lumenal binding protein (BiP), the co-chaperone heat shock protein 40 (HSP40) (also known as DnaJ), protein-disulfide isomerases (PDI), and the lectins, calnexin and calreticulin (Reviewed in Deng et al. 2013; Howell 2013). BiP, a member of the HSP70 family, is a multifunctional chaperone that aids in the folding process of nascent proteins (Lee et al. 1999; Wang et al. 2005; Kim et al. 2013). BiP also facilitates translocation of growing nascent polypeptides into the ER lumen and maintains the permeability barrier of the ER by sealing the luminal side of translocons that are assembled but are not engaged in translocation (Hamman et al. 1998; reviewed in Hebert and Molinari 2006). Similar to other members of HSP70 family, BiP contains an N-terminal nucleotide-binding domain (NBD) that is connected to a C-terminal substrate-binding domain (SBD) by a highly conserved hydrophobic linker (reviewed in Kim et al. 2013). The SBD of BiP binds to the exposed hydrophobic patches of unfolded substrates and thus BiP shields them from aggregation (Mayer and Bukau 2005; Fritz et al. 2013). Conformational changes in the

NBD as a result of ATP binding and hydrolysis regulate peptide binding and release in the SBD region of BiP. Hydrolysis of ATP bound to the NBD leads to high affinity binding of the substrate. Exchange of ADP with ATP releases the substrate from the BiP allowing the substrate to progress further in the folding process (Mayer and Bukau 2005). The co-chaperones of the HSP40 family induce substrate binding to BiP by interacting with the N-terminal ATPase domain of BiP and strongly stimulating ATP hydrolysis (Jiang et al. 2007; Fritz et al. 2013). Oxidative protein folding is mediated by protein-disulfide isomerases (PDI family proteins) (Deng et al. 2013). PDI family proteins are responsible for the formation, reduction, and isomerization of disulfide bonds in newly synthesized proteins and the exchange of bonds between cysteine residues in unfolded proteins (Freedman et al. 1994; Schroder 2008).

Many secretory proteins are co-translationally N-glycosylated by oligosaccharyl transferase (OST) as they are transported into the ER lumen. OST transfers the $Glc_3Man_9Glc\text{-}NAc_2$ triantennary oligosaccharide from the glycolipid $Glc_3Man_9GlcNAc_2$-PP-dolichol to the side-chain amide of the asparagine residue within the Asn-X-Ser/Thr recognition sequence of the nascent polypeptide as described earlier (Crowley et al. 1994; Sanyal and Menon 1994; Powers et al. 2009). The transferred oligosaccharide mediates key interactions between the newly synthesized polypeptide and components of the ER proteostasis network, which leads to proper folding of N-linked glycoproteins (reviewed in Price et al. 2012). Immediately following glycan transfer the outer two glucose (Glc) residues from the A-branch of the N-glycan are removed by the sequential action of glucosidase I and glucosidase II (Ruddock and Molinari 2006). The resulting monoglucosylated oligosaccharide is then recognized by the lectins calnexin and calreticulin (CNX/CRT) (Figure 1-1). This allows N-glycosylated polypeptides to enter the CNX/CRT-assisted folding cycle instead of the degradation cycle (reviewed in Price et al. 2012). These lectins are also known to recruit other components of the ER folding machinery such as oxidoreductase ERp57, which catalyzes disulfide bond isomerisation, facilitating the formation of native disulfide bonds which are required for proper folding of N-glycoproteins (Molinari and Helenius 1999; Price et al. 2012). Upon release of the protein from CNX/CRT, glucosidase II removes the last glucose from $GlcMan_9GlcNAc_2$ of correctly folded N-glycoproteins to prevent their reassociation with CNX/CRT (Figure 1-1). Thus, N-glycosylation accelerates protein folding, facilitates disulfide formation,

reduces protein aggregation and increases protein thermodynamic stability (reviewed in Price et al. 2012).

Properly folded N-glycoproteins are exported from the ER in COPII-coated vesicular carriers (Dancourt and Barlowe 2010; Schmidt and Stephens 2010; Gillon et al. 2012). COPII transport vesicles are assembled at specialized regions of the ER called ER exit sites (Bannykh et al. 1996). The assembly of COPII-coated vesicles starts with activation of the Sar1p GTPase by Sec12p nucleotide exchange factor (Barlowe 2002). The activated Sar1p-GTP binds to the ER membranes to initiate membrane curvature and also interacts with the Sec23p portion of the bi-functional cargo adaptor Sec23-24p. The Sec24p subunit of this activated cargo adaptor captures the cargo protein to form a pre-budding complex (Barlowe and Schekman 1993; Barlowe et al. 1994; Miller et al. 2003). The Sec13-Sec31 complex then assembles around this pre-budding complex to polymerize and further deform the ER membrane bilayer, producing the native COPII-coated structures (reviewed in Dancourt and Barlowe 2010; Kakoi et al. 2013). The ER exit sites are also involved in segregation of different classes of cargo molecules and in ER quality control resulting in exclusion of misfolded proteins from the cargo leaving the ER (Mezzacasa and Helenius 2002). The vesicles carrying correctly folded proteins subsequently move to the pre-Golgi or ER-Golgi intermediate compartment (ERGIC) (also known as vesicular tubular clusters, or VTCs). From the ERGIC, the cargo either traffics to the Golgi complex or is transported back to the ER (reviewed in Lord et al. 2014).

1.5.3. Protein misfolding in the ER: how does the endomembrane system recognize misfolded proteins?

Protein folding is an error-prone process (Mogk et al. 2002). Protein misfolding and aggregation is common and it is not uncommon for proteins to exhibit non-native interactions (Herczenik and Gebbink 2008; Kim et al. 2013). Protein misfolding can occur even under optimal conditions. However, amino acid compositions caused by the presence of certain mutations are known to accelerate misfolding events. During the folding process, substrates with exposed hydrophobic patches may accumulate (Chiti and Dobson 2006; Eichner and Radford 2011; Kim et al. 2013). These substrates are

bound by ER-retained chaperones such as HSP70- and HSP40- chaperones which prevent continued transit along the secretory pathways by retaining the misfolded proteins in a soluble conformation to prevent their aggregation (reviewed in Nishikawa et al. 2005). Non-native N-glycoproteins and incorrectly folded glycoproteins are recognized by UDP-glucose:glycoprotein glucosyltransferase, which places a single glucose back on the N-glycan and thereby restores the ability of the N-glycoprotein to bind CNX and/or CRT (Price et al. 2012). The N-glycan then re-enters the CNX/CRT cycle which serves to retain glycoproteins in the ER until they reach their native conformation just prior to their export from the ER (Caramelo and Parodi 2008). Persistently misfolded N-glycoproteins are eventually detected by α1,2-mannosidases which catalyze the cleavage of a single mannose residue from the N-glycan (Hosokawa et al. 2001; Price et al. 2012; Pan et al. 2013). Such modification blocks reglucosylation of N-glycans and reduces their binding affinity for CNX/CRT. This leads to the association of the misfolded glycoprotein with the ER degradation-enhancing 1,2-mannosidase-like protein (EDEM) lectin (Hosokawa et al. 2001). EDEM is proposed to pull misfolded proteins out from the CNX/CRT cycle for entry into the degradation pathway, which is known as ER-associated degradation (ERAD) and is mediated by ubiquitination and proteasomal degradation (Molinari et al. 2003).

1.5.4. ER-associated degradation (ERAD) and the ubiquitin-proteasome system

Misfolded proteins that fail to achieve their native conformation are degraded by ER-associated degradation (ERAD), a complex process during which misfolded proteins are selected and ultimately eliminated via the ubiquitin-proteasome system (reviewed in Olzmann et al. 2013). ERAD is conducted in four steps involving: substrate recognition, dislocation across the ER membrane (retrotranslocation), polyubiquitination, and proteasomal degradation. Based on the location of the misfolded domains of the protein, three different ERAD pathways have been identified by which a misfolded protein can be eliminated: ERAD-L (defects in luminal regions), ERAD-M (defects in transmembrane domains) and ERAD-C (defects in cytosolic regions) (Nishikawa et al. 2005).

The ER chaperone BiP is a key determinant in substrate recognition and selection. BiP recognizes exposed hydrophobic patches of the protein that are normally buried inside the native protein. Interaction with BiP keeps misfolded proteins in soluble conformations and thus prevents their aggregation prior to the retrotranslocation process (Fewell et al. 2001; Nishikawa et al. 2005). Interestingly, ERAD substrates have high affinity for BiP. Furthermore, soluble ERAD substrates require BiP for their degradation (Nishikawa et al. 2005).

ER-resident lectins also play a key role in the recognition of ERAD substrates in the ER. The lectin Yos9 was recently identified as a mediator that recognizes ERAD substrates and recruits proteins with aberrant N-glycans to the ubiquitin-protein ligases (E3) in *Saccharomyces cerevisiae*. Yos9 and its mammalian homologues, OS-9 and XTP3-B act as gate-keeping complexes through which ERAD substrates must pass prior to retrotranslocation (Gauss et al. 2006; Nakatsukasa and Brodsky 2008; Hosokawa et al. 2010). The OS-9 and XTP3-B lectins selectively recognize mannose trimmed substrates generated by ER ManI and EDEM1-3 through their mannose-6-phosphate receptor homology (MRH) domains (reviewed in Olzmann et al. 2013). In Yeast, Yos9 interacts with Hrd3, a type I transmembrane protein belonging to the Hrd E3 ligase complex. The Hrd3 ubiquitin ligase is thought to be involved in handing over mannose trimmed Man_7-$GlcNAc_2$-containing misfolded substrates to the Hrd1/Der3 ligase for polyubiquitylation (Gardner et al. 2000; Bhamidipati et al. 2005; Quan et al. 2008; reviewed in Eisele et al. 2010). The Hrd1/Der3 enzymes carry out the ubiquitylation of ERAD-L and ERAD-M substrates in yeast, while the ubiquitin ligase, Doa10, mediates ubiquitination of ERAD-C substrates (Kostova et al. 2007, reviewed in Jo et al. 2010). It has been suggested that the Hrd1/Der3- Hrd3 ligase complex without Yos9 might be involved in the recognition of non-glycosylated proteins in the ER lumen that are destined for degradation (Gauss et al. 2006; reviewed in Eisele et al. 2010).

The degradation process is carried out by the 26S proteasome that is localized in the cytosol. Thus, ubiquitinated misfolded proteins have to be retrotranslocated to the cytosol for degradation (Hiller et al. 1996). A crucial event for retrotranslocation may be the reduction and breaking of disulfide bonds in the ERAD substrate. This event is catalyzed by ERdj5, an ER-resident PDI family member protein, which possess four

thioredoxin domains (reviewed in Brodsky 2012). ERdj5 accelerates ERAD by reducing incorrect disulfide bonds in misfolded glycoproteins recognized by EDEM1. Recent studies have shown that ERAD substrates sequentially move from calnexin to the EDEM1-ERdj5 complex and then to the retrotranslocation channel, probably through BiP (Hagiwara et al. 2011). ERdj5 also accelerates the ERAD of non-glycosylated misfolded proteins recruited by BiP (Ushioda et al. 2008).

Retrotranslocation is an energy consuming process. An AAA ATPase (AAA = ATPases Associated with diverse cellular Activities) known as Cdc48 in yeast, or p97 in mammals provides the energy for the retrograde translocation of misfolded proteins from the ER (reviewed in Deng et al. 2013), and this event presumably occurs through channels in the Hrd1 or Doa10 complexes (Hampton and Sommer 2012) or Sec61 channel (Wiertz et al. 1996). In addition, Cdc48/p97 serves as a platform for other enzymes involved in the ERAD process (Brodsky 2012). The Cdc48 complex delivers misfolded proteins to the proteasome through its interaction with the ubiquitin ligase Ufd2 (ubiquitin fusion degradation) and Rad23 (Baek et al. 2011) proteins. Ufd2 is essential for the elongation of an existing ubiquitin chain and can bind to both Cdc48 and Rad23 (Stolz et al. 2011). Rad23 is able to interact with the ubiquitin chains of the substrate and also with the proteasome through its different domains thus serving as a bridge linking ubiquitylated substrates to the proteasome. Cdc48 eventually disassembles Rad23 from the Ufd2-Rad23 complex to allow the transfer of substrates from the ubiquitylation apparatus to the proteasome for degradation (Baek et al. 2011).

1.6. The unfolded protein response (UPR)

Accumulation of unfolded or misfolded proteins inside the ER leads to ER stress. ER stress initiates a series of intracellular signal transduction pathways known as the unfolded protein response (UPR) (Walter and Ron 2011). Activation of the UPR is a cell survival mechanism that potentiates many components of the secretory pathway necessary to restore protein folding capacity and homeostasis (Hetz et al. 2013). When unfolded proteins accumulate in the ER lumen, these sensors regulate protein load by blocking protein translation and by limiting the pool of mRNAs available for translation (reviewed in Osorio et al. 2014). When these mechanisms fail to restore ER

homeostasis, UPR signaling results in cell death by apoptosis (Tabas and Ron 2011). In eukaryotic cells, UPR signaling pathways are initiated via three classes of ER signal sensors/transducers including inositol-requiring protein 1 (IRE1), activating transcription factor 6 (ATF6), and protein kinase RNA-like endoplasmic reticulum kinase (PERK) (Mori 1999) (Figure 1-2). Under physiological conditions, these stress sensors are maintained in an inactive confirmation. Early work suggested that BiP acts as the master regulator of the activation of IRE1, PERK and ATF6. According to this model, BiP interacts with the luminal domains of these ER stress transducers to keep them in a monomeric, inactive state. Competition for BiP by unfolded proteins upon ER stress favors BiP disassociation from these sensor complexes, allowing for their activation (Liu and Kaufman 2003; Walter and Ron 2011). However, subsequent studies show that IRE1 becomes activated when unfolded proteins bind directly to it, indicating that IRE1 regulation can occur independently of BiP (reviewed in Walter and Ron 2011; Gardner et al. 2013).

ATF6 is one of the best-studied stress-transducing transcription factors in mammalian cells. ATF6 is initially synthesized as an ER-resident transmembrane protein bearing a bZIP transcription factor in its cytosolic domain (Howell 2013). Upon accumulation of unfolded proteins, ATF6 is transported to the Golgi complex where it is cleaved sequentially by two Golgi-associated proteases including site 1 protease (S1P) and site 2 protease (S2P). S1P cleaves ATF6 in the luminal domain. The N-terminal portion is subsequently cleaved by S2P releasing the bZIP-containing domain. The liberated N-terminal ATF6 transcription factor then migrates to the nucleus and binds to the ER stress response elements (ERSEs) and unfolded protein response elements (UPREs) located in the promoters of UPR response genes (Yamamoto et al. 2004; Ron and Walter 2007; Wu et al. 2007; Adachi et al. 2008; Teske et al 2011; Walter and Ron 2011; Moore and Hollien 2012; Hetz et al, 2013). ATF6 increases the expression of a network of genes involved in protein folding such as BiP, PDI and components of the ERAD pathway (Teske et al 2011). The luminal domain of ATF6 contains intra- and intermolecular disulfide bonds that might act as redox sensors for monitoring the ER environment (Walter and Ron 2011).

The second branch or arm of UPR is mediated by PERK, a transmembrane kinase that oligomerizes during ER stress. When activated, PERK performs trans-autophosphorylation of its C-terminal cytoplasmic kinase domain as well as phosphorylation of the α-subunit of the eukaryotic initiation factor 2 (eIF2) (Figure 1-2). PERK-mediated eIF2α phosphorylation in turn inhibits the assembly of the 80 S ribosome and inhibits overall protein synthesis (Harding et al. 1999; Saito et al. 2011). In this way, PERK helps to alleviate ER stress by decreasing the load of proteins that enter the ER (Lin et al. 2008). However, although general protein synthesis is repressed when eIF2α is phosphorylated, some mRNAs containing short open reading frames in their 5′-untranslated regions are translated under these conditions (Lee et al. 2009; Saito et al. 2011). One of these includes ATF4 (activating transcription factor 4), which activates the transcription of CHOP (transcription factor C/EBP homologous protein) and GADD34 (growth arrest and DNA damage–inducible 34). CHOP is a transcription factor involved in apoptosis. GADD34 encodes a PERK-inducible regulatory subunit of the protein phosphatase PP1C that relieves the inhibition of translation by dephosphorylateing eIF2α. This mechanism promotes recovery from translational inhibition in the UPR (Novoa et al. 2001; Ma and Hendershot 2003; Harding et al. 2009; Tsaytler et al. 2001; Walter and Ron 2011).

IRE1, another branch of the UPR, is a bi-functional transmembrane kinase/endoribonuclease that senses misfolded proteins in the ER most likely by direct ligand-mediated recognition (Credle et al. 2005; Li et al. 2010; Pincus et al. 2010). Upon UPR activation, oligomerization and subsequent trans-autophosphorylation of IRE1 takes place resulting in the activation of its cytoplasmic kinase and endoribonuclease (RNase) domains (Li et al. 2010; reviewed in Hetz et al. 2013). When activated, IRE1 cleaves the mRNA that encodes transcription factor XBP-1 (x-box binding protein 1) (Figure 1-2); the latter drives the transcription of genes involved in the UPR, ERAD, protein quality control and organelle biogenesis (Yoshida et al. 2001; Calfon et al. 2002; Lee et al. 2003; Acosta-Alvear et al. 2007; Hetz et al. 2013). Prolonged activation of IRE1 may promote apoptosis (Tabas and Ron 2011). The RNase activity of IRE1 can also degrade a subset of mRNAs in a process termed regulated IRE1-dependent decay (RIDD) of mRNA (Hollien and Weissman 2006; Han et al. 2009; Hollien et al. 2009). RIDD is selective. It depends on the cell type and is specific for mRNAs encoding

proteins of the secretory pathway. The selection of target mRNAs for degradation may depend on the tendency of the encoded protein to misfold (Hollien and Weissman 2006; Han et al. 2009; Hollien et al. 2009; Oikawa et al. 2010; reviewed in Hetz et al. 2013).

1.7. Pathways of disposal for missense mutant lysosomal proteins

Missense mutations resulting in the misfolding of lysosomal proteins have the potential to cause disease (Fan 2008). As a quality control mechanism, such misfolded lysosomal proteins are recognized by the ERAD system and are eventually eliminated via the proteasome machinery (Ellgaard and Helenius 2003; Fan 2008). Considerable insight into the ER quality control of missense lysosomal enzymes has come from the analysis of mutations underlying Gaucher disease. The work of Ron and Horowitz, showed that mutant GCase variants often display variable levels of ER retention and undergo ERAD (Ron and Horowitz 2005). Biosynthetic labeling studies combined with immunofluorescence analyses using patient-derived fibroblasts carrying GCase missense mutations reveal retarded transport of GCase containing the N370S substitution and ER retention of the enzyme variants generated by the *GBA* mutations G202R, L444P and D409H (Schmitz et al., 2005). Recently, Wang et al. demonstrated that ERAD prevents native folding of mutated lysosomal enzymes in patient-derived fibroblasts associated with Gaucher and another LSD, Tay-Sachs disease, and found that inhibition of ERAD rescues native folding of mutant protein in these diseases (Wang et al. 2011). Degradation of misfolded mutant α-galactosidase A enzymes by ERAD is evident in Fabry disease (Ishii et al. 2007). These results indicate that ERAD and proteasomal degradation are key determinant pathways for disposal of misfolded lysosomal enzymes.

In addition to ERAD, autophagy can play a role in the disposal of mutant lysosomal enzymes. An induction of autophagy is observed in Niemann-Pick type C (NPC), a LSD caused by mutations in the *NPC1* or *NPC2* gene. The deficiency of NPC1 leads to both an induction of autophagy and an impairment of autophagic flux; the impaired degradation of autophagic substrates may cause the accumulation of ubiquitinated proteins (Pacheco et al. 2007; reviewed in Lieberman et al. 2012). Similar

to NPC, a Gaucher disease mouse model shows both an induction of autophagy and also an accumulation of autophagosomes and autophagic substrates (Vaccaro et al. 2010; Lieberman et al. 2012), processes that contribute to neuronopathy in this Gaucher disease mouse model. Disturbances and blockage of autophagy have been observed in several neurodegenerative LSDs including Mucopolysaccharidosis type IIIA and type VI (Sun and Grabowski 2010; Lieberman et al. 2012).

1.8. Therapeutics for LSDs

1.8.1. General aspects of enzyme replacement therapy, substrate reduction therapy and enzyme enhancement therapy

During the last two decades, advances in understanding some aspects of the underlying pathophysiology of LSDs have led to the development of a variety of innovative therapeutic approaches which address the underlying enzymatic and/or storage problems. These include enzyme replacement therapy (ERT), substrate reduction therapy (SRT) and enzyme enhancement therapy (EET) (Grabowski and Hopkin 2003; Wilcox 2004; Desnick and Schuchman 2012; Ortolano et al. 2013).

ERT is an effective way to restore the deficient lysosomal enzyme and involves weekly infusions for intravenous delivery of the recombinant lysosomal enzyme. The recombinant lysosomal enzymes are typically produced in mammalian cell systems such as in Chinese hamster ovary (CHO) cells or in human fibroblasts (Neufeld 2006). Currently, ERT with recombinant enzymes is available for Gaucher, Fabry, Pompe, and mucopolysaccharidosis I, II, and VI diseases (Desnick and Schuchman 2012; Schulze and Sandhoff 2013). ERT ameliorates many manifestations of LSDs and is both a safe and effective approach (Tropak et al. 2008). However, administration of enzymes in patients with CNS involvement can not improve neurologic manifestations, partly because of an inability of recombinant enzymes to cross the blood-brain barrier (BBB) (Desnick and Schuchman 2012). In addition, ERT is extremely costly (Beutler 2006, Parenti 2009).

SRT is based on the concept that inhibition of specific enzymes involved in the biosynthetic pathways of substrates may reduce the precursor synthesis to a level that can be effectively cleared by residual hydrolytic activity (Pastores and Barnett 2003; Platt and Jeyakumar 2008). This approach restores appropriate substrate synthesis and catabolism (Parenti et al. 2013). The largest clinical application of this approach has been the use of N-butyldeoxynojirimycin (NB-DNJ) or Miglustat for treatment of patients with mild to moderate non-neuronopathic Gaucher disease (Weinreb et al. 2005; Parenti et al. 2013). Miglustat decreases the synthesis of glucosylceramide, the cerebroside that accumulates in Gaucher disease, by inhibiting glucosylceramide synthase (Platt et al. 1994; Pastores et al. 2009; Grabowski 2010). NB-DNJ also functions as a SRT agent in a Tay-Sachs mouse model (Platt et al. 1997) and was shown to be beneficial in NPC patients (Lachmann et al. 2004, Patterson et al. 2007). NB-DNJ is able to cross the BBB (Platt et al. 1997). SRT has also been applied for the treatment of MPSs using the isoflavone genistein and the chemical dye rhodamine B as inhibitors of glycosaminoglycan synthesis (Piotrowska et al. 2006; Roberts et al. 2010; Jakobkiewicz-Banecka et al. 2011; Parenti et al. 2012).

EET is an attractive approach for the treatment of genetic disorders resulting from protein misfolding and/or mistrafficking (Desnick 2004). In some genetic disorders, certain missense mutations do not directly affect protein function, but instead impair the ability of newly synthesized proteins to appropriately fold and traffic out of the ER (Kopito and Ron 2000; Yu et al. 2007; Fan 2008). Such proteins are retained in the ER, where they interact with molecular chaperones such as BiP and calnexin to restore their native conformation or, having failed to form a functional state, undergo ERAD and are rapidly eliminated from the cell through the proteasome machinery (Nishikawa et al. 2005). The aim of EET is to use small molecules such as pharmacological chaperones or proteostasis regulators to restore the stability and trafficking of these proteins (Yu et al. 2007; Mu et al. 2008). Pharmacological chaperones and proteostasis regulators are reviewed in the following section.

1.8.2. EET for LSDs

1.8.2.1. Pharmacological chaperone therapy

For some mutant lysosomal enzymes, pharmacological chaperones (PCs) are being pursued as potential small-molecule 'enzyme enhancement' therapeutics; some of these active-site-specific inhibitors can 'rescue' a proportion of the newly synthesized mutant enzymes when present at sub-inhibitory concentrations by promoting their maturation and lysosomal trafficking. By serving as a folding template in the ER, PCs are thought to allow a small proportion of proteins to fold properly and thus pass the protein quality control system of the ER (Sawkar 2002, 2005; Yu et al. 2007; Fan 2008). Once in the lysosome, it is presumed that the PC will be disassociated from its associated enzyme due to the acidic pH of lysosomes and the high affinity of the accumulated natural substrate for the active site of the associated enzyme (Valenzano et al. 2011). LSDs are considered to be suitable candidates for PC therapy as it is assumed that a threshold activity of approximately 10% is sufficient to prevent substrate storage in several of the LSDs (Conzelmann and Sandhoff 1983; Schueler et al. 2004; reviewed in Parenti 2009; Boyd et al. 2013). Thus, even a minor increase in enzyme activity in response to PCs is likely to have a large impact on disease pathology and to be clinically beneficial for patients (Parenti 2009). In addition PCs can cross the BBB, and therefore provide a potential treatment for diseases with CNS involvement (Boyd et al. 2013).

Fabry disease, an X-linked LSD that is caused by a deficiency of α-galactosidase A (α-Gal A) activity, was the first LSD to be assessed for potential PC therapy. Pioneering work by Fan et al. demonstrated a positive effect of 1-deoxy-galactonojirimycin (DGJ), a potent competitive inhibitor of α-Gal A, on stabilizing and enhancing the activity of mutant α-gal A in Fabry patient lymphoblasts (Fan et al. 1999). Subsequently, various PCs were identified and tested for therapeutic potential for other LSDs including Gaucher disease (Sawkar et al. 2002, 2005, and 2006; Steet et al. 2006; Yu et al. 2007;Tropak et al. 2008; Maegawa et al. 2009). The effect of N-(n-nonyl)deoxynojirimycin (NN-DNJ) on N370S GCase in fibroblasts from patients with type 1 Gaucher disease was investigated by Sawkar et al. (2002). Treatment of patient-derived fibroblasts with sub-inhibitory concentrations of NN-DNJ resulted in a two-fold increase in the activity of N370S GCase. Isofagomine (IFG) was then identified and

characterized as a PC for N370S GCase with an ability to increase GCase activity more than two-fold (Steet et al. 2006). Using a high-throughput screening (HTS) approach, Tropak et al. (2008) identified two putative PCs for mutant GCase - both non-carbohydrate-based inhibitory molecules, a 2,4-diamino-5-substituted quinazoline and a 5-substituted pyridinyl-2-furamide. These compounds enhanced the levels of functional GCase 1.5- 2.5-fold in N370S or F213I Gaucher disease fibroblasts. Using a thermal denaturation assay to screen the NINDS library (National Institute of Neurological Disorders and Stroke, a library of 1,040 drugs previously used to treat humans), a novel compound, Ambroxol (ABX) was identified as a PC for GCase. ABX stabilizes GCase in a pH-dependent manner and significantly increases the activity and protein levels of N370S GCase in Gaucher fibroblasts (Maegawa et al. 2009).

The mechanism by which NN-DNJ or IFG-binding stabilizes GCase has been characterized by X-ray crystallography and hydrogen/deuterium exchange coupled with mass spectrometry (H/D-Ex) (Hamuro et al. 2003; Brumshtein et al. 2007; Lieberman et al. 2007; Kornhaber et al. 2008; Tropak et al. 2008). The general understanding is that similar to natural substrates, PCs fit into the active site pocket and form hydrogen bonds with specific amino acid residues of the active site; this may result in increased stability of the protein (reviewed in Parenti 2009) and/or may facilitate domains of the protein to fold correctly.

After several years of *in vitro* research surrounding the therapeutic potential of PCs for LSDs, research is now moving into clinical translation (Parenti 2009). Several chaperones are in clinical trials for Fabry, Gaucher and Pompe diseases (Parenti et al 2013). IFG is currently undergoing Phase I and II clinical trials by Amicus Therapeutics (reviewed in Tropak et al. 2008; Benjamin et al. 2012). The most promising PC currently in clinical trials is DGJ. Now in phase III clinical trials, DGJ has shown encouraging results in enhancing the residual α-gal A enzyme activity and clearance of its substrate (Fan 2008; Benjamin et al. 2009; Benjamin et al. 2012; reviewed in Clark et al. 2012).

Although promising, one of the challenges associated with the therapeutic use of PCs is that, as a monotherapy, they only ameliorate particular mutant variants. However, the combination of PC therapy and ERT may provide a greater efficacy for patients, as

PCs may improve the pharmacological properties of the infused enzyme. Administration of a PC prior to ERT can help stabilize the recombinant enzyme in the neutral pH environment of the blood, as shown for IFG (Shen et al. 2008; Boyd et al. 2013).

1.8.2.2. Proteostasis regulators

Proteostasis or cellular protein homeostasis refers to highly complex interconnection of pathways that regulate protein synthesis, folding, trafficking, disaggregation, and degradation; proteostasis is often regulated through transcriptional and translational changes (Blach et al. 2008; Mu et al. 2008; Hutt et al. 2009). Proteostasis thus influences specific cellular functions and enables the cell to change its physiology to cope with misfolding-prone proteins, environmental stress, and to prevent disease onset (Balch et al. 2008). Recently, remodeling the proteostasis network by using “proteostasis regulators” (PRs) has been suggested as a strategy to correct proteostatic deficiencies that contribute to a broad range of human diseases including LSDs (Balch et al. 2008; Mu et al. 2009). The ultimate goal of this approach is to enhance the innate cellular folding capacity in order to restore partial folding, trafficking and function of misfolded or ERAD-prone mutant lysosomal proteins (Mu et al. 2008; Wang and Segatori 2013). This approach relies on manipulating signaling pathways, including the unfolded protein response (UPR) which leads to transcriptional and translational up-regulation of components of the proteostasis network in the ER such as molecular chaperones (Mu et al. 2008). Two proteostasis regulators have been reported to restore the function and post-ER trafficking of L444P GCase in patient-derived fibroblasts. These include celastrol, an activator of the heat-shock response (HSR) (Mu et al. 2008) and an inhibitor of the chymotrypsin-like activity of the proteasome (Yang et al. 2006), and MG-132, a well known inhibitor of 26S proteasome activity (Mu et al. 2008). Both of these agents significantly up-regulate the levels of transcripts encoding cytosolic HSP40, HSP70, and HSP90 and ER luminal BiP, and activate all branches of the UPR (IRE1, ATF6, and PERK) (Mu et al. 2008). Activation of the UPR and elevated levels of chaperones/folding enzymes reduce mutant enzyme misfolding and enhance the folding efficiency of L444P GCase. In addition, co-application of PCs with PRs rescues the function of mutant L444P GCase in a synergistic fashion (Mu et al. 2008). However, activation of UPR relies on cellular stress induced by PRs, which can lead to induction of apoptosis (Wang et al. 2011). Thus, recent focus on enhancing the cellular

folding capacity in order to rescue the folding of unstable and degradation-prone proteins has focused on mechanisms that do not induce the apoptotic cascade (Wang and Segatori 2013).

Manipulation of calcium homeostasis using lacidipine, an L-type Ca^{2+} channel blocker enhances folding, trafficking, and activity of the degradation-prone L444P GCase and prevents apoptosis induction (Wang et al. 2011). Interestingly, reprogramming the proteostasis network by combining Ca^{2+} homeostasis modulators and ERAD inhibitors such as Eeryastatin I (EerI), a small molecule previously shown to promote the folding of L444P GCase, dramatically lowers the induction of apoptosis typically associated with ERAD inhibition (Wang et al. 2011; Wang and Segatori 2013). Arm-selective UPR activation has also been assessed as a strategy to restore the aberrant ER proteostasis of destabilized protein variants (Kudo et al. 2008; Shoulders et al. 2013). However, the use of proteostasis remodelling agents has not progressed to any clinical studies.

1.9. The plant cell as a eukaryotic model

1.9.1. General advantages

During the past decades, plant-based systems have emerged as an attractive alternative to mammalian cell culture or microbial cell-based systems for the production of industrial and therapeutic recombinant proteins (da Cunha et al. 2014). Since the production of recombinant human growth hormone (hGH) in transgenic tobacco plants in 1986, a wide range of pharmaceuticals have been expressed in various plant-based expression systems (reviewed in da Cunha et al. 2014). Most relevant to this work, human lysosomal enzymes have been successfully produced as recombinant proteins in plant-based systems (Downing et al., 2006; Shaaltiel et al. 2007; He et al. 2012, 2013) and a carrot-suspension-culture derived recombinant GCase has recently been approved by the US Food and Drug Administration (FDA) for treatment of Gaucher disease (Fox 2012). Plant-based bioreactor systems offer several advantages over the current production systems for recombinant biopharmaceuticals, such as yeasts, fungi, insect cells, mammalian cell cultures and transgenic animals. Some of the advantages include (i) the capacity of plants to fold, assemble and post-translationally modify

complex recombinant pharmaceuticals in a manner similar to that of the native host (Paul and Ma 2011); (ii) potentially cost-effective production of recombinant proteins, due to the ease of scale-up of biomass, and established protocols for harvesting, transport, storage and processing; (iii) a low risk of product contamination by animal/human pathogens, such as prions and viruses which provides a significant manufacturing advantage in terms of safety and quality; (iv) the availability of natural storage organs such as tubers, fruits and seeds to facilitate the stable accumulation of recombinant proteins (reviewed in Downing 2006; Kermode 2006; Kermode 2012); (v) a fast cellular reproduction rate if suspension-cultured plant cells (e.g. tobacco BY2 cells) are used (Fu et al. 2009; Plasson et al. 2009).

1.9.2. Conservation of elements of PTMs, protein folding, PQC, and UPR

The eukaryotic protein synthesis pathway is highly conserved between plants and animals (Ma et al. 2003). Plant cells are also generally able to properly fold, post-translationally modify, and otherwise process recombinant proteins in a manner that is similar to that of mammalian systems (Downing et al. 2006). Similar to mammals, N-linked glycans of folding proteins enter the calnexin/calreticulin protein-folding cycle in the ER, which is necessary for proper protein folding.

N-glycosylation, carried out by plants and some animals is not identical to that in mammals, such that this process requires manipulation to control N-glycan maturation in the Golgi complex (Schillberg et al. 2003). There are no differences in the early events of N-glycosylation (Gomord and Faye, 2004). However, once plant recombinant proteins enter the Golgi complex, Golgi-localized enzymes convert the original high-mannose glycans of proteins to complex glycans. In plants, β(1,2)-xylose and α(1,3)-fucose residues are assembled on to the core $Man_3GlcNAc_2$ structure of glycans instead of core α(1,6)-fucose residues and terminal sialic acid residues, as in mammalian cells (Downing et al. 2006; Gomord et al. 2010). Unless this process is controlled, a recombinant therapeutic protein that is potentially immunogenic is generated (reviewed in Kermode, 2012).

Internal factors such as genetic mutations and external factors such as biotic/abiotic stresses, nutrient deprivation and pharmacological agents, such as tunicamycin can cause ER stress in plant cells (reviewed in Duwi Fanata et al. 2013). Many components of the UPR are conserved among mammals, yeast, and plants, although mammals and plants each have unique UPR factors that lead to unique cellular responses (Ye et al. 2011). In mammalian cells, the genes involved in UPR are up-regulated through the action of transcription factors that act on promoter cis-elements called ER stress response elements (ERSEs; Yoshida et al. 1998). Three types of ERSE including ERSE-I (CCAAT-N9-CCACG), ERSE-II (ATTGG-NCCACG), and XBP1-BS [GA-TGACGT-G(T/G)] have been identified in target genes of mammalian cells (Liu and Howell 2010). RNA profiling studies reveal that ERSEs are also found in the promoters of several genes induced by ER stress in *Arabidopsis thaliana* (Arabidopsis). However, whether ERSEs play roles in the regulation of ER stress-induced genes in plants is as yet unknown (Martinez and Chrispeels 2003; Noh et al. 2003; Kamauchi et al. 2005; Liu and Howell 2010).

Some of the conserved branches of UPR have been identified in plants. In Arabidopsis, bZIP28 is known to be a functional homolog of mammalian ATF6. bZIP28 is thought to sense stress through its ER-lumenal domain and through its interaction with BiP (Srivastava et al. 2013). In response to ER stress, bZIP28 is translocated from the ER to the Golgi, where the cytoplasm-facing N-terminal regions are proteolytically processed by Site 2 protease (S2P) to liberate the active transcription factor. The active transcription factors then migrate into the nucleus where they activate the transcription of downstream genes involved in ER stress and the UPR (Liu and Howell 2010; Chen and Brandizzi 2012; Srivastava et al. 2013). One of the highly upregulated bZIP28-dependent genes is BiP3, which carries an ERSE-I (CCAAT-N9-CCACG) in its promoter. In mammals, the direct binding of ATF6 to the CCACG box of the ERSE element happens when NF-Y, a heterotrimeric complex composed of NF-YA, NF-YB, and NF-YC, initially binds to the CCAAT box of ERSE. Similarly, in plants bZIP28 assembles a larger transcriptional activation complex through interactions with the heterotrimeric CCAAT-binding factors composed of subunits NF-YA4, NF-YB3 and NF-YC2 (Duwi Fanata et al. 2013; Howell 2013).

Arabidopsis also has two sequence homologs of IRE1 - *AtIRE1A* and *AtIRE1B* (Koizumi et al., 2001). Similar to its yeast and mammalian counterparts, Arabidopsis IRE1 plays a key role in the ER stress response by catalyzing unconventional splicing of bZIP60 mRNA to produce the active transcription factor (bZIP60), which is eventually transported to the nucleus to activate transcription of UPR-associated genes (Deng et al. 2011; Nagashima et al. 2011; Duwi Fanata et al. 2013). A number of bZIP60-responsive genes contain plant-specific unfolded protein response elements and ERSEs. Thus, it seems likely that during ER stress the active bZIP60 transcription factor binds to the cis-elements of the promoters of target genes to activate their transcription (Iwata and Koizumi 2005; Iwata et al. 2008).

While no orthologs of PERK have been identified in plants, the presence of a general control non-depressible 2 (GCN2) that is activated by various stresses including amino acid starvation, UV irradiation, wounding, cold shock, salicylic acid and methyl jasmonate has been reported in Arabidopsis (Zhang et al. 2003; Lageix et al. 2008; Sormani et al. 2011). Arabidopsis GCN2 binds to uncharged tRNA molecules and functions as an eIF2α kinase and its activity results in strong inhibition of global protein synthesis in plants (Lageix et al. 2008; Li et al. 2013). In mammalian cells, $P58^{IPK}$, an HSP40 family member, is induced during ER stress and is known to interact with the kinase domain of PERK and inhibit its activity (Yan et al. 2002; van Huizen et al. 2003). Interestingly, a homologue of $P58^{IPK}$ has been identified in Arabidopsis and its role in plant survival during viral infection has been demonstrated (Bilgin et al. 2003; Duwi Fanata et al. 2013). However, its role in the plant response to ER stress needs to be elucidated.

Molecular components of ERAD including the homologs of EDEMs, OS9, Hrd1, Hrd3/Sel1L, Derlin-1 and ubiquitin-conjugating enzyme, UBC32, are also found in plants (Kamauchi et al. 2005; Martinez and Chrispeels 2003; Kirst et al. 2005; Cui et al. 2012; Duwi Fanata et al. 2013). Another ERAD player, SUD1 of Arabidopsis, encodes a putative E3 ubiquitin ligase. SUD1 shows sequence and structural similarity to yeast Degradation of α factor (Doα10) and to the human ER-resident ubiquitin ligase TEB4, both of which are components of the ERAD-C (Doblas et al. 2013). The induction of these components by ER stress in plants suggests that an ERAD system similar to those

of yeast or mammalian cells may remove misfolded proteins produced in the ER lumen of plant cells. Together, these results reveal that the pathways that exist in yeast and mammals to manage UPR and ERAD are also conserved in plants. However, little is known about the pathways and mechanisms by which ERAD functions in plants.

1.9.3. Application of plant cells as model organisms for studying disease processes

Arabidopsis is an important model organism for studying plant biology. Emerging evidence demonstrates that this model plant also is a valuable tool for understanding molecular mechanisms that underpin human disease states (Jones et al. 2008). The completion of the Arabidopsis and human genome sequencing projects allowed researchers to discover the similarities and differences existing between the two genomes. Comparative analysis of the two genomes revealed that the majority of human genes that were suspected or known to play a role in disease had conserved orthologs in Arabidopsis (Jones et al. 2008; Xu and Moller 2011). For instance, 71% and 70% of the genes implicated in neurological disorders and cancer, respectively, have Arabidopsis orthologs (Rubin et al. 2000). Interestingly, the percentage of cancer gene orthologs in the fruit fly *Drosophila melanogaster* is 67%, in the worm *Caenorhabditis elegans* is 72%, and in the yeast *Saccharomyces cerevisiae* is 41% (Jones et al. 2008). In addition to the genetic similarity to mammals, Arabidopsis has several advantages that make it an adaptable model system for studying human diseases. These include short generation time, the availability of large collection of knockout lines and genomic resources, and few ethical constraints (Xu and Moller 2011). A few select examples of the use of Arabidopsis to dissect molecular mechanisms associated with human diseases are discussed below.

Arabidopsis has been used as a new model to understand Parkinson disease (PD). The Parkinson disease-related DJ-1 protein (the product of the PARK7 gene) has been implicated in protection against oxidative stress in dopaminergic neurons (Ottolini et al. 2013). However, the precise mode of action of DJ-1 remains unknown. To understand its function, DJ-1 was characterized in Arabidopsis which harbor homologs of human DJ-1. Superoxide dismutase 1 (SOD1) and glutathione peroxidase 2 (GPX2)

interact with both AtDJ-1a and human DJ-1, and this interaction results in AtDJ-1a and DJ-1-mediated cytosolic SOD1 activation in a copper-dependent fashion (Xu et al. 2010; Xu and Moller 2010). Based on this initial research in Arabidopsis, the authors propose that DJ1 acts in both plants and humans as a scaffolding protein that brings together SOD and GPX to mediate and control ROS scavenging, and thus ultimately prevent oxidative stress-induced cell death (Xu and Moller 2010).

Alzheimer's disease (AD), the most common cause of dementia, is characterized by neuronal degeneration and the extensive deposition of amyloid-β peptide (Aβ) plaques in the brains of affected individuals (Lublin and Gandy 2010). Mitochondrial damage results from accumulation of Aβ in the mitochondria; the subsequent increase of reactive oxygen species (ROS) is thought to be one of the main causes of AD (Reddy and Beal 2008). A number of genes associated with AD have been reported (Bettens et al. 2010) among which half have orthologs in Arabidopsis (Xu and Moller 2011). In humans, *PITRM1*, encoding human pre-sequence protease (hPreP), is involved in clearing toxic Aβ in human brain mitochondria (Falkevall et al. 2006; Alikhani et al. 2009). PreP first was identified and characterized in Arabidopsis as the protease involved in the degradation of the pre-sequences (or sorting peptides) of mitochondrial proteins and of the freed transit peptides (TP) of chloroplast proteins (Stahl et al. 2000, 2002). There are two isoforms of this protease in Arabidopsis (AtPreP1 and AtPreP2) and they mediate the degradation of a wide range of unstructured peptides ranging between 10 and 65 amino acids in length (Stahl et al. 2005). Later studies led to the identification of AtPreP orthologs in the mitochondrial matrix of mammalian cells and a characterization of their roles in Aβ degradation (Falkevall et al. 2006; Alikhani et al. 2011). Although there are differences between PreP of Arabidopsis and PreP of humans, the fundamental research carried out on plant PrePs in terms of substrate specificity, subcellular localization, and structural analysis has shaped human PreP research (Xu and Moller 2011).

Recently a novel organellar oligopeptidase (OOP) of Arabidopsis has been identified and shown to be localized dually in mitochondria and chloroplasts. OOP degrades small peptide fragments within the range of 8-23 aa and acts not only in parallel but also downstream of PreP to cleave peptides partially degraded by PreP

(Kmiec et al. 2013). This finding could impact AD research as a human variant of OOP might also play a role in the degradation of Aβ peptides.

Plants have also been used to investigate the function of presenilin (PS) mutations associated with familial forms of AD (Levy-Lahad et al. 1995; reviewed in Gama Sosa et al. 2012). Presenilin forms the catalytic center of γ-secretase, an aspartate protease complex that cleaves a range of substrate proteins in animals including the amyloid precursor protein (APP) (Schroeter et al. 2003; Khandelwal et al. 2007). However, presenilins have long been suggested to have γ-secretase-independent functions. Interestingly, homologs of presenilin exist in plants. To characterize the function of presenilin an early land plant, the moss *Physcomitrella patens* has been used. These studies showed that presenilin is involved in maintenance and/or formation of a proper cytoskeletal network. Importantly, human presenilin 1 rescues abnormal growth and light responses in *P. patens*; presenilin knock-out plants and introduction of moss presenilin into PS-deficient mouse embryonic fibroblasts rescues the normal growth rates of these cells (Khandelwal et al. 2007; reviewed in Gama Sosa et al. 2012).

In another study, Arabidopsis was used to reveal the molecular defects involved in Lafora disease, an autosomal recessive disorder resulting from mutations of the gene encoding laforin, a glucan phosphatase (Gentry et al. 2007; Gentry and Pace 2009; Vander Kooi et al. 2010). Lafora disease involves progressive myoclonus epilepsy and is characterized by the accumulation of water insoluble glucans (Niittyla et al. 2006; Gentry et al 2009; Gama Sosa et al. 2012). Niittyla et al. found a protein homolog of laforin in Arabidopsis called glucan phosphatase starch excess 4 (SEX4). Strikingly, mutations in *AtSEX4* result in an increase in insoluble glucans similar to what is observed in Lafora disease patients (Gentry et al 2009; reviewed in Gama Sosa et al. 2012). Laforin and *AtSEX4* have similar biochemical properties such as glucan binding and phosphatase activity. Moreover, laforin is the functional equivalent of *AtSEX4*, as human laforin rescues the phenotype of plants with *SEX4* mutations (Gentry et al 2009; Vander Kooi et al. 2010; Gama Sosa et al. 2012).

1.9.4. Use of plant cells for screening small molecule libraries

Over the last decade, chemical biology has been used as a powerful tool by plant scientists to understand diverse cellular processes in plants, including endomembrane trafficking, hormone transport, gravitropism and cell wall biosynthesis (Hicks and Raikhe 2009). In many cases, plant phenotype-based chemical screens led to the identification of small molecules that target a particular process (Hicks and Raikhe 2009). For instance screening of a diverse chemical library resulted in the identification of gravacin, an inhibitor of root and shoot gravitropism (Rojas-Pierce et al. 2007). In another approach screening of LACTA (Library of Active Compounds in Arabidopsis) identified compounds that disturb cortical microtubule alignment in tobacco BY-2 cells and cause a swollen cell phenotype (Yoneda et al. 2007).

To dissect the endomembrane system and to understand the function of proteins involved in protein trafficking, high-throughput confocal-based screens were carried out to identify compounds that inhibited tobacco pollen germination *in vitro* or affected polar growth (pollen tube tip swelling), processes which depend on vesicle trafficking and transport (Robert at al. 2008). More than 46,000 compounds were screened in a tobacco pollen germination/growth assay and 360 of these compounds were found to be bioactive on the basis of resulting morphological or developmental defects (Robert at al. 2008; Drakakaki et al. 2011; reviewed in Brown et al. 2014). Secondary screening identified compounds affecting protein trafficking or the morphology of endomembrane compartments (Drakakaki et al. 2011). Interestingly, Endosidin 5 (ES5) which blocks protein recycling and induces a strong accumulation of plasma membrane (PM) cargoes in the plant vacuole also targets the recycling of plasma membrane proteins in HeLa cells (Drakakaki et al. 2011). This shows that certain pathways involved in endomembrane trafficking are evolutionarily conserved in both plants and humans. Similar effects of certain chemicals in both plant- and human- cells demonstrate the potential utility of plant-based systems for drug discovery (Drakakaki et al. 2011; reviewed in Hicks and Raikhel 2012).

1.10. The Present Study: Objectives

The aim of this study was to establish a plant-based system for identifying potential therapeutics for LSDs - namely small molecules that are able to 'rescue' mutant lysosomal enzymes (so-called 'pharmacological chaperones', PCs). The most common approach of identifying PCs is to purify the lysosomal enzyme under study and conduct *in vitro* analyses to identify substrate analogues that bind tightly to its active site (e.g. in vitro inhibition assays). However, potentially effective PCs need not necessarily be substrate analogues possessing inhibitory activity at higher concentrations. Thus, some important small molecules may be missed by present screening systems. My research was focussed on establishing an alternative plant-based system for identifying PCs – one with advantages over present screening assays. I targeted two human LSDs - Gaucher disease and MPS I disease - that involve deficiencies of the lysosomal hydrolases glucocerebrosidase (GCase) and alpha-L-iduronidase (IDUA), respectively. On a more basic level, I sought to uncover potentially novel aspects of protein quality control involved in the disposal of mutant forms of GCase by examining their fate in plant cells.

My specific objectives were:

1. To develop a plant-based system to generate recombinant wild-type and missense-mutant forms of GCase (N370S and L444P) and IDUA (P533R and R383H) and characterize their expression in the plant host.
2. To conduct proof-of-principle studies for the efficacy of the plant-based system as a screening tool by the use of known PCs to determine their ability to increase the activity and secretion of mutant GCase in transgenic plant cells.
3. To assess the fate of mutant GCase proteins in the plant cell environment in the absence and presence of the ERAD inhibitors and proteostasis regulators.
4. To utilize the plant-based system to screen small molecule libraries in order to identify lead compounds for mutant IDUA that could serve as putative PCs for MPS I.

The studies undertaken are reported in three chapters, two of which are published in peer-reviewed journals; the last chapter represents research that is in preparation for publication.

Chapter 2 describes proof-of-concept studies using plant cells expressing N370S mutant protein (Babajani et al. 2012; *Molecular Genetics and Metabolism*). Chapter 3 reports on the proteostasis of recombinant L444P GCase in plant cells (Babajani and Kermode 2014; *Plant Signaling & Behaviour*). Finally, Chapter 4 describes the implementation of the plant-based screening system to identify putative pharmacological chaperones for treatment of MPS I disease (manuscript in preparation). I end my thesis with concluding remarks, and future questions and directions.

1.11. References

Acosta-Alvear D, Zhou Y, Blais A, Tsikitis M, Lents NH, Arias C, Lennon CJ, Kluger Y, Dynlacht BD. XBP1 controls diverse cell type- and condition-specific transcriptional regulatory networks. Mol Cell 2007; 27:53-66.

Adachi Y, Yamamoto K, Okada T, Yoshida H, Harada A, Mori K. ATF6 is a transcription factor specializing in the regulation of quality control proteins in the endoplasmic reticulum. Cell Struct Funct 2008; 33:75-89.

Aerts JM, Kallemeijn WW, Wegdam W, Joao Ferraz M, van Breemen MJ, Dekker N, Kramer G, Poorthuis BJ, Groener JE, Cox-Brinkman J, Rombach SM, Hollak CE, Linthorst GE, Witte MD, Gold H, van der Marel GA, Overkleeft HS, Boot RG. Biomarkers in the diagnosis of lysosomal storage disorders: Proteins, lipids, and inhibodies. J Inherit Metab Dis 2011; 34:605-619.

Alif N, Hess K, Straczek J, Sebbar S, Belahsen Y, Mouane N, Abkari A, Nabet P, Gelot MA. Mucopolysaccharidosis type I in morocco: Clinical features and genetic profile. Arch Pediatr 2000; 7:597-604.

Alikhani N, Ankarcrona M, Glaser E. Mitochondria and alzheimer's disease: Amyloid-beta peptide uptake and degradation by the presequence protease, hPreP. J Bioenerg Biomembr 2009; 41:447-451.

Alikhani N, Berglund AK, Engmann T, Spanning E, Vogtle FN, Pavlov P, Meisinger C, Langer T, Glaser E. Targeting capacity and conservation of PreP homologues localization in mitochondria of different species. J Mol Biol 2011; 410:400-410.

Baek GH, Kim I, Rao H. The Cdc48 ATPase modulates the interaction between two proteolytic factors Ufd2 and Rad23. Proc Natl Acad Sci U S A 2011; 108:13558-13563.

Balch WE, Morimoto RI, Dillin A, Kelly JW. Adapting proteostasis for disease intervention. Science 2008; 319:916-919.

Bannykh SI, Rowe T, Balch WE. The organization of endoplasmic reticulum export complexes. J Cell Biol 1996; 135:19-35.

Barlowe C. COPII-dependent transport from the endoplasmic reticulum. Curr Opin Cell Biol 2002; 14:417-422.

Barlowe C, Orci L, Yeung T, Hosobuchi M, Hamamoto S, Salama N, Rexach MF, Ravazzola M, Amherdt M, Schekman R. COPII: A membrane coat formed by sec proteins that drive vesicle budding from the endoplasmic reticulum. Cell 1994; 77:895-907.

Barlowe C, Schekman R. SEC12 encodes a guanine-nucleotide-exchange factor essential for transport vesicle budding from the ER. Nature 1993; 365:347-349.

Benjamin ER, Flanagan JJ, Schilling A, Chang HH, Agarwal L, Katz E, Wu X, Pine C, Wustman B, Desnick RJ, Lockhart DJ, Valenzano KJ. The pharmacological chaperone 1-deoxygalactonojirimycin increases alpha-galactosidase A levels in fabry patient cell lines. J Inherit Metab Dis 2009; 32:424-440.

Benjamin ER, Khanna R, Schilling A, Flanagan JJ, Pellegrino LJ, Brignol N, Lun Y, Guillen D, Ranes BE, Frascella M, Soska R, Feng J, Dungan L, Young B, Lockhart DJ, Valenzano KJ. Co-administration with the pharmacological chaperone AT1001 increases recombinant human alpha-galactosidase A tissue uptake and improves substrate reduction in fabry mice. Mol Ther 2012; 20:717-726.

Berg-Fussman A, Grace ME, Ioannou Y, Grabowski GA. Human acid beta-glucosidase. N-glycosylation site occupancy and the effect of glycosylation on enzymatic activity. J Biol Chem 1993; 268:14861-14866.

Bernfield M, Gotte M, Park PW, Reizes O, Fitzgerald ML, Lincecum J, Zako M. Functions of cell surface heparan sulfate proteoglycans. Annu Rev Biochem 1999; 68:729-777.

Bettens K, Sleegers K, Van Broeckhoven C. Current status on alzheimer disease molecular genetics: From past, to present, to future. Hum Mol Genet 2010; 19:R4-R11.

Beutler E. Lysosomal storage diseases: Natural history and ethical and economic aspects. Mol Genet Metab 2006; 88:208-215.

Beutler E, Grabowski GA. Glucosylceramide lipidoses: Gaucher disease. In: Scriver CR, Beaudet AL, Sly WS, Valle D, editors. The Metabolic and Molecular Basis of Inherited Diseases. New York: McGraw-Hill 1995; 2641-70.

Beutler E, Grabowski G. Gaucher disease. In: Scriver C, Beaudet A, Sly W, Valle D (eds). The metabolic and molecular bases of inherited disease, 8th Ed. New York, New York: McGraw-Hill 2001; 3635-68.

Bhamidipati A, Denic V, Quan EM, Weissman JS. Exploration of the topological requirements of ERAD identifies Yos9p as a lectin sensor of misfolded glycoproteins in the ER lumen. Mol Cell 2005; 19:741-751.

Bie H, Yin J, He X, Kermode AR, Goddard-Borger ED, Withers SG, James MN. Insights into mucopolysaccharidosis I from the structure and action of alpha-L-iduronidase. Nat Chem Biol 2013; 9:739-745.

Bilgin DD, Liu Y, Schiff M, Dinesh-Kumar SP. P58(IPK), a plant ortholog of double-stranded RNA-dependent protein kinase PKR inhibitor, functions in viral pathogenesis. Dev Cell 2003; 4:651-661.

Blanz J, Groth J, Zachos C, Wehling C, Saftig P, Schwake M. Disease-causing mutations within the lysosomal integral membrane protein type 2 (LIMP-2) reveal the nature of binding to its ligand beta-glucocerebrosidase. Hum Mol Genet 2010; 19:563-572.

Boustany RM. Lysosomal storage diseases--the horizon expands. Nat Rev Neurol 2013; 9:583-598.

Boyd RE, Lee G, Rybczynski P, Benjamin ER, Khanna R, Wustman BA, Valenzano KJ. Pharmacological chaperones as therapeutics for lysosomal storage diseases. J Med Chem 2013; 56:2705-2725.

Braakman I, Bulleid NJ. Protein folding and modification in the mammalian endoplasmic reticulum. Annu Rev Biochem 2011; 80:71-99.

Brady RO, Barton NW, Grabowski GA. The role of neurogenetics in gaucher disease. Arch Neurol 1993; 50:1212-1224.

Braulke T, Bonifacino JS. Sorting of lysosomal proteins. Biochim Biophys Acta 2009; 1793:605-614.

Brodsky JL. Cleaning up: ER-associated degradation to the rescue. Cell 2012; 151:1163-1167.

Brooks DA, Fabrega S, Hein LK, Parkinson EJ, Durand P, Yogalingam G, Matte U, Giugliani R, Dasvarma A, Eslahpazire J, Henrissat B, Mornon JP, Hopwood JJ, Lehn P. Glycosidase active site mutations in human alpha-L-iduronidase. Glycobiology 2001; 11:741-750.

Brown MQ, Ung N, Raikhel NV, Hicks GR. High-throughput identification of chemical endomembrane cycling disruptors utilizing tobacco pollen. Methods Mol Biol 2014; 1056:111-114.

Brumshtein B, Greenblatt HM, Butters TD, Shaaltiel Y, Aviezer D, Silman I, Futerman AH, Sussman JL. Crystal structures of complexes of N-butyl- and N-nonyl-deoxynojirimycin bound to acid beta-glucosidase: Insights into the mechanism of chemical chaperone action in gaucher disease. J Biol Chem 2007; 282:29052-29058.

Bryngelson JD, Onuchic JN, Socci ND, Wolynes PG. Funnels, pathways, and the energy landscape of protein folding: A synthesis. Proteins 1995; 21:167-195.

Bunge S, Clements PR, Byers S, Kleijer WJ, Brooks DA, Hopwood JJ. Genotype-phenotype correlations in mucopolysaccharidosis type I using enzyme kinetics, immunoquantification and in vitro turnover studies. Biochim Biophys Acta 1998; 1407:249-256.

Bunge S, Kleijer WJ, Steglich C, Beck M, Schwinger E, Gal A. Mucopolysaccharidosis type I: Identification of 13 novel mutations of the alpha-L-iduronidase gene. Hum Mutat 1995; 6:91-94.

Calfon M, Zeng H, Urano F, Till JH, Hubbard SR, Harding HP, Clark SG, Ron D. IRE1 couples endoplasmic reticulum load to secretory capacity by processing the XBP-1 mRNA. Nature 2002; 415:92-96.

Caramelo JJ, Parodi AJ. Getting in and out from calnexin/calreticulin cycles. J Biol Chem 2008; 283:10221-10225.

Chen Y, Brandizzi F. AtIRE1A/AtIRE1B and AGB1 independently control two essential unfolded protein response pathways in arabidopsis. Plant J 2012; 69:266-277.

Chen Y, Zhang Y, Yin Y, Gao G, Li S, Jiang Y, Gu X, Luo J. SPD--a web-based secreted protein database. Nucleic Acids Res 2005; 33:D169-73.

Chiti F, Dobson CM. Protein misfolding, functional amyloid, and human disease. Annu Rev Biochem 2006; 75:333-366.

Clark NE, Metcalf MC, Best D, Fleet GW, Garman SC. Pharmacological chaperones for human alpha-N-acetylgalactosaminidase. Proc Natl Acad Sci U S A 2012; 109:17400-17405.

Clarke LA. Pathogenesis of skeletal and connective tissue involvement in the mucopolysaccharidoses: Glycosaminoglycan storage is merely the instigator. Rheumatology (Oxford) 2011; 50 Suppl 5:v13-8.

Collins ML, Traboulsi EI, Maumenee IH. Optic nerve head swelling and optic atrophy in the systemic mucopolysaccharidoses. Ophthalmology 1990; 97:1445-1449.

Conzelmann E, Sandhoff K. Partial enzyme deficiencies: Residual activities and the development of neurological disorders. Dev Neurosci 1983; 6:58-71.

Credle JJ, Finer-Moore JS, Papa FR, Stroud RM, Walter P. On the mechanism of sensing unfolded protein in the endoplasmic reticulum. Proc Natl Acad Sci U S A 2005; 102:18773-18784.

Crowley KS, Liao S, Worrell VE, Reinhart GD, Johnson AE. Secretory proteins move through the endoplasmic reticulum membrane via an aqueous, gated pore. Cell 1994; 78:461-471.

Cui F, Liu L, Zhao Q, Zhang Z, Li Q, Lin B, Wu Y, Tang S, Xie Q. Arabidopsis ubiquitin conjugase UBC32 is an ERAD component that functions in brassinosteroid-mediated salt stress tolerance. Plant Cell 2012; 24:233-244.

da Cunha NB, Vianna GR, da Almeida Lima T, Rech E. Molecular farming of human cytokines and blood products from plants: Challenges in biosynthesis and detection of plant-produced recombinant proteins. Biotechnol J 2014; 9:39-50.

Dancourt J, Barlowe C. Protein sorting receptors in the early secretory pathway. Annu Rev Biochem 2010; 79:777-802.

Dancourt J, Barlowe C. Protein sorting receptors in the early secretory pathway. Annu Rev Biochem 2010; 79:777-802.

de Duve C. The lysosome turns fifty. Nat Cell Biol 2005; 7:847-849.

de Duve C, Pressman BC, Gianetto R, Wattiaux R, Appelmans F. Tissue fractionation studies. 6. intracellular distribution patterns of enzymes in rat-liver tissue. Biochem J 1955; 60:604-617.

Deng Y, Humbert S, Liu JX, Srivastava R, Rothstein SJ, Howell SH. Heat induces the splicing by IRE1 of a mRNA encoding a transcription factor involved in the unfolded protein response in arabidopsis. Proc Natl Acad Sci U S A 2011; 108:7247-7252.

Deng Y, Srivastava R, Howell SH. Endoplasmic reticulum (ER) stress response and its physiological roles in plants. Int J Mol Sci 2013; 14:8188-8212.

Desnick RJ. Enzyme replacement and enhancement therapies for lysosomal diseases. J Inherit Metab Dis 2004; 27:385-410.

Desnick RJ, Schuchman EH. Enzyme replacement therapy for lysosomal diseases: Lessons from 20 years of experience and remaining challenges. Annu Rev Genomics Hum Genet 2012; 13:307-335.

Dinner AR, Sali A, Smith LJ, Dobson CM, Karplus M. Understanding protein folding via free-energy surfaces from theory and experiment. Trends Biochem Sci 2000; 25:331-339.

Doblas VG, Amorim-Silva V, Pose D, Rosado A, Esteban A, Arro M, Azevedo H, Bombarely A, Borsani O, Valpuesta V, Ferrer A, Tavares RM, Botella MA. The SUD1 gene encodes a putative E3 ubiquitin ligase and is a positive regulator of 3-hydroxy-3-methylglutaryl coenzyme a reductase activity in arabidopsis. Plant Cell 2013; 25:728-743.

Dobson CM, Ellis RJ. Protein folding and misfolding inside and outside the cell. EMBO J 1998; 17:5251-5254.

Downing WL, Galpin JD, Clemens S, Lauzon SM, Samuels AL, Pidkowich MS, Clarke LA, Kermode AR. Synthesis of enzymatically active human alpha-L-iduronidase in arabidopsis cgl (complex glycan-deficient) seeds. Plant Biotechnol J 2006; 4:169-181.

Drakakaki G, Robert S, Szatmari AM, Brown MQ, Nagawa S, Van Damme D, Leonard M, Yang Z, Girke T, Schmid SL, Russinova E, Friml J, Raikhel NV, Hicks GR. Clusters of bioactive compounds target dynamic endomembrane networks in vivo. Proc Natl Acad Sci U S A 2011; 108:17850-17855.

Duwi Fanata WI, Lee SY, Lee KO. The unfolded protein response in plants: A fundamental adaptive cellular response to internal and external stresses. J Proteomics 2013; 93:356-368.

Dvir H, Harel M, McCarthy AA, Toker L, Silman I, Futerman AH, Sussman JL. X-ray structure of human acid-beta-glucosidase, the defective enzyme in gaucher disease. EMBO Rep 2003; 4:704-709.

Eichner T, Radford SE. A diversity of assembly mechanisms of a generic amyloid fold. Mol Cell 2011; 43:8-18.

Eisele F, Schafer A, Wolf DH. Ubiquitylation in the ERAD pathway. Subcell Biochem 2010; 54:136-148.

Ellgaard L, Helenius A. ER quality control: Towards an understanding at the molecular level. Curr Opin Cell Biol 2001; 13:431-437.

Falkevall A, Alikhani N, Bhushan S, Pavlov PF, Busch K, Johnson KA, Eneqvist T, Tjernberg L, Ankarcrona M, Glaser E. Degradation of the amyloid beta-protein by the novel mitochondrial peptidasome, PreP. J Biol Chem 2006; 281:29096-29104.

Fan JQ, Ishii S, Asano N, Suzuki Y. Accelerated transport and maturation of lysosomal alpha-galactosidase A in fabry lymphoblasts by an enzyme inhibitor. Nat Med 1999; 5:112-115.

Febbraio M, Hajjar DP, Silverstein RL. CD36: A class B scavenger receptor involved in angiogenesis, atherosclerosis, inflammation, and lipid metabolism. J Clin Invest 2001; 108:785-791.

Fewell SW, Travers KJ, Weissman JS, Brodsky JL. The action of molecular chaperones in the early secretory pathway. Annu Rev Genet 2001; 35:149-191.

Fox J. First plant-made biologic approved. Nat Bio 2012; 30: 472

Freedman RB, Hirst TR, Tuite MF. Protein disulphide isomerase: Building bridges in protein folding. Trends Biochem Sci 1994; 19:331-336.

Fritz JM, Dong M, Apsley KS, Martin EP, Na CL, Sitaraman S, Weaver TE. Deficiency of the BiP cochaperone ERdj4 causes constitutive endoplasmic reticulum stress and metabolic defects. Mol Biol Cell 2013.

Fu L H, Miao Y, Lo SW, Seto TC, Sun S, Xu Z, Clemens S, Clarke L, Kermode AR, Production and characterization of soluble human lysosomal enzyme α-iduronidase with high activity from culture media of transgenic tobacco BY-2 cells Original Research. Plant Science 2009; 177: 668-675.

Fujita H, Saeki M, Yasunaga K, Ueda T, Imoto T, Himeno M. In vitro binding study of adaptor protein complex (AP-1) to lysosomal targeting motif (LI-motif). Biochem Biophys Res Commun 1999; 255:54-58.

Fuller M, Brooks DA, Evangelista M, Hein LK, Hopwood JJ, Meikle PJ. Prediction of neuropathology in mucopolysaccharidosis I patients. Mol Genet Metab 2005; 84:18-24.

Gama Sosa MA, De Gasperi R, Elder GA. Modeling human neurodegenerative diseases in transgenic systems. Hum Genet 2012; 131:535-563.

Gardner BM, Pincus D, Gotthardt K, Gallagher CM, Walter P. Endoplasmic reticulum stress sensing in the unfolded protein response. Cold Spring Harb Perspect Biol 2013; 5:a013169.

Gardner RG, Swarbrick GM, Bays NW, Cronin SR, Wilhovsky S, Seelig L, Kim C, Hampton RY. Endoplasmic reticulum degradation requires lumen to cytosol signaling. transmembrane control of Hrd1p by Hrd3p. J Cell Biol 2000; 151:69-82.

Gatti R, DiNatale P, Villani GR, Filocamo M, Muller V, Guo XH, Nelson PV, Scott HS, Hopwood JJ. Mutations among italian mucopolysaccharidosis type I patients. J Inherit Metab Dis 1997; 20:803-806.

Gauss R, Jarosch E, Sommer T, Hirsch C. A complex of Yos9p and the HRD ligase integrates endoplasmic reticulum quality control into the degradation machinery. Nat Cell Biol 2006; 8:849-854.

Gentry MS, Dixon JE, Worby CA. Lafora disease: Insights into neurodegeneration from plant metabolism. Trends Biochem Sci 2009; 34:628-639.

Gentry MS, Dowen RH,3rd, Worby CA, Mattoo S, Ecker JR, Dixon JE. The phosphatase laforin crosses evolutionary boundaries and links carbohydrate metabolism to neuronal disease. J Cell Biol 2007; 178:477-488.

Gentry MS, Pace RM. Conservation of the glucan phosphatase laforin is linked to rates of molecular evolution and the glucan metabolism of the organism. BMC Evol Biol 2009; 9:138-2148-9-138.

Ghosh P, Dahms NM, Kornfeld S. Mannose 6-phosphate receptors: New twists in the tale. Nat Rev Mol Cell Biol 2003; 4:202-212.

Ghosh P, Kornfeld S. The GGA proteins: Key players in protein sorting at the trans-golgi network. Eur J Cell Biol 2004; 83:257-262.

Gillon AD, Latham CF, Miller EA. Vesicle-mediated ER export of proteins and lipids. Biochim Biophys Acta 2012; 1821:1040-1049.

Ginns EI, Choudary PV, Martin BM, Winfield S, Stubblefield B, Mayor J, Merkle-Lehman D, Murray GJ, Bowers LA, Barranger JA. Isolation of cDNA clones for human beta-glucocerebrosidase using the lambda gt11 expression system. Biochem Biophys Res Commun 1984; 123:574-580.

Gomord V, Faye L. Posttranslational modification of therapeutic proteins in plants. Curr Opin Plant Biol 2004; 7:171-181.

Gomord V, Fitchette AC, Menu-Bouaouiche L, Saint-Jore-Dupas C, Plasson C, Michaud D, Faye L. Plant-specific glycosylation patterns in the context of therapeutic protein production. Plant Biotechnol J 2010; 8:564-587.

Gort L, Chabas A, Coll MJ. Analysis of five mutations in 20 mucopolysaccharidois type 1 patients: High prevalence of the W402X mutation. mutations in brief no. 121. online. Hum Mutat 1998; 11:332-333.

Grabowski GA. Gaucher disease and other storage disorders. Hematology Am Soc Hematol Educ Program 2012; 2012:13-18.

Grabowski GA. Gaucher disease: Gene frequencies and genotype/phenotype correlations. Genet Test 1997; 1:5-12.

Grabowski GA, Hopkin RJ. Enzyme therapy for lysosomal storage disease: Principles, practice, and prospects. Annu Rev Genomics Hum Genet 2003; 4:403-436.

Grace ME, Newman KM, Scheinker V, Berg-Fussman A, Grabowski GA. Analysis of human acid beta-glucosidase by site-directed mutagenesis and heterologous expression. J Biol Chem 1994; 269:2283-2291.

Hagiwara M, Maegawa K, Suzuki M, Ushioda R, Araki K, Matsumoto Y, Hoseki J, Nagata K, Inaba K. Structural basis of an ERAD pathway mediated by the ER-resident protein disulfide reductase ERdj5. Mol Cell 2011; 41:432-444.

Hamman BD, Hendershot LM, Johnson AE. BiP maintains the permeability barrier of the ER membrane by sealing the lumenal end of the translocon pore before and early in translocation. Cell 1998; 92:747-758.

Hampton RY, Sommer T. Finding the will and the way of ERAD substrate retrotranslocation. Curr Opin Cell Biol 2012; 24:460-466.

Hamuro Y, Coales SJ, Southern MR, Nemeth-Cawley JF, Stranz DD, Griffin PR. Rapid analysis of protein structure and dynamics by hydrogen/deuterium exchange mass spectrometry. J Biomol Tech 2003; 14:171-182.

Han D, Lerner AG, Vande Walle L, Upton JP, Xu W, Hagen A, Backes BJ, Oakes SA, Papa FR. IRE1alpha kinase activation modes control alternate endoribonuclease outputs to determine divergent cell fates. Cell 2009; 138:562-575.

Harding HP, Zhang Y, Ron D. Protein translation and folding are coupled by an endoplasmic-reticulum-resident kinase. Nature 1999; 397:271-274.

Harding HP, Zhang Y, Scheuner D, Chen JJ, Kaufman RJ, Ron D. Ppp1r15 gene knockout reveals an essential role for translation initiation factor 2 alpha (eIF2alpha) dephosphorylation in mammalian development. Proc Natl Acad Sci U S A 2009; 106:1832-1837.

Hartl FU. Molecular chaperones in cellular protein folding. Nature 1996; 381:571-579.

Hartl FU, Hayer-Hartl M. Converging concepts of protein folding in vitro and in vivo. Nat Struct Mol Biol 2009; 16:574-581.

Hatsuzawa K, Tagaya M, Mizushima S. The hydrophobic region of signal peptides is a determinant for SRP recognition and protein translocation across the ER membrane. J Biochem 1997; 121:270-277.

He X, Galpin JD, Tropak MB, Mahuran D, Haselhorst T, von Itzstein M, Kolarich D, Packer NH, Miao Y, Jiang L, Grabowski GA, Clarke LA, Kermode AR. Production of active human glucocerebrosidase in seeds of arabidopsis thaliana complex-glycan-deficient (cgl) plants. Glycobiology 2012; 22:492-503.

He X, Haselhorst T, von Itzstein M, Kolarich D, Packer NH, Gloster TM, Vocadlo DJ, Clarke LA, Qian Y, Kermode AR. Production of alpha-L-iduronidase in maize for the potential treatment of a human lysosomal storage disease. Nat Commun 2012; 3:1062.

He X, Pierce O, Haselhorst T, von Itzstein M, Kolarich D, Packer NH, Gloster TM, Vocadlo DJ, Qian Y, Brooks D, Kermode AR. Characterization and downstream mannose phosphorylation of human recombinant alpha-L-iduronidase produced in arabidopsis complex glycan-deficient (cgl) seeds. Plant Biotechnol J 2013; 11:1034-1043.

Hebert DN, Molinari M. In and out of the ER: Protein folding, quality control, degradation, and related human diseases. Physiol Rev 2007; 87:1377-1408.

Hein LK, Bawden M, Muller VJ, Sillence D, Hopwood JJ, Brooks DA. Alpha-L-iduronidase premature stop codons and potential read-through in mucopolysaccharidosis type I patients. J Mol Biol 2004; 338:453-462.

Herczenik E, Gebbink MF. Molecular and cellular aspects of protein misfolding and disease. FASEB J 2008; 22:2115-2133.

Hers HG. Alpha-glucosidase deficiency in generalized glycogenstorage disease (pompe's disease). Biochem J 1963; 86:11-16.

Hetz C, Chevet E, Harding HP. Targeting the unfolded protein response in disease. Nat Rev Drug Discov 2013; 12:703-719.

Hicks GR, Raikhel NV. Small molecules present large opportunities in plant biology. Annu Rev Plant Biol 2012; 63:261-282.

Hiller MM, Finger A, Schweiger M, Wolf DH. ER degradation of a misfolded luminal protein by the cytosolic ubiquitin-proteasome pathway. Science 1996; 273:1725-1728.

Hollien J, Lin JH, Li H, Stevens N, Walter P, Weissman JS. Regulated Ire1-dependent decay of messenger RNAs in mammalian cells. J Cell Biol 2009; 186:323-331.

Hollien J, Weissman JS. Decay of endoplasmic reticulum-localized mRNAs during the unfolded protein response. Science 2006; 313:104-107.

Horowitz M, Wilder S, Horowitz Z, Reiner O, Gelbart T, Beutler E. The human glucocerebrosidase gene and pseudogene: Structure and evolution. Genomics 1989; 4:87-96.

Hosokawa N, Kamiya Y, Kato K. The role of MRH domain-containing lectins in ERAD. Glycobiology 2010; 20:651-660.

Hosokawa N, Wada I, Hasegawa K, Yorihuzi T, Tremblay LO, Herscovics A, Nagata K. A novel ER alpha-mannosidase-like protein accelerates ER-associated degradation. EMBO Rep 2001; 2:415-422.

Howell SH. Endoplasmic reticulum stress responses in plants. Annu Rev Plant Biol 2013; 64:477-499.

Hruska KS, LaMarca ME, Scott CR, Sidransky E. Gaucher disease: Mutation and polymorphism spectrum in the glucocerebrosidase gene (GBA). Hum Mutat 2008; 29:567-583.

Hutt DM, Powers ET, Balch WE. The proteostasis boundary in misfolding diseases of membrane traffic. FEBS Lett 2009; 583:2639-2646.

Ishii S, Chang HH, Kawasaki K, Yasuda K, Wu HL, Garman SC, Fan JQ. Mutant alpha-galactosidase A enzymes identified in fabry disease patients with residual enzyme activity: Biochemical characterization and restoration of normal intracellular processing by 1-deoxygalactonojirimycin. Biochem J 2007; 406:285-295.

Iwata Y, Fedoroff NV, Koizumi N. Arabidopsis bZIP60 is a proteolysis-activated transcription factor involved in the endoplasmic reticulum stress response. Plant Cell 2008; 20:3107-3121.

Iwata Y, Koizumi N. An arabidopsis transcription factor, AtbZIP60, regulates the endoplasmic reticulum stress response in a manner unique to plants. Proc Natl Acad Sci U S A 2005; 102:5280-5285.

Jakobkiewicz-Banecka J, Piotrowska E, Gabig-Ciminska M, Borysiewicz E, Slominska-Wojewodzka M, Narajczyk M, Wegrzyn A, Wegrzyn G. Substrate reduction therapies for mucopolysaccharidoses. Curr Pharm Biotechnol 2011; 12:1860-1865.

Janda CY, Li J, Oubridge C, Hernandez H, Robinson CV, Nagai K. Recognition of a signal peptide by the signal recognition particle. Nature 2010; 465:507-510.

Jiang J, Maes EG, Taylor AB, Wang L, Hinck AP, Lafer EM, Sousa R. Structural basis of J cochaperone binding and regulation of Hsp70. Mol Cell 2007; 28:422-433.

Jo Y, Debose-Boyd RA. Control of cholesterol synthesis through regulated ER-associated degradation of HMG CoA reductase. Crit Rev Biochem Mol Biol 2010; 45:185-198.

Jones AM, Chory J, Dangl JL, Estelle M, Jacobsen SE, Meyerowitz EM, Nordborg M, Weigel D. The impact of arabidopsis on human health: Diversifying our portfolio. Cell 2008; 133:939-943.

Journet A, Chapel A, Kieffer S, Roux F, Garin J. Proteomic analysis of human lysosomes: Application to monocytic and breast cancer cells. Proteomics 2002; 2:1026-1040.

Kakkis ED, Matynia A, Jonas AJ, Neufeld EF. Overexpression of the human lysosomal enzyme alpha-L-iduronidase in chinese hamster ovary cells. Protein Expr Purif 1994; 5:225-232.

Kakoi S, Yorimitsu T, Sato K. COPII machinery cooperates with ER-localized Hsp40 to sequester misfolded membrane proteins into ER-associated compartments. Mol Biol Cell 2013; 24:633-642.

Kamauchi S, Nakatani H, Nakano C, Urade R. Gene expression in response to endoplasmic reticulum stress in arabidopsis thaliana. FEBS J 2005; 272:3461-3476.

Kermode AR. Plants as factories for production of biopharmaceutical and bioindustrial proteins: lessons from cell biology. Can J Bot 2006; 84:679-694.

Kermode AR. Seed Expression Systems for Molecular Farming. In: Wang A, Ma S, editors. Molecular farming in plants: recent advances and future prospects. New York: Springer 2012; 89-123.

Khandelwal A, Chandu D, Roe CM, Kopan R, Quatrano RS. Moonlighting activity of presenilin in plants is independent of gamma-secretase and evolutionarily conserved. Proc Natl Acad Sci U S A 2007; 104:13337-13342.

Kim YE, Hipp MS, Bracher A, Hayer-Hartl M, Hartl FU. Molecular chaperone functions in protein folding and proteostasis. Annu Rev Biochem 2013; 82:323-355.

Kirst ME, Meyer DJ, Gibbon BC, Jung R, Boston RS. Identification and characterization of endoplasmic reticulum-associated degradation proteins differentially affected by endoplasmic reticulum stress. Plant Physiol 2005; 138:218-231.

Kleizen B, Braakman I. Protein folding and quality control in the endoplasmic reticulum. Curr Opin Cell Biol 2004; 16:343-349.

Kmiec B, Teixeira PF, Berntsson RP, Murcha MW, Branca RM, Radomiljac JD, Regberg J, Svensson LM, Bakali A, Langel U, Lehtio J, Whelan J, Stenmark P, Glaser E. Organellar oligopeptidase (OOP) provides a complementary pathway for targeting peptide degradation in mitochondria and chloroplasts. Proc Natl Acad Sci U S A 2013; 110:E3761-9.

Koizumi N, Martinez IM, Kimata Y, Kohno K, Sano H, Chrispeels MJ. Molecular characterization of two arabidopsis Ire1 homologs, endoplasmic reticulum-located transmembrane protein kinases. Plant Physiol 2001; 127:949-962.

Kopito RR, Ron D. Conformational disease. Nat Cell Biol 2000; 2:E207-9.

Koprivica V, Stone DL, Park JK, Callahan M, Frisch A, Cohen IJ, Tayebi N, Sidransky E. Analysis and classification of 304 mutant alleles in patients with type 1 and type 3 gaucher disease. Am J Hum Genet 2000; 66:1777-1786.

Kornfeld S, Mellman I. The biogenesis of lysosomes. Annu Rev Cell Biol 1989; 5:483-525.

Kornhaber GJ, Tropak MB, Maegawa GH, Tuske SJ, Coales SJ, Mahuran DJ, Hamuro Y. Isofagomine induced stabilization of glucocerebrosidase. Chembiochem 2008; 9:2643-2649.

Kostova Z, Tsai YC, Weissman AM. Ubiquitin ligases, critical mediators of endoplasmic reticulum-associated degradation. Semin Cell Dev Biol 2007; 18:770-779.

Kudo T, Kanemoto S, Hara H, Morimoto N, Morihara T, Kimura R, Tabira T, Imaizumi K, Takeda M. A molecular chaperone inducer protects neurons from ER stress. Cell Death Differ 2008; 15:364-375.

Lachmann RH, te Vruchte D, Lloyd-Evans E, Reinkensmeier G, Sillence DJ, Fernandez-Guillen L, Dwek RA, Butters TD, Cox TM, Platt FM. Treatment with miglustat reverses the lipid-trafficking defect in niemann-pick disease type C. Neurobiol Dis 2004; 16:654-658.

Lageix S, Lanet E, Pouch-Pelissier MN, Espagnol MC, Robaglia C, Deragon JM, Pelissier T. Arabidopsis eIF2alpha kinase GCN2 is essential for growth in stress conditions and is activated by wounding. BMC Plant Biol 2008; 8:134-2229-8-134.

Laradi S, Tukel T, Erazo M, Shabbeer J, Chkioua L, Khedhiri S, Ferchichi S, Chaabouni M, Miled A, Desnick RJ. Mucopolysaccharidosis I: Alpha-L-iduronidase mutations in three tunisian families. J Inherit Metab Dis 2005; 28:1019-1026.

Le Borgne R, Hoflack B. Mannose 6-phosphate receptors regulate the formation of clathrin-coated vesicles in the TGN. J Cell Biol 1997; 137:335-345.

Lee AH, Iwakoshi NN, Glimcher LH. XBP-1 regulates a subset of endoplasmic reticulum resident chaperone genes in the unfolded protein response. Mol Cell Biol 2003; 23:7448-7459.

Lee YK, Brewer JW, Hellman R, Hendershot LM. BiP and immunoglobulin light chain cooperate to control the folding of heavy chain and ensure the fidelity of immunoglobulin assembly. Mol Biol Cell 1999; 10:2209-2219.

Lee YY, Cevallos RC, Jan E. An upstream open reading frame regulates translation of GADD34 during cellular stresses that induce eIF2alpha phosphorylation. J Biol Chem 2009; 284:6661-6673.

Leighton SE, Papsin B, Vellodi A, Dinwiddie R, Lane R. Disordered breathing during sleep in patients with mucopolysaccharidoses. Int J Pediatr Otorhinolaryngol 2001; 58:127-138.

Leonova T, Grabowski GA. Fate and sorting of acid beta-glucosidase in transgenic mammalian cells. Mol Genet Metab 2000; 70:281-294.

Levy-Lahad E, Wasco W, Poorkaj P, Romano DM, Oshima J, Pettingell WH, Yu CE, Jondro PD, Schmidt SD, Wang K. Candidate gene for the chromosome 1 familial alzheimer's disease locus. Science 1995; 269:973-977.

Li H, Korennykh AV, Behrman SL, Walter P. Mammalian endoplasmic reticulum stress sensor IRE1 signals by dynamic clustering. Proc Natl Acad Sci U S A 2010; 107:16113-16118.

Li MW, AuYeung WK, Lam HM. The GCN2 homologue in arabidopsis thaliana interacts with uncharged tRNA and uses arabidopsis eIF2alpha molecules as direct substrates. Plant Biol (Stuttg) 2013; 15:13-18.

Lieberman AP, Puertollano R, Raben N, Slaugenhaupt S, Walkley SU, Ballabio A. Autophagy in lysosomal storage disorders. Autophagy 2012; 8:719-730.

Lieberman RL, Wustman BA, Huertas P, Powe AC,Jr, Pine CW, Khanna R, Schlossmacher MG, Ringe D, Petsko GA. Structure of acid beta-glucosidase with pharmacological chaperone provides insight into gaucher disease. Nat Chem Biol 2007; 3:101-107.

Lin JH, Walter P, Yen TS. Endoplasmic reticulum stress in disease pathogenesis. Annu Rev Pathol 2008; 3:399-425.

Little L, Alcouloumre M, Drotar AM, Herman S, Robertson R, Yeh RY, Miller AL. Properties of N-acetylglucosamine 1-phosphotransferase from human lymphoblasts. Biochem J 1987; 248:151-159.

Little LE, Mueller OT, Honey NK, Shows TB, Miller AL. Heterogeneity of N-acetylglucosamine 1-phosphotransferase within mucolipidosis III. J Biol Chem 1986; 261:733-738.

Liu CY, Kaufman RJ. The unfolded protein response. J Cell Sci 2003; 116:1861-1862.

Liu JX, Howell SH. bZIP28 and NF-Y transcription factors are activated by ER stress and assemble into a transcriptional complex to regulate stress response genes in arabidopsis. Plant Cell 2010; 22:782-796.

Lord C, Ferro-Novick S, Miller EA. The highly conserved COPII coat complex sorts cargo from the endoplasmic reticulum and targets it to the golgi. Cold Spring Harb Perspect Biol 2013; 5:10.1101/cshperspect.a013367.

Lublin AL, Gandy S. Amyloid-beta oligomers: Possible roles as key neurotoxins in alzheimer's disease. Mt Sinai J Med 2010; 77:43-49.

Ma JK, Drake PM, Christou P. The production of recombinant pharmaceutical proteins in plants. Nat Rev Genet 2003; 4:794-805.

Ma Y, Hendershot LM. Delineation of a negative feedback regulatory loop that controls protein translation during endoplasmic reticulum stress. J Biol Chem 2003; 278:34864-34873.

Maegawa GH, Tropak M, Buttner J, Stockley T, Kok F, Clarke JT, Mahuran DJ. Pyrimethamine as a potential pharmacological chaperone for late-onset forms of GM2 gangliosidosis. J Biol Chem 2007; 282:9150-9161.

Martin R, Martin C, Escobedo S, Suarez JE, Quiros LM. Surface glycosaminoglycans mediate adherence between HeLa cells and lactobacillus salivarius Lv72. BMC Microbiol 2013; 13:210-2180-13-210.

Martinez IM, Chrispeels MJ. Genomic analysis of the unfolded protein response in arabidopsis shows its connection to important cellular processes. Plant Cell 2003; 15:561-576.

Matte U, Yogalingam G, Brooks D, Leistner S, Schwartz I, Lima L, Norato DY, Brum JM, Beesley C, Winchester B, Giugliani R, Hopwood JJ. Identification and characterization of 13 new mutations in mucopolysaccharidosis type I patients. Mol Genet Metab 2003; 78:37-43.

Mayer MP, Bukau B. Hsp70 chaperones: Cellular functions and molecular mechanism. Cell Mol Life Sci 2005; 62:670-684.

Meikle PJ, Ranieri E, Simonsen H, Rozaklis T, Ramsay SL, Whitfield PD, Fuller M, Christensen E, Skovby F, Hopwood JJ. Newborn screening for lysosomal storage disorders: Clinical evaluation of a two-tier strategy. Pediatrics 2004; 114:909-916.

Mellman I. Endocytosis and molecular sorting. Annu Rev Cell Dev Biol 1996; 12:575-625.

Menendez-Sainz C, Gonzalez-Quevedo A, Gonzalez-Garcia S, Pena-Sanchez M, Giugliani R. High proportion of mannosidosis and fucosidosis among lysosomal storage diseases in cuba. Genet Mol Res 2012; 11:2352-2359.

Mezzacasa A, Helenius A. The transitional ER defines a boundary for quality control in the secretion of tsO45 VSV glycoprotein. Traffic 2002; 3:833-849.

Miller EA, Beilharz TH, Malkus PN, Lee MC, Hamamoto S, Orci L, Schekman R. Multiple cargo binding sites on the COPII subunit Sec24p ensure capture of diverse membrane proteins into transport vesicles. Cell 2003; 114:497-509.

Mindell JA. Lysosomal acidification mechanisms. Annu Rev Physiol 2012; 74:69-86.

Mogk A, Mayer MP, Deuerling E. Mechanisms of protein folding: Molecular chaperones and their application in biotechnology. Chembiochem 2002; 3:807-814.

Mohan UR, Hay AA, Cleary MA, Wraith JE, Patel RG. Cardiovascular changes in children with mucopolysaccharide disorders. Acta Paediatr 2002; 91:799-804.

Molinari M, Calanca V, Galli C, Lucca P, Paganetti P. Role of EDEM in the release of misfolded glycoproteins from the calnexin cycle. Science 2003; 299:1397-1400.

Molinari M, Helenius A. Glycoproteins form mixed disulphides with oxidoreductases during folding in living cells. Nature 1999; 402:90-93.

Moore KA, Hollien J. The unfolded protein response in secretory cell function. Annu Rev Genet 2012; 46:165-183.

Mori K. Signalling pathways in the unfolded protein response: Development from yeast to mammals. J Biochem 2009; 146:743-750.

Moskowitz SM, Tieu PT, Neufeld EF. Mutation in scheie syndrome (MPS IS): A G-->A transition creates new splice site in intron 5 of one IDUA allele. Hum Mutat 1993; 2:141-144.

Mu T, Ong D, Wang Y, Balch WE, Yates JR, Segatori L, Kelly JW. Proteostasis regulators and pharmacologic chaperones synergize to correct protein misfolding diseases. Cell 2008; 134: 769-781.

Nagashima Y, Mishiba K, Suzuki E, Shimada Y, Iwata Y, Koizumi N. Arabidopsis IRE1 catalyses unconventional splicing of bZIP60 mRNA to produce the active transcription factor. Sci Rep 2011; 1:29.

Neufeld EF. Enzyme replacement therapy - a brief history. In: Mehta A, Beck M, Sunder-Plassmann G, eds. Fabry Disease: Perspectives from 5 Years of FOS. Oxford: Oxford Pharma Genesis; 2006.

Neufeld EF, Muenzer J. The Mucopolysaccharidoses. In: Scriver C, Beaudet A, Sly W, Valle D (eds). The metabolic and molecular bases of inherited disease, 8th Ed. New York, New York: McGraw-Hill 2001; 3421-52

Niittyla T, Comparot-Moss S, Lue WL, Messerli G, Trevisan M, Seymour MD, Gatehouse JA, Villadsen D, Smith SM, Chen J, Zeeman SC, Smith AM. Similar protein phosphatases control starch metabolism in plants and glycogen metabolism in mammals. J Biol Chem 2006; 281:11815-11818.

Nishikawa S, Brodsky JL, Nakatsukasa K. Roles of molecular chaperones in endoplasmic reticulum (ER) quality control and ER-associated degradation (ERAD). J Biochem 2005; 137:551-555.

Noh SJ, Kwon CS, Oh DH, Moon JS, Chung WI. Expression of an evolutionarily distinct novel BiP gene during the unfolded protein response in arabidopsis thaliana. Gene 2003; 311:81-91.

Novoa I, Zeng H, Harding HP, Ron D. Feedback inhibition of the unfolded protein response by GADD34-mediated dephosphorylation of eIF2alpha. J Cell Biol 2001; 153:1011-1022.

Oikawa D, Tokuda M, Hosoda A, Iwawaki T. Identification of a consensus element recognized and cleaved by IRE1 alpha. Nucleic Acids Res 2010; 38:6265-6273.

Olzmann JA, Kopito RR, Christianson JC. The mammalian endoplasmic reticulum-associated degradation system. Cold Spring Harb Perspect Biol 2013; 5:10.1101/cshperspect.a013185.

Ortolano S, Vieitez I, Navarro C, Spuch C. Treatment of lysosomal storage diseases: Recent patents and future strategies. Recent Pat Endocr Metab Immune Drug Discov 2014.

Osorio F, Tavernier SJ, Hoffmann E, Saeys Y, Martens L, Vetters J, Delrue I, De Rycke R, Parthoens E, Pouliot P, Iwawaki T, Janssens S, Lambrecht BN. The unfolded-protein-response sensor IRE-1alpha regulates the function of CD8alpha dendritic cells. Nat Immunol 2014.

Ottolini D, Cali T, Negro A, Brini M. The parkinson disease-related protein DJ-1 counteracts mitochondrial impairment induced by the tumour suppressor protein p53 by enhancing endoplasmic reticulum-mitochondria tethering. Hum Mol Genet 2013; 22:2152-2168.

Pacheco CD, Kunkel R, Lieberman AP. Autophagy in niemann-pick C disease is dependent upon beclin-1 and responsive to lipid trafficking defects. Hum Mol Genet 2007; 16:1495-1503.

Pan S, Cheng X, Sifers RN. Golgi-situated endoplasmic reticulum alpha-1, 2-mannosidase contributes to the retrieval of ERAD substrates through a direct interaction with gamma-COP. Mol Biol Cell 2013; 24:1111-1121.

Parenti G. Treating lysosomal storage diseases with pharmacological chaperones: From concept to clinics. EMBO Mol Med 2009; 1:268-279.

Parenti G, Pignata C, Vajro P, Salerno M. New strategies for the treatment of lysosomal storage diseases (review). Int J Mol Med 2013; 31:11-20.

Pastores GM, Barnett NL. Substrate reduction therapy: Miglustat as a remedy for symptomatic patients with gaucher disease type 1. Expert Opin Investig Drugs 2003; 12:273-281.

Pastores GM, Giraldo P, Cherin P, Mehta A. Goal-oriented therapy with miglustat in gaucher disease. Curr Med Res Opin 2009; 25:23-37.

Patterson MC, Vecchio D, Prady H, Abel L, Wraith JE. Miglustat for treatment of niemann-pick C disease: A randomised controlled study. Lancet Neurol 2007; 6:765-772.

Paul M, Ma JK. Plant-made pharmaceuticals: Leading products and production platforms. Biotechnol Appl Biochem 2011; 58:58-67.

Pincus D, Chevalier MW, Aragon T, van Anken E, Vidal SE, El-Samad H, Walter P. BiP binding to the ER-stress sensor Ire1 tunes the homeostatic behavior of the unfolded protein response. PLoS Biol 2010; 8:e1000415.

Piotrowska E, Jakobkiewicz-Banecka J, Baranska S, Tylki-Szymanska A, Czartoryska B, Wegrzyn A, Wegrzyn G. Genistein-mediated inhibition of glycosaminoglycan synthesis as a basis for gene expression-targeted isoflavone therapy for mucopolysaccharidoses. Eur J Hum Genet 2006; 14:846-852.

Piotrowska E, Jakobkiewicz-Banecka J, Baranska S, Tylki-Szymanska A, Czartoryska B, Wegrzyn A, Wegrzyn G. Genistein-mediated inhibition of glycosaminoglycan synthesis as a basis for gene expression-targeted isoflavone therapy for mucopolysaccharidoses. Eur J Hum Genet 2006; 14:846-852.

Plasson C, Michel R, Lienard D, Saint-Jore-Dupas C, Sourrouille C, de March GG, Gomord V. Production of recombinant proteins in suspension-cultured plant cells. Methods Mol Biol 2009; 483:145-161.

Platt FM, Boland B, van der Spoel AC. The cell biology of disease: Lysosomal storage disorders: The cellular impact of lysosomal dysfunction. J Cell Biol 2012; 199:723-734.

Platt FM, Jeyakumar M. Substrate reduction therapy. Acta Paediatr Suppl 2008; 97:88-93.

Platt FM, Neises GR, Dwek RA, Butters TD. N-butyldeoxynojirimycin is a novel inhibitor of glycolipid biosynthesis. J Biol Chem 1994; 269:8362-8365.

Platt FM, Neises GR, Reinkensmeier G, Townsend MJ, Perry VH, Proia RL, Winchester B, Dwek RA, Butters TD. Prevention of lysosomal storage in tay-sachs mice treated with N-butyldeoxynojirimycin. Science 1997; 276:428-431.

Powers ET, Morimoto RI, Dillin A, Kelly JW, Balch WE. Biological and chemical approaches to diseases of proteostasis deficiency. Annu Rev Biochem 2009; 78:959-991.

Price JL, Culyba EK, Chen W, Murray AN, Hanson SR, Wong CH, Powers ET, Kelly JW. N-glycosylation of enhanced aromatic sequons to increase glycoprotein stability. Biopolymers 2012; 98:195-211.

Quan EM, Kamiya Y, Kamiya D, Denic V, Weibezahn J, Kato K, Weissman JS. Defining the glycan destruction signal for endoplasmic reticulum-associated degradation. Mol Cell 2008; 32:870-877.

Rapraeger A, Jalkanen M, Bernfield M. Cell surface proteoglycan associates with the cytoskeleton at the basolateral cell surface of mouse mammary epithelial cells. J Cell Biol 1986; 103:2683-2696.

Reczek D, Schwake M, Schroder J, Hughes H, Blanz J, Jin X, Brondyk W, Van Patten S, Edmunds T, Saftig P. LIMP-2 is a receptor for lysosomal mannose-6-phosphate-independent targeting of beta-glucocerebrosidase. Cell 2007; 131:770-783.

Reddy PH, Beal MF. Amyloid beta, mitochondrial dysfunction and synaptic damage: Implications for cognitive decline in aging and alzheimer's disease. Trends Mol Med 2008; 14:45-53.

Reitman ML, Kornfeld S. Lysosomal enzyme targeting. N-acetylglucosaminylphosphotransferase selectively phosphorylates native lysosomal enzymes. J Biol Chem 1981*a*; 256:11977-11980.

Reitman ML, Kornfeld S. UDP-N-acetylglucosamine:Glycoprotein N-acetylglucosamine-1-phosphotransferase. proposed enzyme for the phosphorylation of the high mannose oligosaccharide units of lysosomal enzymes. J Biol Chem 1981*b*; 256:4275-4281.

Robert S, Chary SN, Drakakaki G, Li S, Yang Z, Raikhel NV, Hicks GR. Endosidin1 defines a compartment involved in endocytosis of the brassinosteroid receptor BRI1 and the auxin transporters PIN2 and AUX1. Proc Natl Acad Sci U S A 2008; 105:8464-8469.

Roberts AL, Fletcher JM, Moore L, Byers S. Trans-generational exposure to low levels of rhodamine B does not adversely affect litter size or liver function in murine mucopolysaccharidosis type IIIA. Mol Genet Metab 2010; 101:208-213.

Rojas-Pierce M, Titapiwatanakun B, Sohn EJ, Fang F, Larive CK, Blakeslee J, Cheng Y, Cutler SR, Peer WA, Murphy AS, Raikhel NV. Arabidopsis P-glycoprotein19 participates in the inhibition of gravitropism by gravacin. Chem Biol 2007; 14:1366-1376.

Ron D, Walter P. Signal integration in the endoplasmic reticulum unfolded protein response. Nat Rev Mol Cell Biol 2007; 8:519-529.

Ron I, Horowitz M. ER retention and degradation as the molecular basis underlying gaucher disease heterogeneity. Hum Mol Genet 2005; 14:2387-2398.

Rubin GM, Yandell MD, Wortman JR, Gabor Miklos GL, Nelson CR, Hariharan IK, Fortini ME, Li PW, Apweiler R, Fleischmann W, Cherry JM, Henikoff S, Skupski MP, Misra S, Ashburner M, Birney E, Boguski MS, Brody T, Brokstein P, Celniker SE, Chervitz SA, Coates D, Cravchik A, Gabrielian A, Galle RF, Gelbart WM, George RA, Goldstein LS, Gong F, Guan P, Harris NL, Hay BA, Hoskins RA, Li J, Li Z, Hynes RO, Jones SJ, Kuehl PM, Lemaitre B, Littleton JT, Morrison DK, Mungall C, O'Farrell PH, Pickeral OK, Shue C, Vosshall LB, Zhang J, Zhao Q, Zheng XH, Lewis S. Comparative genomics of the eukaryotes. Science 2000; 287:2204-2215.

Ruddock LW, Molinari M. N-glycan processing in ER quality control. J Cell Sci 2006; 119:4373-4380.

Ruddock LW, Molinari M. N-glycan processing in ER quality control. J Cell Sci 2006; 119:4373-4380.

Saftig P, Klumperman J. Lysosome biogenesis and lysosomal membrane proteins: Trafficking meets function. Nat Rev Mol Cell Biol 2009; 10:623-635.

Saito A, Ochiai K, Kondo S, Tsumagari K, Murakami T, Cavener DR, Imaizumi K. Endoplasmic reticulum stress response mediated by the PERK-eIF2(alpha)-ATF4 pathway is involved in osteoblast differentiation induced by BMP2. J Biol Chem 2011; 286:4809-4818.

Sanyal S, Menon AK. Specific transbilayer translocation of dolichol-linked oligosaccharides by an endoplasmic reticulum flippase. Proc Natl Acad Sci U S A 2009; 106:767-772.

Sawkar AR, Adamski-Werner SL, Cheng WC, Wong CH, Beutler E, Zimmer KP, Kelly JW. Gaucher disease-associated glucocerebrosidases show mutation-dependent chemical chaperoning profiles. Chem Biol 2005; 12:1235-1244.

Sawkar AR, Cheng WC, Beutler E, Wong CH, Balch WE, Kelly JW. Chemical chaperones increase the cellular activity of N370S beta -glucosidase: A therapeutic strategy for gaucher disease. Proc Natl Acad Sci U S A 2002; 99:15428-15433.

Sawkar AR, D'Haeze W, Kelly JW. Therapeutic strategies to ameliorate lysosomal storage disorders--a focus on gaucher disease. Cell Mol Life Sci 2006; 63:1179-1192.

Schillberg S, Fischer R, Emans N. 'Molecular farming' of antibodies in plants. Naturwissenschaften 2003; 90:145-155.

Schmidt K, Stephens DJ. Cargo loading at the ER. Mol Membr Biol 2010; 27:398-411.

Schmitz M, Alfalah M, Aerts JM, Naim HY, Zimmer KP. Impaired trafficking of mutants of lysosomal glucocerebrosidase in gaucher's disease. Int J Biochem Cell Biol 2005; 37:2310-2320.

Schroder M. Endoplasmic reticulum stress responses. Cell Mol Life Sci 2008; 65:862-894.

Schroeter EH, Ilagan MX, Brunkan AL, Hecimovic S, Li YM, Xu M, Lewis HD, Saxena MT, De Strooper B, Coonrod A, Tomita T, Iwatsubo T, Moore CL, Goate A, Wolfe MS, Shearman M, Kopan R. A presenilin dimer at the core of the gamma-secretase enzyme: Insights from parallel analysis of notch 1 and APP proteolysis. Proc Natl Acad Sci U S A 2003; 100:13075-13080.

Schueler UH, Kolter T, Kaneski CR, Zirzow GC, Sandhoff K, Brady RO. Correlation between enzyme activity and substrate storage in a cell culture model system for gaucher disease. J Inherit Metab Dis 2004; 27:649-658.

Schulze H, Sandhoff K. Sphingolipids and lysosomal pathologies. Biochim Biophys Acta 2013.

Scott HS, Anson DS, Orsborn AM, Nelson PV, Clements PR, Morris CP, Hopwood JJ. Human alpha-L-iduronidase: CDNA isolation and expression. Proc Natl Acad Sci U S A 1991; 88:9695-9699.

Scott HS, Guo XH, Hopwood JJ, Morris CP. Structure and sequence of the human alpha-L-iduronidase gene. Genomics 1992; 13:1311-1313.

Scott HS, Litjens T, Nelson PV, Brooks DA, Hopwood JJ, Morris CP. Alpha-L-iduronidase mutations (Q70X and P533R) associate with a severe hurler phenotype. Hum Mutat 1992; 1:333-339.

Scott JE. Elasticity in extracellular matrix 'shape modules' of tendon, cartilage, etc. A sliding proteoglycan-filament model. J Physiol 2003; 553:335-343.

Shaaltiel Y, Bartfeld D, Hashmueli S, Baum G, Brill-Almon E, Galili G, Dym O, Boldin-Adamsky SA, Silman I, Sussman JL, Futerman AH, Aviezer D. Production of glucocerebrosidase with terminal mannose glycans for enzyme replacement therapy of gaucher's disease using a plant cell system. Plant Biotechnol J 2007; 5:579-590.

Shen JS, Edwards NJ, Hong YB, Murray GJ. Isofagomine increases lysosomal delivery of exogenous glucocerebrosidase. Biochem Biophys Res Commun 2008; 369:1071-1075.

Shinhar SY, Zablocki H, Madgy DN. Airway management in mucopolysaccharide storage disorders. Arch Otolaryngol Head Neck Surg 2004; 130:233-237.

Shoulders MD, Ryno LM, Genereux JC, Moresco JJ, Tu PG, Wu C, Yates JR,3rd, Su AI, Kelly JW, Wiseman RL. Stress-independent activation of XBP1s and/or ATF6 reveals three functionally diverse ER proteostasis environments. Cell Rep 2013; 3:1279-1292.

Song W, Wang F, Savini M, Ake A, di Ronza A, Sardiello M, Segatori L. TFEB regulates lysosomal proteostasis. Hum Mol Genet 2013; 22:1994-2009.

Sorge J, West C, Westwood B, Beutler E. Molecular cloning and nucleotide sequence of human glucocerebrosidase cDNA. Proc Natl Acad Sci U S A 1985; 82:7289-7293.

Srivastava R, Deng Y, Shah S, Rao AG, Howell SH. BINDING PROTEIN is a master regulator of the endoplasmic reticulum stress sensor/transducer bZIP28 in arabidopsis. Plant Cell 2013; 25:1416-1429.

Stahl A, Moberg P, Ytterberg J, Panfilov O, Brockenhuus Von Lowenhielm H, Nilsson F, Glaser E. Isolation and identification of a novel mitochondrial metalloprotease (PreP) that degrades targeting presequences in plants. J Biol Chem 2002; 277:41931-41939.

Stahl A, Nilsson S, Lundberg P, Bhushan S, Biverstahl H, Moberg P, Morisset M, Vener A, Maler L, Langel U, Glaser E. Two novel targeting peptide degrading proteases, PrePs, in mitochondria and chloroplasts, so similar and still different. J Mol Biol 2005; 349:847-860.

Stahl A, Pavlov PF, Szigyarto C, Glaser E. Rapid degradation of the presequence of the f1beta precursor of the ATP synthase inside mitochondria. Biochem J 2000; 349 Pt 3:703-707.

Steet RA, Chung S, Wustman B, Powe A, Do H, Kornfeld SA. The iminosugar isofagomine increases the activity of N370S mutant acid beta-glucosidase in gaucher fibroblasts by several mechanisms. Proc Natl Acad Sci U S A 2006; 103:13813-13818.

Stolz A, Hilt W, Buchberger A, Wolf DH. Cdc48: A power machine in protein degradation. Trends Biochem Sci 2011; 36:515-523.

Sun Y, Grabowski GA. Impaired autophagosomes and lysosomes in neuronopathic gaucher disease. Autophagy 2010; 6:648-649.

Tabas I, Ron D. Integrating the mechanisms of apoptosis induced by endoplasmic reticulum stress. Nat Cell Biol 2011; 13:184-190.

Tabas I, Ron D. Integrating the mechanisms of apoptosis induced by endoplasmic reticulum stress. Nat Cell Biol 2011; 13:184-190.

Tamargo RJ, Velayati A, Goldin E, Sidransky E. The role of saposin C in gaucher disease. Mol Genet Metab 2012; 106:257-263.

Terlato NJ, Cox GF. Can mucopolysaccharidosis type I disease severity be predicted based on a patient's genotype? A comprehensive review of the literature. Genet Med 2003; 5:286-294.

Teske BF, Wek SA, Bunpo P, Cundiff JK, McClintick JN, Anthony TG, Wek RC. The eIF2 kinase PERK and the integrated stress response facilitate activation of ATF6 during endoplasmic reticulum stress. Mol Biol Cell 2011; 22:4390-4405.

Tropak MB, Kornhaber GJ, Rigat BA, Maegawa GH, Buttner JD, Blanchard JE, Murphy C, Tuske SJ, Coales SJ, Hamuro Y, Brown ED, Mahuran DJ. Identification of pharmacological chaperones for gaucher disease and characterization of their effects on beta-glucocerebrosidase by hydrogen/deuterium exchange mass spectrometry. Chembiochem 2008; 9:2650-2662.

Tsai CJ, Kumar S, Ma B, Nussinov R. Folding funnels, binding funnels, and protein function. Protein Sci 1999; 8:1181-1190.

Tsaytler P, Harding HP, Ron D, Bertolotti A. Selective inhibition of a regulatory subunit of protein phosphatase 1 restores proteostasis. Science 2011; 332:91-94.

Ung N, Brown MQ, Hicks GR, Raikhel NV. An approach to quantify endomembrane dynamics in pollen utilizing bioactive chemicals. Mol Plant 2013; 6:1202-1213.

Ushioda R, Hoseki J, Araki K, Jansen G, Thomas DY, Nagata K. ERdj5 is required as a disulfide reductase for degradation of misfolded proteins in the ER. Science 2008; 321:569-572.

Vaccaro AM, Motta M, Tatti M, Scarpa S, Masuelli L, Bhat M, Vanier MT, Tylki-Szymanska A, Salvioli R. Saposin C mutations in gaucher disease patients resulting in lysosomal lipid accumulation, saposin C deficiency, but normal prosaposin processing and sorting. Hum Mol Genet 2010; 19:2987-2997.

Valenzano KJ, Khanna R, Powe AC, Boyd R, Lee G, Flanagan JJ, Benjamin ER. Identification and characterization of pharmacological chaperones to correct enzyme deficiencies in lysosomal storage disorders. Assay Drug Dev Technol 2011; 9:213-235.

van Huizen R, Martindale JL, Gorospe M, Holbrook NJ. P58IPK, a novel endoplasmic reticulum stress-inducible protein and potential negative regulator of eIF2alpha signaling. J Biol Chem 2003; 278:15558-15564.

van Meel E, Klumperman J. Imaging and imagination: Understanding the endo-lysosomal system. Histochem Cell Biol 2008; 129:253-266.

Vander Kooi CW, Taylor AO, Pace RM, Meekins DA, Guo HF, Kim Y, Gentry MS. Structural basis for the glucan phosphatase activity of starch Excess4. Proc Natl Acad Sci U S A 2010; 107:15379-15384.

von Heijne G. Protein targeting signals. Curr Opin Cell Biol 1990; 2:604-608.

Waguri S, Dewitte F, Le Borgne R, Rouille Y, Uchiyama Y, Dubremetz JF, Hoflack B. Visualization of TGN to endosome trafficking through fluorescently labeled MPR and AP-1 in living cells. Mol Biol Cell 2003; 14:142-155.

Walter P, Johnson AE. Signal sequence recognition and protein targeting to the endoplasmic reticulum membrane. Annu Rev Cell Biol 1994; 10:87-119.

Walter P, Ron D. The unfolded protein response: From stress pathway to homeostatic regulation. Science 2011; 334:1081-1086.

Wang F, Agnello G, Sotolongo N, Segatori L. Ca2+ homeostasis modulation enhances the amenability of L444P glucosylcerebrosidase to proteostasis regulation in patient-derived fibroblasts. ACS Chem Biol 2011; 6:158-168.

Wang F, Chou A, Segatori L. Lacidipine remodels protein folding and ca 2+ homeostasis in gaucher's disease fibroblasts: A mechanism to rescue mutant glucocerebrosidase. Chem Biol 2011; 18:766-776.

Wang F, Segatori L. Remodeling the proteostasis network to rescue glucocerebrosidase variants by inhibiting ER-associated degradation and enhancing ER folding. PLoS One 2013; 8:e61418.

Wang F, Song W, Brancati G, Segatori L. Inhibition of endoplasmic reticulum-associated degradation rescues native folding in loss of function protein misfolding diseases. J Biol Chem 2011; 286:43454-43464.

Wang N, Daniels R, Hebert DN. The cotranslational maturation of the type I membrane glycoprotein tyrosinase: The heat shock protein 70 system hands off to the lectin-based chaperone system. Mol Biol Cell 2005; 16:3740-3752.

Weinreb NJ, Barranger JA, Charrow J, Grabowski GA, Mankin HJ, Mistry P. Guidance on the use of miglustat for treating patients with type 1 gaucher disease. Am J Hematol 2005; 80:223-229.

Wiertz EJ, Tortorella D, Bogyo M, Yu J, Mothes W, Jones TR, Rapoport TA, Ploegh HL. Sec61-mediated transfer of a membrane protein from the endoplasmic reticulum to the proteasome for destruction. Nature 1996; 384:432-438.

Wilcox WR. Lysosomal storage disorders: The need for better pediatric recognition and comprehensive care. J Pediatr 2004; 144:S3-14.

Wu J, Rutkowski DT, Dubois M, Swathirajan J, Saunders T, Wang J, Song B, Yau GD, Kaufman RJ. ATF6alpha optimizes long-term endoplasmic reticulum function to protect cells from chronic stress. Dev Cell 2007; 13:351-364.

Xu XM, Lin H, Maple J, Bjorkblom B, Alves G, Larsen JP, Moller SG. The arabidopsis DJ-1a protein confers stress protection through cytosolic SOD activation. J Cell Sci 2010; 123:1644-1651.

Xu XM, Moller SG. The value of arabidopsis research in understanding human disease states. Curr Opin Biotechnol 2011; 22:300-307.

Xu XM, Moller SG. ROS removal by DJ-1: Arabidopsis as a new model to understand parkinson's disease. Plant Signal Behav 2010; 5:1034-1036.

Yamamoto K, Yoshida H, Kokame K, Kaufman RJ, Mori K. Differential contributions of ATF6 and XBP1 to the activation of endoplasmic reticulum stress-responsive cis-acting elements ERSE, UPRE and ERSE-II. J Biochem 2004; 136:343-350.

Yan W, Frank CL, Korth MJ, Sopher BL, Novoa I, Ron D, Katze MG. Control of PERK eIF2alpha kinase activity by the endoplasmic reticulum stress-induced molecular chaperone P58IPK. Proc Natl Acad Sci U S A 2002; 99:15920-15925.

Yang H, Chen D, Cui QC, Yuan X, Dou QP. Celastrol, a triterpene extracted from the chinese "thunder of god vine," is a potent proteasome inhibitor and suppresses human prostate cancer growth in nude mice. Cancer Res 2006; 66:4758-4765.

Ye C, Dickman MB, Whitham SA, Payton M, Verchot J. The unfolded protein response is triggered by a plant viral movement protein. Plant Physiol 2011; 156:741-755.

Yoneda A, Higaki T, Kutsuna N, Kondo Y, Osada H, Hasezawa S, Matsui M. Chemical genetic screening identifies a novel inhibitor of parallel alignment of cortical microtubules and cellulose microfibrils. Plant Cell Physiol 2007; 48:1393-1403.

Yoshida H, Haze K, Yanagi H, Yura T, Mori K. Identification of the cis-acting endoplasmic reticulum stress response element responsible for transcriptional induction of mammalian glucose-regulated proteins. involvement of basic leucine zipper transcription factors. J Biol Chem 1998; 273:33741-33749.

Yoshida H, Matsui T, Yamamoto A, Okada T, Mori K. XBP1 mRNA is induced by ATF6 and spliced by IRE1 in response to ER stress to produce a highly active transcription factor. Cell 2001; 107:881-891.

Yu Z, Sawkar AR, Kelly JW. Pharmacologic chaperoning as a strategy to treat gaucher disease. FEBS J 2007; 274:4944-4950.

Zachos C, Blanz J, Saftig P, Schwake M. A critical histidine residue within LIMP-2 mediates pH sensitive binding to its ligand beta-glucocerebrosidase. Traffic 2012; 13:1113-1123.

Zhang Y, Dickinson JR, Paul MJ, Halford NG. Molecular cloning of an arabidopsis homologue of GCN2, a protein kinase involved in co-ordinated response to amino acid starvation. Planta 2003; 217:668-675.

Zhao KW, Faull KF, Kakkis ED, Neufeld EF. Carbohydrate structures of recombinant human alpha-L-iduronidase secreted by chinese hamster ovary cells. J Biol Chem 1997; 272:22758-22765.

1.12. Figures

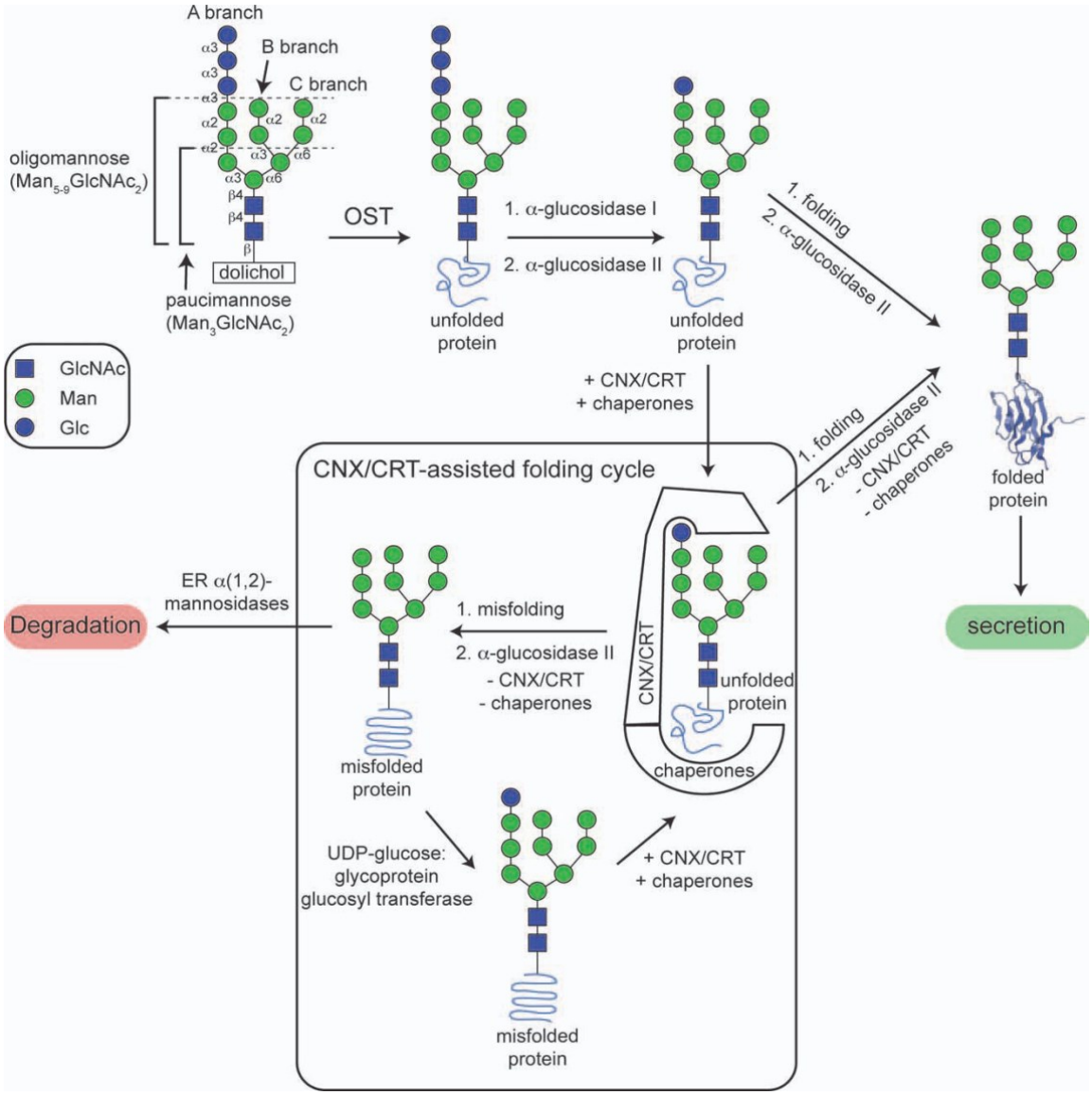


Figure 1-1: Cellular N-glycosylation allows a glycoprotein to enter the calnexin/calreticulin-assisted folding vs. degradation cycle in the ER.

These processes allow the N-glycoprotein to fold and be secreted or to be targeted for degradation if attempts to fold the N-glycoprotein are unsuccessful. Price et al. 2012, Biopolymers. 98(3):195-211. Figure reproduced with permission from John Wiley and Sons.

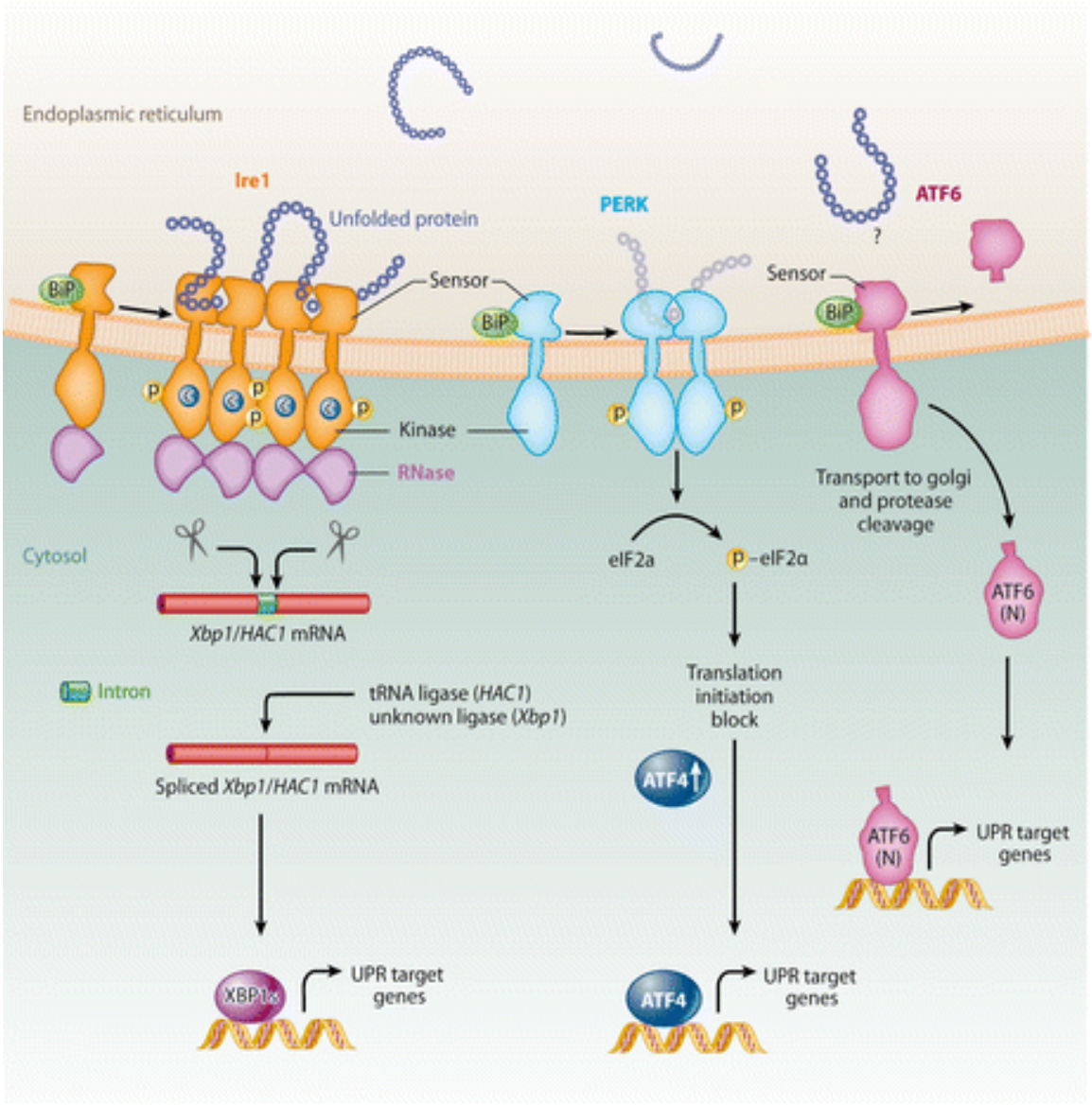


Figure 1-2: The three branches of unfolded protein response (UPR) in higher eukaryotes.

The UPR is controlled by three sensors including Ire1, PERK, and ATF6 in the ER membrane. To relieve stress, these sensors activate transcriptional and translational programs that collectively re-establish the homeostasis of protein folding in the ER. Korennykh and Walter, Ann. Rev. Cell. Dev. Biol. 2012. 28: 251-277.

Chapter 2.

Pharmacological chaperones facilitate the post-ER transport of recombinant N370S mutant β-glucocerebrosidase in plant cells: Evidence that N370S is a folding mutant

Results from this chapter were published: Babajani G., Tropak M.B., Mahuran D., and Kermode A.R. (2012). Molecular Genetics and Metabolism, 106 (3): 323-329.

2.1. Abstract

Gaucher disease is a prevalent lysosomal storage disease in which affected individuals inherit mutations in the gene (*GBA1*) encoding lysosomal acid β-glucosidase (glucocerebrosidase, GCase, EC 3.2.1.45). One of the most prevalent disease-causing mutations in humans is a N370S missense mutation in the GCase protein. As part of a larger endeavour to study the fate of mutant human proteins expressed in plant cells, the N370S mutant protein along with the wild-type- (WT)-GCase, both equipped with a signal peptide, were synthesized in transgenic tobacco BY2 cells, which do not possess lysosomes. The enzymatic activity of plant-recombinant N370S GCase lines was significantly lower (by 81-95%) than that of the WT-GCase lines. In contrast to the WT-GCase protein, which was efficiently secreted from tobacco BY2 cells, and detected in large amounts in the culture medium, only a small proportion of the N370S GCase was secreted. Pharmacological chaperones such as *N*-(*n*-nonyl) deoxynojirimycin and ambroxol increased the steady-state mutant protein levels both inside the plant cells and in the culture medium. These findings contradict the assertion that small molecule chaperones increase N370S GCase activity (as assayed in treated patient cell lysates) by stabilizing the enzyme in the lysosome, and suggest that the mutant protein is

impaired in its ability to obtain its functional folded conformation, which is a requirement for exiting the lumen of the ER.

2.2. Introduction

Gaucher disease is a prevalent lysosomal storage disease caused by a deficiency of the lysosomal hydrolase acid β-glucosidase (glucocerebrosidase, GCase, EC 3.2.1.45). This membrane-associated lysosomal enzyme cleaves the β-glucosidic linkage of glucosylceramide; thus the biochemical hallmark of Gaucher disease is the excessive storage of glucosylceramide, primarily in the cells and tissues of the reticuloendothelial system (subtype 1), and additionally in the brain and other nervous tissues, in subtypes 2 and 3 (Tropak et al. 2008). Almost 300 disease-causing mutations in the *GBA1* gene encoding human GCase have been identified to date. Most cause a deficiency of GCase protein and activity in the lysosome (Sawkar et al. 2006; Hruska et al. 2008). In some GCase variants, the protein is thought to be impaired in its folding in the ER and thus becomes subjected to endoplasmic reticulum-associated degradation (ERAD) involving retrotranslocation of the defective protein to the cytosol and its subsequent disposal by the cytosolic ubiquitin-proteasome machinery. Consequently the cells of an affected patient exhibit a decreased concentration of the hydrolase in the lysosome as a result of an inability of a large percentage of the newly synthesized mutant protein molecules to properly fold in the ER, which in turn prevents post-ER trafficking to the lysosome (Nagy and Sanders 2004; Schmitz et al. 2005).

The N370S missense mutation of the *GBA1* gene is associated with the non-neuropathic subtype (subtype 1), and it is one of the most prevalent mutations causing Gaucher disease, second only to the L444P mutation. For some mutant lysosomal enzymes, including N370S GCase, pharmacological chaperones are being pursued as potential small-molecule '*enzyme enhancement'* therapeutics; some of these active-site-specific inhibitors can 'rescue' a proportion of the newly synthesized mutant enzymes when present at sub-inhibitory concentrations, promoting their maturation and lysosomal trafficking. By serving as a folding template in the ER, the pharmacological chaperone is

thought to allow a small proportion of the proteins to fold properly and thus pass the protein quality control system of the ER (Sawkar et al. 2002, 2005; Fan 2008).

There is some controversy about whether N370S GCase suffers solely from a reduced catalytic activity or whether this mutant hydrolase is also subject to turnover by ERAD. There is considerable evidence that N370S GCase shows reduced catalytic activity, for example when expressed as a recombinant protein in cells of various heterologous hosts – both mammalian (e.g. NIH 3T3 fibroblast) and insect (e.g. *Spodoptera frugiperda*; Sf9) (Grace et al. 1990, 1994). Various studies provide evidence that N370S is an unstable protein, which is destabilized in the environment of the ER (neutral pH) (Sawkar et al. 2002; Yu et al. 2007), and targeted for efficient degradation by ERAD. Sub-inhibitory concentrations of N-(n-nonyl)deoxynojirimycin (NN-DNJ) and the iminosugar isofagomine (IFG) increase the activity of N370S GCase by 2- to 3- fold in cultured N370S-patient fibroblasts; the authors conclude that the chaperones help the mutant protein to fold in the neutral pH of the ER lumen, thus allowing some proportion of the protein to pass ER quality control and undergo transport to the lysosome (Sawkar et al. 2002; Yu et al. 2007). Similarly others have provided evidence that N370S GCase is impaired in its ability to exit from the ER (Steet et al. 2006). Ambroxol (ABX) was uncovered as an enzyme-enhancing therapeutic agent for GCase using a thermal denaturation assay to screen the NINDS, National Institutes of Health, library of 1,040 drugs that have been previously used to treat humans. This chemical stabilizes GCase in a pH-dependent manner and significantly increases the activity and protein levels of N370S GCase in deficient fibroblasts; in ABX-treated Gaucher disease (N370S/N370S) lymphoblasts, the enzyme reaches therapeutically significant levels such that storage of glucosylceramide is reduced (Maegawa et al. 2009). Collectively these data imply that the N370S mutation affects both the catalytic activity, as well as the conformational stability of the enzyme. Yet others still contend that the N370S GCase mutant protein is primarily a catalytically impaired mutant. Based on crystallization of an insect- recombinant human N370S GCase, these authors assert that the mutant protein has an identical folding to that of its normal recombinant counterpart. Interestingly, the authors further speculate that the active site-specific inhibitor NN-DNJ

stabilizes and increases the lysosomal levels of N370S GCase by reducing its degradation in the lysosome, rather than in the ER lumen (Wei et al. 2011).

To resolve this conflicting literature and to determine whether pharmacological chaperones facilitate the post-ER transport of N370S GCase, the recombinant mutant and wild-type (WT) proteins, each equipped with a signal peptide, were produced in transgenic tobacco BY2 cells. The fact that plant cells do not have lysosomes eliminates the possibility of a chaperone-mediated stabilization of N370S GCase in these organelles. As in mammalian cells, the signal peptide effects the co-translational translocation of the recombinant protein into the ER lumen of the plant cell, which similarly provides an oxidizing environment of neutral pH (Denecke et al. 1990; Liua and Howell 2010). In the absence of additional topogenic information, the protein (if transport-competent) will transit to the plant cell surface by a default mechanism.

We show that in the absence of a pharmacological chaperone, N370S GCase is impaired in its secretion presumably because of its inability to pass the ER protein quality control system. Further, treatment of the transgenic tobacco BY-2 cells with the ABX and NN-DNJ promoted the post-ER transport of N370S GCase, as shown by an increased amount of mutant protein in the media surrounding cultured BY-2 protoplasts. Thus the N370S GCase mutant protein is indeed capable of being ‘chaperoned’, which further suggest that the mutant protein is somehow impaired in its ability to obtain a functional folded conformation. The steady-state levels of mutant proteins inside the plant cells were also increased in the presence of these chemicals, suggestive of an increase in the concentration of folded mutant GCase in the ER, and/or in transit to the cell surface via the secretory pathway. Similar to the findings of others, there was additionally a significant impairment of the catalytic activity of the recombinant N370S GCase.

2.3. Materials and Methods

2.3.1. Generation of constructs for production of WT and mutant GCase proteins in tobacco BY-2 cells

The N370S mutation was engineered into the WT cDNA (*GBA1*) clone using the Quick Change site-directed mutagenesis kit (Stratagene, Santa Clara, USA). To allow for secretion of mutant and WT GCase into the culture medium, a sequence encoding the signal peptide of proaleurain (MAHARVLLLALAVLATAAVAVA) was fused to the coding sequences specifying the WT and mutant GCase using the following pair of primers: 5'-GCTCGCCGTCCTGGCCACGGCCGCCGTCGCCGTCGCCGCCCGCCCCTGCATCCC TAAAAG-3' (GBA-SPp-F1), 5'-CACCATGGCCCACGCCCGCGTCC TCCTCCTGGCGCTCGCCGTCCTGGCCACGG-3' (GBA-SPp-F2), and 5'-TCACTGGCGATGCCACAGGTAGG-3' (GBA-SPp-R). Re-amplified cDNA products were cloned into a Gateway entry vector pENTR/D-TOPO (Invitrogen, Burlington, Canada). The nucleotide sequences were then determined using the M13 reverse and forward universal primers. The resulting constructs containing sequences encoding WT and N370S GCase were sub-cloned into a Gateway expression vector (pSITE-0B) (Chakrabarty et al. 2007), which carries the neomycin phosphotransferase II gene (for kanamycin resistance) and the cauliflower mosaic virus (CaMV) 35S promoter (with a duplicated enhancer element; 2X35S) to drive expression of the WT and mutant genes.

2.3.2. Transformation of tobacco BY-2 cells

The expression constructs for production of WT-GCase and N370S-GCase were introduced into *Agrobacterium tumefaciens* (GV 3101) by electroporation. Transformed *Agrobacterium* cells were selected and used to transform tobacco BY-2 cells. Transformed tobacco BY-2 cells were transferred onto solid Murashige and Skoog (MS) media (Sigma, St. Louis, MO) containing kanamycin (100 µg/mL) and cefotaxime (300 µg/mL) and incubated at 28 °C for 3 to 4 weeks until transformed colonies were formed (Mayo et al. 2006; Fu et al. 2009). Resistant cell colonies (at least 30 per construct) were maintained on solid MS culture medium and sub-cultured twice a week.

2.3.3. Northern blot analyses of transgenic BY-2 lines

Total RNA was extracted (from 13 individual lines per construct) using Trizol reagent following the manufacturer's instructions (Invitrogen). Bulk RNA samples (15 µg) were separated on formaldehyde-denaturing agarose gels (1.2%, w/v) and blotted onto positively charged nylon membranes as described in Sambrook et al. (Sambrook et al. 1989). RNA was fixed to the nylon membranes and the blots were pre-hybridized in 10 mL hybridization solution (100 mM NaH_2PO_4, 50 mM $Na_2P_2O_7$, 1 mM EDTA and 7% SDS) for 2 h at 65 °C with hybridization proceeding in the presence of a radiolabelled full-length cDNA probe for human *GBA1* at 65 °C overnight. Following hybridization, the blot was washed three times in 2X SSC, 0.1% SDS at room temperature, twice in 1X SSC, 0.1% SDS at 65 °C, and once in 0.5X SSC, 0.1% SDS at 65 °C. The blot was exposed to X-ray film (Kodak, Rochester, USA) and developed after 1 h.

2.3.4. Protein isolation from tobacco BY2 cells grown on solid media and from BY2 protoplasts

BY2 cells (100 mg) grown on solid media were ground on ice in a 1.5-mL microcentrifuge tube using glass beads in 100 µL GCase extraction buffer (50 mM sodium phosphate pH 6.0, 50 mM NaCl, 0.1% sodium taurocholic acid, 1 mM EDTA, 0.5 mM PMSF). After centrifugation at 14 000 ***g*** for 15 min at 4 °C, the supernatant was removed and transferred to a new microcentrifuge tube. To isolate protein from BY2 protoplasts, protoplasts (2 X 10^5) were vortexed in the presence of glass beads in 100 µL GCase extraction buffer. Cell debris was removed by centrifugation at 14000 ***g*** for 15 min at 4 °C and the supernatant used for western blot analysis. Protein concentrations were determined using the Bio-Rad DC™ (Bio-Rad Laboratories, Missasauga, Canada) protein assay kit and BSA as a standard.

2.3.5. Immunoprecipitation of GCase and GCase activity assays

GCase proteins (WT and N370S) were immunoprecipitated using a mouse polyclonal anti-GCase antibody (Novus Biologicals, Oakville, Canada) prior to GCase activity assays. Ten µg of anti-GCase antibody was incubated with protein G sepharose beads (BioVision, Mountain View, USA) in PBS pH 7.4, containing 0.02% Tween-20 for

1 h at 4 ºC with rotation. Beads were plated and incubated with 100 µg of total soluble protein extracts from WT- and N370S-GCase (high expressing lines) for 1 h at 4 ºC. Following three washes (5 min each) in wash buffer (PBS pH 7.4 containing 0.5% Tween-20), the beads were re-suspended in 25 µL citrate phosphate buffer (200 mM, pH 5.5). GCase assays were initiated by adding buffer comprised of citrate phosphate (200 mM, pH 5.5), 0.4% taurocholic acid and 10 mM substrate (4-MUGP) to each sample and incubating at 37 ºC for 1 h. The assay was terminated by increasing the pH to 10.5 by adding 200 µL of 0.1 M 2-amino-2-methyl-1-propanol (Sigma, Oakville, Canada). GCase activities were determine by the rate of release of the fluorescent product 4-methylumbelliferone (4-MU) monitored by a Spectramax M2e (Molecular Devices Corp, Sunnyvale, USA) fluorometer (excitation, 360 nm; emission, 450 nm). One unit of enzyme activity was defined as 1 nmol of 4-MU produced per minute. To demonstrate the efficiency of the immunocapture step (particularly its equivalence for the WT versus the N370S GCase), protein G beads were boiled for 5 min in 30 µL of SDS sample loading buffer to elute the captured GCase. Similar amounts of eluted proteins (WT and N370S GCase) were fractionated on 12% SDS-PAGE followed by western blot using a mouse monoclonal anti-GCase 2E2 antibody (Novus Biologicals, Oakville, Canada).

2.3.6. Western blot analyses

Protein extracts from BY2 transgenic lines (10 µg total soluble protein) were separated by 12% SDS-PAGE and transferred onto Amersham Hybond-P (PVDF) membranes. The blots were blocked overnight at room temperature using 5% (w/v) non-fat dry milk in 10 mM Na_2HPO_4, 150 mM NaCl, and 0.1% (v/v) Tween-20 (PBST) followed by 3 washes (15 min each) with PBST. Blots were incubated with the primary, polyclonal anti-GCase antibodies (1: 5,000 dilution) for 3 h at room temperature. After 3 washes with PBST (15 min each) the membrane was incubated with an anti-rabbit HRP (horseradish peroxidase) secondary antibody (1:10,000) for 1 h at room temperature. An enhanced chemiluminescence (ECL) advance western blotting kit (GE Healthcare, Quebec, Canada) was used to detect WT- and N370S- GCase. The same blot was then stripped with guanidine hydrochloride stripping buffer (6 M GnHCl, 0.2% Nonidet (NP-40), 0.1 M β-Mercaptoethanol, 20 mM Tris-HCl, pH 7.5) for 10 min (Yeung and Stanley

2009) and re-probed with an anti-tubulin or an anti-GAPDH (plant cytosolic NAD-specific glyceraldehyde-3-phosphate dehydrogenase) primary antibody as a loading control.

2.3.7. Protoplast isolation from tobacco BY2 solid cultures

Tobacco BY2 cells were grown on solid MS medium for 10 d. Ten g of cells were incubated in 25 mL of enzyme solution (1.2% cellulase "Onozuka RS", 0.6% Macerozyme R-10 (Yakult Pharmaceutical Ind. Co. Ltd., Tokyo, Japan), 0.5 M mannitol, 10 mM $CaCl_2$, 12 mM NaOAc) overnight at 28 °C on a rotary shaker at 40 rpm. Protoplasts were filtered through a 40-µm mesh (Sigma) and centrifuged in a 50 ml conical tube at 200 ***g*** for 5 min. The supernatant was discarded and protoplasts were washed with W5 solution (154 mM NaCl, 125 mM $CaCl_2$, 5 mM KCl, 2 mM MES, pH 5.7) at 200 ***g*** for 5 min. Protoplasts were collected and re-suspended in protoplast culture medium (4.3 g/L MS salts, 100mg/L myo-inositol, 1 mg/L thiamine, 0.2 mg/L 2,4,D (2,4-dichlorophenoxyacetic acid), 255 mg/L KH_2PO_4, 30 g/L sucrose, pH 5.7) (Lee et al. 2008). The number of protoplasts was estimated by counting with a haemocytometer (Hausser Scientific, Horsham, USA). Viability of protoplasts was determined with 0.02% solution of Evans blue (Sigma) prepared in protoplast culture medium.

2.3.8. Precipitation of secreted protein from the medium of cultured BY2 protoplasts

For protein precipitation from the cultured media of protoplasts, the culture medium was mixed with chilled acetone in a ratio of 1:5 with gentle shaking and incubated at -20 °C for 1 h, followed by centrifugation at 10,000 ***g*** for 30 min. The resulting precipitates were re-suspended in GCase extraction buffer and fractionated by SDS-PAGE, followed by western blot analysis as previously described.

2.3.9. Determination of IC50 values of ambroxol for human WT- and N370S- GCase at pH 5.5 and 7.0

The inhibitory activity of ambroxol (ABX) against WT- and N370S- GCase was evaluated using either purified human GCase (Cerezyme™, Genzyme, USA) or GCase bearing the N370S mutation present in lysates of Gaucher disease patient fibroblasts

homozygous for mutation, c.1226A3/c.1226A3G (Hospital for Sick Children, Tissue Culture Facility, Toronto, ON), as described previously (Maegawa et al. 2008). Lysates of Gaucher disease patient fibroblasts from a confluent 175 cm^2 tissue culture flask were prepared by 10 freeze-thaw cycles in 10 mM sodium phosphate (pH 6.5), 5% glycerol (100 µL) and then diluted 3-fold in 100 mM citrate phosphate buffer pH 5.5 or 7.0 containing 0.4% taurodeoxycholate. An aliquot (25 µL) of patient lysate or purified Cerezyme™ containing the same number of units was used to determine the IC_{50} of ABX in 100 mM citrate-phosphate buffer, 0.2% taurodeoxychlate and 2.5 mM 4-methylumbelliferyl-β-D-glucopyranoside (4-MUGP) according to Maegawa et al. 2009.

2.4. Results

2.4.1. Steady-state levels of transcripts and protein (WT-GCase and N370S-GCase) in tobacco BY2 cell lines are variable

Tobacco BY2 cells were stably transformed with the GCase gene constructs (Figure 2-1) and regenerated lines were screened by PCR. Northern blot analysis of the PCR-positive transgenic cell lines showed that the steady-state level of transcripts corresponding to both WT- and N370-GCase was variable amongst the population of lines in which abundant transcript was detected (Figure 2-2 A, B). Of thirty lines screened (for WT-GCase and N370S GCase), all were PCR positive, and about 25% and 30% of the lines expressed abundant transcripts corresponding to N370S and WT GCase, respectively. Western blot analysis of selected high-expressing lines transgenic for WT- and mutant- GCase (6 lines per construct) detected variable levels of an ~ 60 kDa protein (Figure 2-2 A, B). In general N370S protein levels were equivalent or slightly lower than those of WT-GCase in individual transgenic lines, particularly in light of the steady-state levels of transcripts in these lines (Figure 2-2 A, B).

2.4.2. Plant recombinant N370S-GCase is catalytically impaired

GCase activities of WT- and N370S- transgenic lines were determined using the same lines selected for western blot analyses (Figure 2-2 A, B). In the tobacco BY-2 cells there are several endogenous plant β-glucosidases with activities that interfere with

detection of that corresponding to the bonafide (i.e. human) GCase. Therefore the WT and N370S GCase proteins were immunoprecipitated with a mouse polyclonal anti-GCase antibody prior to determination of GCase activities. Untransformed tobacco BY2 cells served as the control for immunocaptured GCase activity; these values (0.05 nmol/mg protein/min) were subtracted from the values presented in Figure 2-2. Purified WT-GCase immunocaptured from the selected transgenic lines exhibited specific activity levels of 0.51-1.36 units per mg total soluble protein (TSP) (Figure 2-2 A). The purified N370S GCase enzymes were catalytically active, but exhibited much lower specific activities as compared to the WT-GCase enzymes, which ranged from 0.024 to 0.24 units per mg protein (Figure 2-2 B). Western blot analyses confirmed that there was equal immunocapture of the WT GCase and the mutant GCase prior to the enzyme activity determinations (Figure 2-2 C).

Of the various lines expressing GCase, N370S line 21 and GCase line 60 were selected for further analyses to examine the effects of pharmacological chaperones (Sections 3.4 and 3.5). For verification of general trends, the same analyses were conducted on an additional N370S GCase line (line 24).

2.4.3. Confirmation that ambroxol is an effective active-site directed inhibitor of N370S GCase

As compared to the WT GCase, the N370S mutation has been reported to increase the Kiapp of some active-site directed inhibitors by ~4-fold (Liou and Grabowski 2009). To determine if ambroxol (ABX) behaved in a manner consistent with it being an active-site directed pharmacological chaperone for the N370S GCase, we compared the IC50s of ABX at neutral and acidic pH, against purified commercial GCase (Cerezyme) and the N370S GCase from the lysate of a patient fibroblast cell line homozygous for the mutation (Figure 2-3). At the acidic pH of 5.5 the IC50 of ABX for the WT GCase was 26 μM, as compared to 58 μM for the N370S GCase (~2-fold increase). Shifting to pH 7.0, the neutral pH of the ER, the IC50 shift between the two forms of the enzyme was only barely significant, 1.1-fold. However the magnitude of the decrease in IC50s caused by the increased pH was ~5-fold for the WT, 5.7 μM, and ~9-fold for the N370S, 6.4 μM (Figure 2-3 A). Thus, at the neutral pH of the ER, ABX binds

with similar strength to either the WT GCase or the N370S mutant. Additionally its pH-dependent advantage as a pharmacological chaperone, i.e. tighter binding at neutral pH than at acidic pH, is even more pronounced with the mutant.

It could be argued that undegraded glucocerebrosides present in the Gaucher disease fibroblast lysates may interefere with the IC50 determinations in Fig. 3 A, as they could bind to the catalytic site of mutant GCase. To confirm that this was not the case, the lysate from a L444P homozygous fibroblast line, containing ~3% of normal endogenous GCase activity, was spiked with a small amount of Cerezyme, such that its specific activity resembled that of the N370S homozygous cell lysate (~20% of normal). This was followed by construction of an IC50 curve with ABX; notably the two curves corresponding to the spiked lysate versus pure Cerezyme were superimposable (Figure 2-3 B). Thus if stored glucocerebrosides exist in the lysate of Gaucher cells, they are insufficient to affect the GCase assay that uses saturating levels of an artificial substrate (Figure 2-3 B).

2.4.4. Pharmacological chaperones facilitate the post-ER secretion of N370S GCase into the surrounding media of transgenic protoplasts

To standardize cell numbers and allow for secretion studies, protoplasts (plant cells without their surrounding cell walls) were isolated from the tobacco BY2 transgenic cells. The viability of protoplasts and the secretion of WT GCase into the medium was determined daily from days 1-5. Day 2 and day 3 protoplasts retained reasonable viability (~80%), and further exhibited efficient protein secretion, and so these time points were selected as optimum incubation times for subsequent experiments.

To determine whether pharmacological chaperones facilitate the post-ER transport of mutant GCase, we examined the effects of the previously characterized chaperones, NN-DNJ and ABX, on the secretion of GCase into the culture medium surrounding the transgenic protoplasts. In contrast to the WT-GCase protein, which was efficiently secreted from tobacco BY2 cells, and detected in large amounts in the culture medium, only a small proportion of the N370S GCase was secreted in the absence of pharmacological chaperone (Figure 2-4 A and B; 0 μM ABX and 0 μM NN-DNJ).

Treatment of N370S-GCase transgenic protoplasts with ABX or NN-DNJ promoted the secretion of N370S mutant protein into the surrounding media, with the maximum amounts of GCase protein present in the culture medium as a result of the 30 μM ABX (3-d) treatment and the 20 μM NN-DNJ (2-d) treatment. Interestingly ABX and NN-DNJ also mediated an increase in the secretion of WT GCase into the culture media (e.g. when applied at 40-50 μM and at 10 μM, respectively). These results clearly indicate that the pharmacological chaperones enhanced the amount of N370S GCase that is transported beyond the ER along the secretory pathway.

The inclusion of a protease inhibitor cocktail in the protoplast culture medium during incubation had no effect on the stability of the secreted GCase (data not shown).

2.4.5. Pharmacological chaperones increase the accumulation of N370S GCase protein inside protoplasts

The positive effect of pharmacological chaperones on facilitating the secretion of mutant GCase is presumably mediated through facilitating protein folding in the ER. We further sought to determine whether these compounds stabilize the mutant protein inside the protoplasts. The 3-d ABX-treated and 2-d NN-DNJ-treated protoplasts showed significantly higher levels of N370S GCase protein as compared with untreated controls; a positive and similar effect was noted across the range of concentrations of the two pharmacological chaperones that were tested (Figure 2-5 A). Interestingly, significant cytotoxicity was observed when the plant cell protoplasts were treated with high concentrations of the chemicals (>30 μM NN-DNJ and > 60 μM ABX) (data not shown).

Treatment with the chemical chaperones had no significant effect on the steady-state intracellular protein levels of WT GCase in ABX- and NN-DNJ-treated protoplasts (Figure 2-5 B).

2.5. Discussion

Gaucher disease is a prevalent lysosomal storage disease that affects 1 in 60,000 people in the general population; its incidence is greater in the Ashkenazi Jewish

population, in which it affects 1 in 800 people. One of the most prevalent disease-causing mutations in human *GBA1*, the gene encoding lysosomal GCase, is a missense mutation, N370S. There are two views currently in the literature concerning the function and fate/homeostasis of this mutant enzyme. One view is that the N370S mutant GCase possesses reduced catalytic activity, but is able to fold normally; affected cells exhibit normal steady-state GCase protein levels. This view is partly based on the comparative analysis of the crystal structure of an insect recombinant human N370S GCase as compared to the WT protein. These authors also assert that the major mechanism by which pharmacological chaperones enhance lysosomal levels of N370S GCase is by reducing its degradation within the lysosome rather than by assisting the mutant protein with folding in the ER, a process which facilitates its subsequent trafficking out of this compartment (Wei et al. 2011). The other camp contends that the N370S GCase protein is compromised in its folding in the ER, making it subject to ERAD, instead of undergoing proper folding and trafficking to the lysosome (Bergmann and Grabowski 1989; Schmitz et al. 2005; Sawkar et al. 2005; Steet et al. 2006). Pharmacologic chaperones, such as NN-DNJ and isofagomine, are thought to be effective as *enzyme enhancement therapies* for this disease-causing mutation because they stabilize the folded and near-folded forms of the N370S enzymes in the ER. This in turn increases the population of folded mutant GCase enzymes that can pass ER quality control and undergo subsequent trafficking to the lysosome. The enzyme becomes active upon its arrival in the lysosome because the small molecule is replaced (out-competed) by the large accumulation of the natural GCase substrate (glucosylceramide); displacement of the pharmacological chaperone may also be facilitated by the low pH of this organelle. For example, ABX loses its ability to inhibit the enzyme below pH 4.7 (Maegawa et al. 2009). In order to confirm that the interaction of ABX with the N370S form of GCase was comparable to that of the WT GCase, we determined the IC50s of each enzyme at pH 5.5 and 7.7. At pH 5.5 the binding of ABX to the WT enzyme was ~2-fold greater than the N370S mutant form. However, at pH 7.0, near the pH of the ER, the IC50s of the two forms of the enzyme were nearly identical.

To rule out the possibility of chaperone-mediated lysosomal stabilization of mutant GCase, we generated the N370S GCase and its WT counterpart in transgenic tobacco BY2 cells, eukaryotic cells in which lysosomes do not exist. To allow for efficient

secretion of the WT and mutant recombinant human proteins in the plant host (Hiatt et al. 1989; Hein et al. 1991), the constructs utilized sequences specifying the signal peptide of the barley cysteine protease proaleurain (Tse et al. 2004) in place of those encoding the native signal peptide. As no additional targeting information was added, the recombinant proteins (if transport-competent) are expected to transit along the secretory pathway to the plant cell surface by a default mechanism.

We were most interested in the N370S GCase transgenic lines that exhibited robust levels of steady-state GCase transcripts; for several of the lines the transcript levels approximated those of the WT-GCase lines. Western blot detection of GCase protein in total soluble protein (TSP) extracts from these lines (10 µg TSP) indicated that GCase protein levels associated with the mutant GCase lines were generally equivalent or slightly lower than those of the WT lines, but to a surprisingly little extent. Because we did not examine the rate of turnover/degradation of the two proteins we cannot state that the N370S-GCase and normal GCase protein are equally stable. Certainly there was considerable variability across all lines (regardless of the GCase construct) in terms of the transcript to protein ratios. A comparative analysis of the activities of the recombinant N370S versus WT GCase (in GCase-immunopurified extracts of BY-2 cells), revealed that the N370S GCase is catalytically impaired, with the specific activities associated with the N370S transgenic lines significantly lower than those of the WT-GCase lines. In transfected NIH 3T3 (mouse embryonic fibroblast) cells, transcript and protein levels corresponding to the N370S GCase versus the WT-GCase are comparable (Ohashi et al. 1991), although this work presented only a single transfected 3T3 cell line. The N370S GCase of 3T3 cells exhibits reduced catalytic activity (Ohashi et al. 1991) similar to the mutant protein generated in Sf9 insect cells, with the specific activities in both hosts being ~ 10-20% of those associated with the recombinant WT-GCase Grace et al 1990, 1994). Thus in many ways our findings parallel those of others and further support the contention that catalytic deficiency (impaired function) is likely a contributing factor underlying Gaucher disease pathophysiology in N370S/N370S patients.

Our findings of the effects of two pharmacological chaperones, NN-DNJ and ABX, show that both of these small molecules facilitate the post-ER trafficking of N370S GCase, resulting in enhanced secretion of the mutant protein into the culture medium of

the transgenic protoplasts. This effect likely occurs through the pharmacological chaperone increasing the proportion of stabilized (folded and near-folded) forms of the N370S enzymes in the ER; a greater proportion of the synthesized enzymes are able to avoid ERAD and to pass the quality control systems of the ER. Thus these transport-competent enzymes undergo subsequent trafficking along the endomembrane system, which in the plant heterologous host cells leads to secretion. In the absence of pharmacological chaperone, only a small proportion of the mutant protein was secreted by the cultured protoplasts, in contrast to the WT-GCase, which was efficiently secreted. Impaired lysosomal trafficking and slower transport of mutant GCase as compared to the WT-GCase is evident in cultured fibroblast cells from two patients homozygous for the N370S mutation (Schmitz et al. 2005). More compelling evidence comes from subcellular localization studies of GCase in N370S/N370S fibroblasts, in which the mutant GCase co-localizes with protein disulfide isomerase, a marker of the ER lumen. The pharmacological chaperone ABX (in which cells are treated with 40 μM ABX for 5 d) leads to co-localization of the N370S GCase with a lysosomal marker protein, and a corresponding reduction in its ER localization, indicative of transport of the mutant GCase from the ER to lysosomes (Maegawa et al. 2009).

ABX enhances the GCase activity of deficient (patient) fibroblasts when applied at 5-60 μM (Maegawa et al. 2009). The concentrations effective in promoting secretion of N370S GCase from the plant protoplasts were 10-60 μM, with 30 μM showing the greatest effect. NN-DNJ is most effective in enhancing the GCase activity of mutant fibroblasts when applied at concentrations below 30 μM; a treatment of 10 μM for 9 d leads to a 2-fold increase in the GCase activity in these cells (Sawkar et al. 2002). Similarly, in the present study, enhanced secretion of the N370S GCase from cultured protoplasts occurred as a result of treatments with 10-30 μM NN-DNJ, with 20 μM appearing to be the most effective concentration.

ABX and NN-DNJ not only facilitated secretion of N370S GCase from protoplasts, but further increased the steady-state levels of intracellular GCase in these cells. This could be the result of a stabilization of N370S GCase through direct interactions, since these pharmacological chaperones are active site-specific inhibitors. These direct interactions likely stabilize the folded and partly folded mutant GCase

enzymes in the ER, allowing a greater proportion of the synthesized protein to escape ERAD (Sawkar et al. 2006; Steet et al. 2006; Yu et al. 2007). Upon passing the ER quality control mechanisms the proteins are able to traffic out of the ER to the cell surface via transit through the secretory pathway.

2.6. Conclusions

Our present work provides evidence that N370S GCase is a folding mutant and is impaired in its ability to exit the lumen of the ER. Further, N370S GCase is capable of being 'chaperoned'.

Acknowledgements

We thank G. Grabowski (University of Cincinnati) for generously providing the cDNA clone of *GBA1*. We are also grateful to Ms. Sayuri Yonekawa for her technical assistance in generating the data of Figure 3. This work was supported by Natural Sciences and Engineering Research Council of Canada (NSERC) Strategic and Michael Smith Foundation for Health Research grants awarded to A.R.K.

2.7. References

Bergmann JE, Grabowski GA. Posttranslational processing of human lysosomal acid beta-glucosidase: A continuum of defects in gaucher disease type 1 and type 2 fibroblasts. Am J Hum Genet 1989; 44:741-750.

Chakrabarty R, Banerjee R, Chung SM, Farman M, Citovsky V, Hogenhout SA, Tzfira T, Goodin M. PSITE vectors for stable integration or transient expression of autofluorescent protein fusions in plants: Probing nicotiana benthamiana-virus interactions. Mol Plant Microbe Interact 2007; 20:740-750.

Denecke J, Botterman J, Deblaere R. Protein secretion in plant cells can occur via a default pathway. Plant Cell 1990; 2:51-59.

Fan JQ. A counterintuitive approach to treat enzyme deficiencies: Use of enzyme inhibitors for restoring mutant enzyme activity. Biol Chem 2008; 389:1-11.

Fu LH, Miao Y, Lo SW, Seto TC, Sun SSM., Xu ZF, Clemens S, Clarke LA, Kermode AR, Jiang L. Production and characterization of soluble human lysosomal enzyme α-iduronidase with high activity from culture media of transgenic tobacco BY-2 cells, Plant Sci 2009; 177: 668-675.

Grace ME, Graves PN, Smith FI, Grabowski GA. Analyses of catalytic activity and inhibitor binding of human acid beta-glucosidase by site-directed mutagenesis. identification of residues critical to catalysis and evidence for causality of two Ashkenazi Jewish Gaucher disease type 1 mutations. J Biol Chem 1990; 265:6827-6835.

Grace ME, Newman KM, Scheinker V, Berg-Fussman A, Grabowski GA. Analysis of human acid beta-glucosidase by site-directed mutagenesis and heterologous expression. J Biol Chem 1994; 269:2283-2291.

Hein MB, Tang Y, McLeod DA, Janda KD, Hiatt A. Evaluation of immunoglobulins from plant cells, Biotechnol. Prog. 1991; 7; 455-461.

Hiatt A, Cafferkey R, Bowdish K. Production of antibodies in transgenic plants, Nature 1989; 342: 76-78.

Hruska KS, LaMarca ME, Scott CR, Sidransky E. Gaucher disease: Mutation and polymorphism spectrum in the glucocerebrosidase gene (GBA). Hum Mutat 2008; 29:567-583.

Lee LY, Fang MJ, Kuang LY, Gelvin SB. Vectors for multi-color bimolecular fluorescence complementation to investigate protein-protein interactions in living plant cells. Plant Methods 2008; 4:24-4811-4-24.

Liou B, Grabowski GA. Participation of asparagine 370 and glutamine 235 in the catalysis by acid beta-glucosidase: The enzyme deficient in gaucher disease. Mol Genet Metab 2009; 97:65-74.

Liu JX, Howell SH. Endoplasmic reticulum protein quality control and its relationship to environmental stress responses in plants. Plant Cell 2010; 22:2930-2942.

Maegawa GH, Tropak MB, Buttner JD, Rigat BA, Fuller M, Pandit D, Tang L, Kornhaber GJ, Hamuro Y, Clarke JT, Mahuran DJ. Identification and characterization of ambroxol as an enzyme enhancement agent for gaucher disease. J Biol Chem 2009; 284:23502-23516.

Mayo KJ, Gonzales BJ, Mason HS. Genetic transformation of tobacco NT1 cells with agrobacterium tumefaciens. Nat Protoc 2006; 1:1105-1111.

Nagy JK, Sanders CR. Destabilizing mutations promote membrane protein misfolding. Biochemistry 2004; 43:19-25.

Ohashi T, Hong CM, Weiler S, Tomich JM, Aerts JM, Tager JM, Barranger JA. Characterization of human glucocerebrosidase from different mutant alleles. J Biol Chem 1991; 266:3661-3667.

Sambrook J, Fritsch EF, Maniatis T. Molecular cloning. A laboratory manual, second ed., Cold Spring Harbor Laboratory Press, New York, 1989.

Sawkar AR, Adamski-Werner SL, Cheng WC, Wong CH, Beutler E, Zimmer KP, Kelly JW. Gaucher disease-associated glucocerebrosidases show mutation-dependent chemical chaperoning profiles. Chem Biol 2005; 12:1235-1244.

Sawkar AR, Cheng WC, Beutler E, Wong CH, Balch WE, Kelly JW. Chemical chaperones increase the cellular activity of N370S beta -glucosidase: A therapeutic strategy for gaucher disease. Proc Natl Acad Sci U S A 2002; 99:15428-15433.

Sawkar AR, Schmitz M, Zimmer KP, Reczek D, Edmunds T, Balch WE, Kelly JW. Chemical chaperones and permissive temperatures alter localization of gaucher disease associated glucocerebrosidase variants. ACS Chem Biol 2006; 1:235-251.

Schmitz M, Alfalah M, Aerts JM, Naim HY, Zimmer KP. Impaired trafficking of mutants of lysosomal glucocerebrosidase in gaucher's disease. Int J Biochem Cell Biol 2005; 37:2310-2320.

Steet RA, Chung S, Wustman B, Powe A, Do H, Kornfeld SA. The iminosugar isofagomine increases the activity of N370S mutant acid beta-glucosidase in gaucher fibroblasts by several mechanisms. Proc Natl Acad Sci U S A 2006; 103:13813-13818.

Tropak MB, Kornhaber GJ, Rigat BA, Maegawa GH, Buttner JD, Blanchard JE, Murphy C, Tuske SJ, Coales SJ, Hamuro Y, Brown ED, Mahuran DJ. Identification of pharmacological chaperones for gaucher disease and characterization of their effects on beta-glucocerebrosidase by hydrogen/deuterium exchange mass spectrometry. Chembiochem 2008; 9:2650-2662.

Tse YC, Mo B, Hillmer S, Zhao M, Lo SW, Robinson DG, Jiang L. Identification of multivesicular bodies as prevacuolar compartments in nicotiana tabacum BY-2 cells. Plant Cell 2004; 16:672-693.

Wei RR, Hughes H, Boucher S, Bird JJ, Guziewicz N, Van Patten SM, Qiu H, Pan CQ, Edmunds T. X-ray and biochemical analysis of N370S mutant human acid beta-glucosidase. J Biol Chem 2011; 286:299-308.

Yeung YG, Stanley ER. A solution for stripping antibodies from polyvinylidene fluoride immunoblots for multiple reprobing. Anal Biochem 2009; 389:89-91.

Yu Z, Sawkar AR, Kelly JW. Pharmacologic chaperoning as a strategy to treat gaucher disease. FEBS J 2007; 274:4944-4950.

2.8. Figures

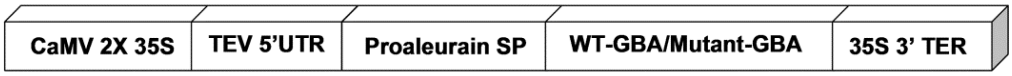


Figure 2-1: Expression constructs in pSITE-0B vector for generating recombinant WT and N370S GCase in tobacco BY-2 cells.

The 5' upstream regulatory sequences included a cauliflower mosaic virus (CaMV) 35S promoter with a duplicated enhancer element, and the translational enhancer element (5' UTR) from the tobacco etch virus (TEV). Both WT and N370S GCase constructs contained in-frame sequences encoding the proaleurain signal peptide, and a 3' end comprised of the CaMV 35S transcription terminator and associated downstream sequences. The *GBA1* coding sequences encoding WT or N370S mutant GCase are also shown.

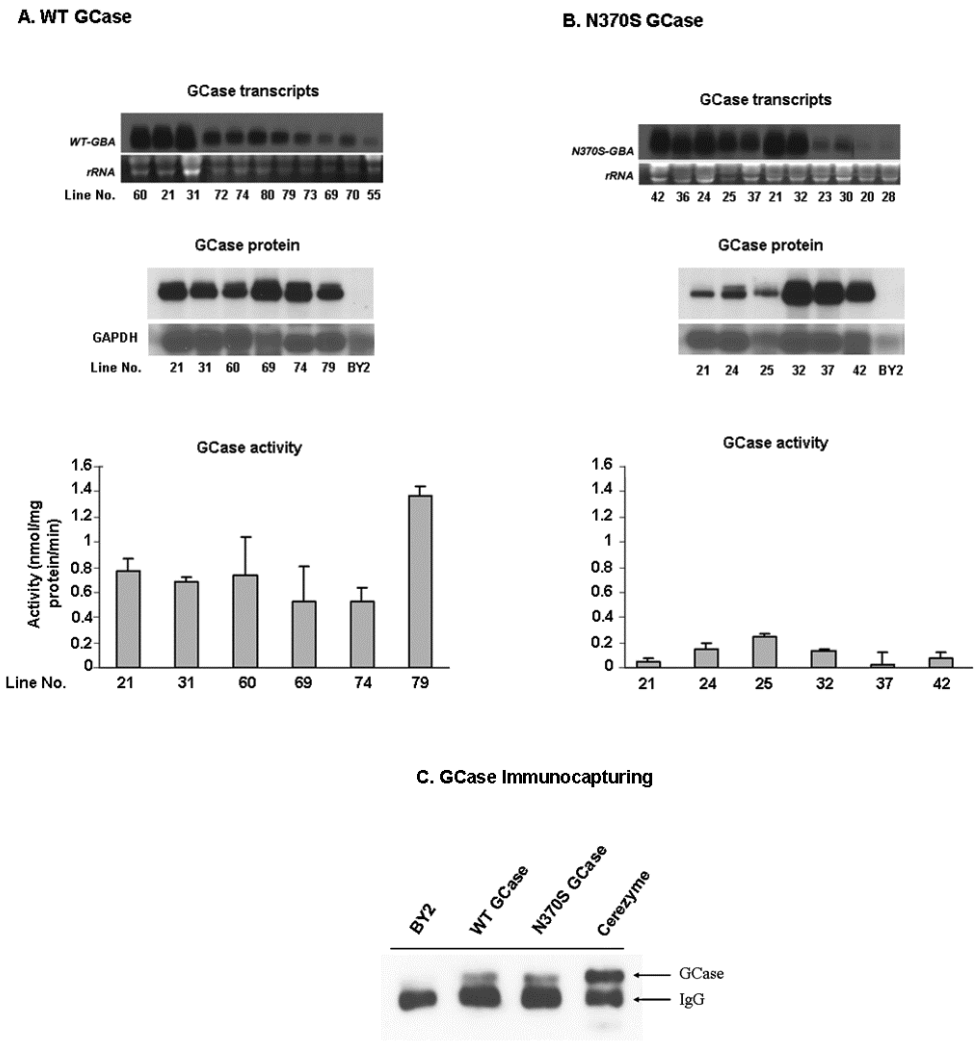


Figure 2-2: Steady-state GCase transcripts and protein levels and specific activities associated with independent transgenic BY2 tobacco lines expressing the WT (A) and N370S mutant (B) gene constructs.

Northern blot analyses (top panels) were based on total RNA (15 µg) isolated from 10-d-old individual transgenic lines. A radiolabeled full-length cDNA probe was used to detect the GCase transcripts. Ethidium bromide staining of ribosomal RNA is shown at the bottom. For western blot analysis (middle panels), total soluble protein (10 µg) from 6 selected lines was fractionated by 12% SDS-PAGE, prior to analysis using a polyclonal anti-GCase antibody. The marker protein GAPDH (NAD-specific glyceraldehyde-3-phosphate dehydrogenase) was used as a control for loading on the same membranes following stripping of membranes and incubation with GAPDH antibodies. Western blots were repeated three times with similar results. For determination of GCase specific activities (lower panels), GCase of total soluble extracts from the selected lines was immunocaptured using protein G beads and a polyclonal mouse anti-GCase antibody. The activity of immobilized GCase on the protein G beads was determined by GCase assays; enzyme specific activities are expressed as nmoles/mg/min of total soluble protein. Untransformed tobacco BY2 cells served as the control for immunocaptured GCase activity; these values (0.05 nmol/mg protein/min) were subtracted from the values presented. Error bars represent the SE derived from three independent experiments. (C) To ensure that there was an equal immunocapture of the WT GCase and the mutant GCase, purified proteins from representative lines (N370S line 21 and GCase line 60) were separately analysed on a western blot following the immunocapture step. Cerezyme and untransformed tobacco BY2 cells were used as positive and negative controls, respectively.

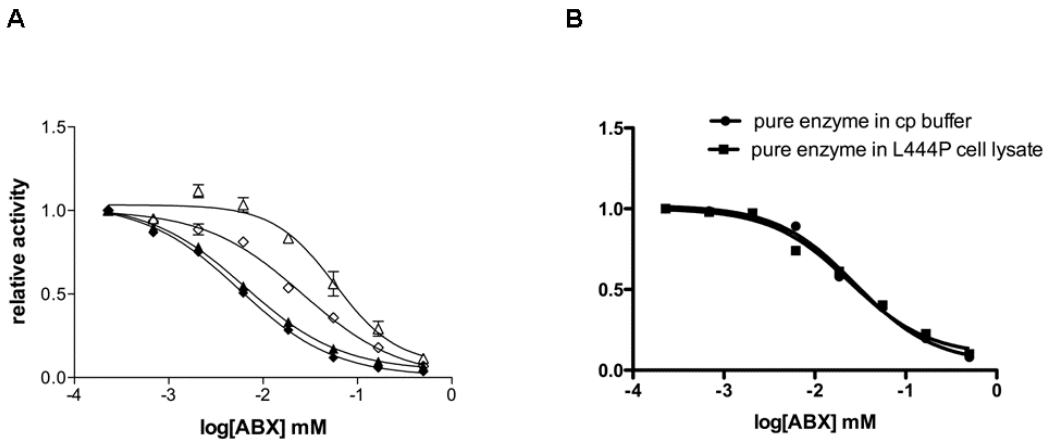


Figure 2-3: A. Dose response curves used to calculate IC50 values of ABX for the WT GCase (Cerezyme) and N370S GCase from a homozygous Gaucher patient fibroblast line.

Activities, relative to those obtained without ABX (set at 1.0 on the Y-axis) are plotted versus the log of the ABX concentration (X-axis) contained in the assay using 2.5 mM 4-MUGP. Triangles represent the values obtained from the N370S cell lysates and diamonds represent those from the WT GCase. Open triangles and diamonds correspond to data obtained at pH 5.5, solid markers to data obtained at pH 7.0. **B.** Control experiment to assess potential interference of any stored glucocerebrosides on the IC50 determinations for ABX of Gaucher disease fibroblast lysates. The lysate from a L444P homozygous fibroblast line was spiked with a small amount of Cerezyme, such that its specific activity resembled that of the N370S homozygous cell lysate. IC50 determinations were conducted and the curves of the spiked lysate versus the pure Cerezyme were compared.

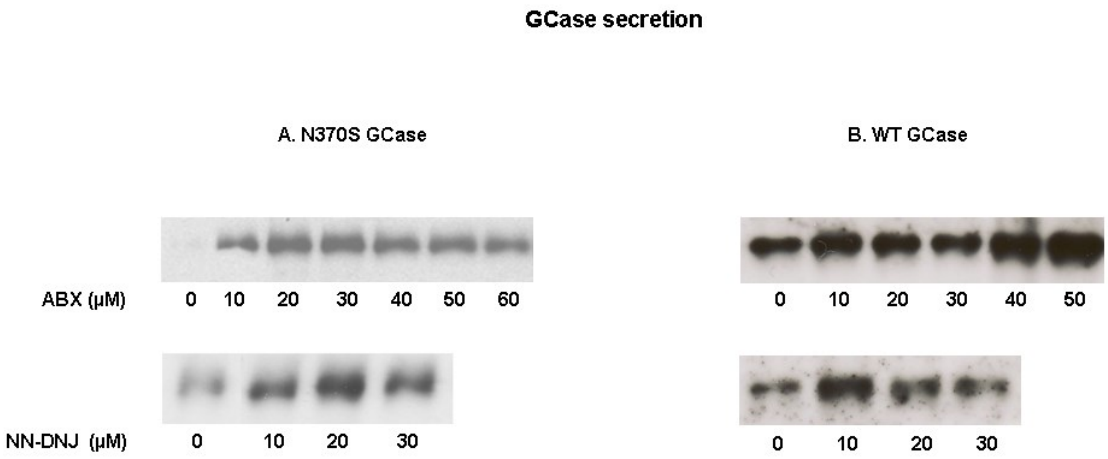


Figure 2-4: Effect of ABX and NN-DNJ on N370S GCase (A) and WT GCase (B) secretion from transgenic protoplasts.

Protoplasts (200,000 per mL) were incubated in culture medium with no pharmacological chaperone or in culture medium containing different concentrations of NN-DNJ or ABX (as indicated) for 2 and 3 days, respectively. Acetone-precipitated proteins from 0.5 mL media were subjected to western blot analyses. The experiment was repeated two times with similar results.

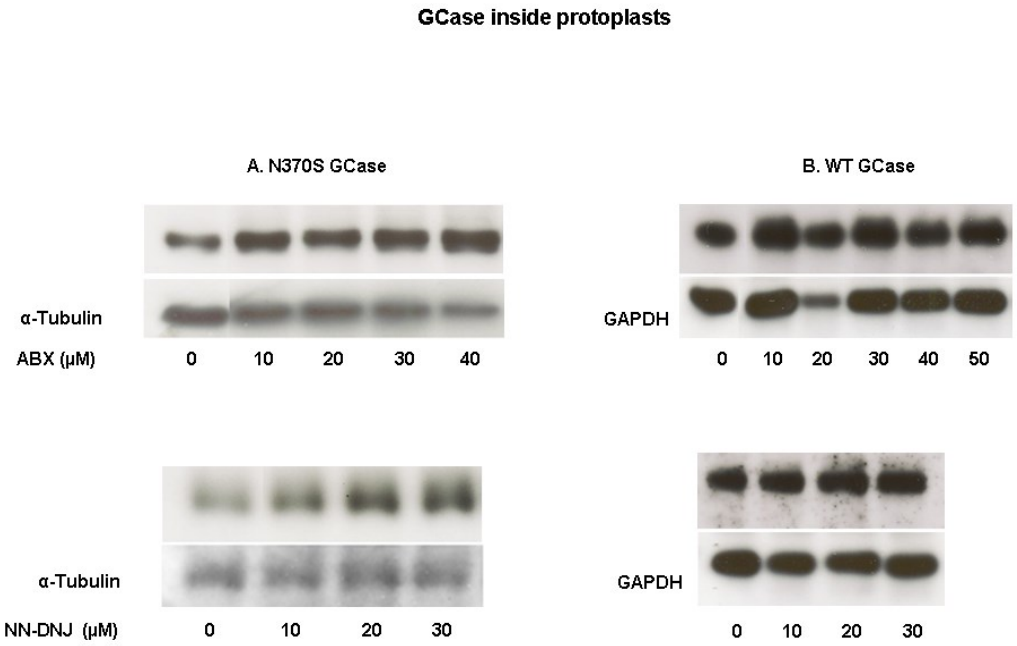


Figure 2-5: The effect of ABX and NN-DNJ on intracellular levels of N370S GCase (A) and WT GCase (B).

Protoplasts (200,000 per mL) were incubated in culture medium with no pharmacological chaperone or in culture medium containing different concentrations of NN-DNJ or ABX (as indicated) for 2 and 3 days, respectively, prior to western blot analysis. Extracts from the transgenic protoplasts were used to determine the intracellular levels of N370S GCase and WT GCase. The marker proteins α-tubulin or GAPDH (NAD-specific glyceraldehyde-3-phosphate dehydrogenase) were used as controls for loading on the same membranes following stripping of membranes and incubation with α-tubulin or GAPDH antibodies. This experiment was repeated two times with similar results.

Chapter 3.

Alteration of the proteostasis network of plant cells promotes the post-endoplasmic reticulum trafficking of recombinant mutant (L444P) human β-glucocerebrosidase

Results from this chapter were published: Babajani G. and Kermode A.R. (2014). Plant Signaling & Behavior 9, e28714.

3.1. Abstract

Gaucher disease is a prevalent lysosomal storage disease characterized by a deficiency in the activity of lysosomal acid β-glucosidase (glucocerebrosidase, GCase, EC 3.2.1.45). One of the most prevalent disease-causing mutations in humans is a L444P missense mutation in the GCase protein, which results in its disrupted folding in the endoplasmic reticulum (ER) and impaired post-ER trafficking. To determine whether the post-ER trafficking of this severely malfolded protein can be restored, we expressed the mutant L444P GCase as a recombinant protein in transgenic tobacco (*Nicotiana tabacum* L. cv Bright Yellow 2 [BY2]) cells, in which the GCase variant was equipped with a plant signal peptide to allow for secretion upon rescued trafficking out of the ER. The recombinant L444P mutant GCase was retained in the plant endoplasmic reticulum (ER). Kifunensine and Eeyarestatin I, both inhibitors of ER-associated degradation (ERAD), and the proteostasis regulators, celastrol and MG-132, increased the steady-state levels of the mutant protein inside the plant cells and further promoted the post-ER trafficking of L444P GCase, as indicated by endoglycosidase-H sensitivity- and secretion- analyses. Transcript profiling of genes encoding ER-molecular chaperones, ER stress responsive proteins, and cytoplasmic heat shock response proteins, revealed

insignificant or only very modest changes in response to the ERAD inhibitors and proteostasis regulators. An exception was the marked response to celastrol which reduced the steady-state levels of cytoplasmic HSP90 transcripts and protein. As HSP90 participates in the targeting of misfolded proteins to the proteasome pathway, its down-modulation in response to celastrol may partly account for the mechanism of improved homeostasis of L444P GCase mediated by this triterpene.

3.2. Introduction

Lysosomal storage diseases are a group of over 70 rare inherited diseases that are characterized by deficiencies of specific hydrolytic enzymes that reside in the lysosome. Consequently, the aberrant accumulation of macromolecules in lysosomes causes pathology by primary and secondary processes (Futerman and van Meer 2004; Ballabio and Gieselmann 2009). Gaucher disease is a prevalent lysosomal storage disease caused by a deficiency of lysosomal glucocerebrosidase (GCase) that results in pathological storage of glucosylceramide (Tropak et al. 2008). Almost 300 disease-causing mutations have been characterized in the *GBA1* gene encoding human GCase (Hruska et al. 2008). Some GCase variants carry missense mutations that destabilize the native structure of the GCase proteins and lead to their misfolding and endoplasmic reticulum-associated degradation (ERAD). ERAD plays a critical role in protein quality control by degrading unfolded and misfolded nascent proteins and involves retrotranslocation of the defective protein to the cytosol and its subsequent disposal by the cytosolic ubiquitin-proteasome machinery (Nagy et al. 2004; Schmitz et al. 2005). One of the most prevalent disease-causing mutations in humans is a L444P missense mutation in the GCase protein, which is linked to the neuronopathic form of the disease in homozygous patients as there is a complete loss of GCase activity (Wang et al. 2011). L444P GCase is severely destabilized due to its defective folding, and consequently it undergoes extensive ERAD (Grabowski 1997; Bendikov-Bar et al. 2011).

The use of pharmacological agents to correct the impairment in lysosomal trafficking of the disease-causing mutant proteins has recently been studied (Mu et al. 2008; Wang et al. 2011). This concept is based on the finding that a mutant protein may

be able to adopt a functionally competent conformation and be permitted to transit beyond the ER, thus passing the ER protein quality control system. Some of the efficacious small molecules include pharmacological chaperones, proteasome inhibitors and proteostasis regulators that “rescue” the GCase variants that otherwise would be unstable (Sawkar et al. 2002, 2005; Fan 2008; Mu et al. 2008; Bendikov-Bar et al. 2011). For instance, the pharmacological chaperones isofagomine and ambroxol are able to increase the lysosomal activity of L444P GCase (Khanna et al. 2010; Bendikov-Bar et al. 2011). Likewise the proteasome inhibitor MG-132 aids in stabilizing L444P GCase in patient-derived fibroblasts (Ron and Horowitz 2005). Augmenting the pool of mutant GCase in the ER is critical to enable its folding and post-ER trafficking (Wang et al. 2011). However, extensive ERAD and rapid clearance of mutant L444P GCase from the lumen of the ER reduces the pool of “restorable” GCase. ERAD inhibitors including kifunensine (Kif) and eeyarestatin I (EerI) increase the steady-state pool of L444P GCase in the ER lumen (Wang et al. 2011). Kif inhibits ER mannosidase I, a key component of the quality control mechanism that recognizes malfolded proteins (Fagioli and Sitia 2001; Avezov et al. 2008); EerI acts later in the pathway to inhibit the activity of p97 ATPase which plays a role in retro-translocation of misfolded substrates (Fiebiger et al. 2004; Wang et al. 2010). Treatment of patient-derived fibroblasts with EerI restored the folding and increased the lysosomal activity of the L444P GCase variant; however, the cells responded by inducing genes of the “unfolded protein response” (UPR), and exhibited cytotoxicity and apoptosis. Kif on the other hand was less efficient at “salvaging” L444P GCase, but mediated minimal UPR activation and had no effect on apoptosis (Wang et al. 2011).

Similar to the modulation of ERAD by chemicals such as Kif and EerI, modulation of the proteostasis network by proteostasis regulators has been explored as a therapeutic approach for the treatment of a variety of protein conformational diseases including neuronopathic subtypes of Gaucher disease (Balch et al. 2008; Mu et al. 2008; Hutt et al. 2009; Calamini et al. 2012). Agents such as celastrol, an inducer of the heat-shock response (HSR) and an inhibitor of the chymotrypsin-like activity of the proteasome (Yang et al. 2006; Mu et al. 2008), and MG-132, a well known inhibitor of 26S proteasome activity, have been used for this endeavor (Mu et al. 2008). The components of the proteostasis network include the UPR, HSR, and Ca^{2+} sensing and

inflammatory pathways (Hutt et al. 2009). These components operate in conjunction with the ubiquitin-proteasome system, and the lysosomal and autophagic degradation pathways to monitor and control the protein folding environment in different cell-types (Balch et al. 2008).

Many components of protein quality control are conserved in eukaryotic organisms from plants to humans. This includes ER quality control mechanisms, including the ER molecular chaperones that aid in the folding and assembly of nascent polypeptides, and recognize and retain malfolded proteins, and the processes and machinery of ERAD and ubiquitin-proteasome-mediated degradation of defective proteins. We previously showed that tobacco Bright Yellow 2 (BY2) cells can be used as a tool to investigate the efficacy of pharmacological chaperones in rescuing mutant (N370S) GCase (Babajani et al. 2012). Because the mutant lysosomal hydrolase is equipped with a plant signal peptide, an assessment of post-ER/post-Golgi trafficking can be conveniently monitored by secretion assays using plant protoplasts. In the present study, we sought to investigate whether ERAD inhibitors and proteostasis regulators can promote the post-ER trafficking of L444P GCase in transgenic BY2 cells. We further investigated transcriptional changes in the transgenic plant cells expressing L444P GCase by profiling the expression levels of genes encoding ER-resident molecular chaperones, ER-stress responsive proteins, and cytoplasmic HSR proteins. Our findings uncover some of the mechanisms by which the various small molecules are able to “rescue” mutant L444P GCase, promoting its post-ER trafficking and secretion.

3.3. Materials and Methods

3.3.1. Generation of constructs for production of L444P GCase protein in tobacco BY-2 cells

The L444P mutation was introduced into the WT cDNA (*GBA1*) clone using the Quick Change site-directed mutagenesis kit (Stratagene, Santa Clara, CA USA). To allow for secretion of mutant GCase into the culture medium, the signal peptide of proaleurain was fused to the coding sequence of L444P GCase as described in Babajani et al. 2012. Re-amplified cDNA products were cloned into the pENTR/D-TOPO vector

(Life Technologies, Burlington, ON CA) and subsequently inserted into the Gateway expression vector pSITE-0B (Chakrabarty et al. 2007) by LR recombination based on the manufacturer's instructions (Life Technologies). The expression vector carries the kanamycin resistance gene expression cassette (neomycin phosphotransferase II) for the selection of transgenic plant cells, and the cauliflower mosaic virus (CaMV) 35S promoter (with a duplicated enhancer element; 2X35S), which is used to drive constitutive expression of the mutant gene encoding L444P GCase in the transgenic plant cells.

3.3.2. Transformation of tobacco BY-2 cells

The L444P GCase expression constructs were introduced into *Agrobacterium tumefaciens* (GV 3101) by electroporation. Transformed *Agrobacterium* cells were selected and used to generate transgenic tobacco BY-2 cells as described previously (Mayo et al. 2006; Babajani 2012). Transgenic BY2 cells lines were maintained on solid Murashige and Skoog (MS) culture medium (PhytoTechnology Laboratories, Overland Park, KS USA) and sub-cultured twice a month. All experiments were initiated using vigorously growing BY2 cells at equivalent stages during log growth.

3.3.3. Northern blot analyses of transgenic BY-2 lines

Northern hybridization analysis was performed using 15 µg of total RNA isolated from transgenic BY2 cells using Trizol reagent (Sigma-Aldrich, Oakville, ON CA). A radiolabelled full-length cDNA probe for human *GBA1* was used to detect transcripts for L444P mutant GCase, and northern blot analyses were performed as described previously (Babajani et al. 2012).

3.3.4. RNA extraction and cDNA synthesis

Tobacco BY2 cells were ground in liquid nitrogen and total RNA was extracted using the RNeasy Plant Mini Kit (Qiagen, Toronto, ON CA) according to the manufacturer's instructions. The purity of RNA was determined with a nanodrop spectrophotometer (ND-2000C, Thermo Scientific) to assess the OD260/280 ratios. One

µg RNA was reverse transcribed using the ProtoScript II First Strand cDNA Synthesis Kit (New England Biolabs Ltd, Whitby, ON CA) with a mixture of random hexamers and oligo-dT primers. cDNA from 3 biological replicate RNA samples was used for qPCR.

3.3.5. Quantitative RT-PCR

Quantitative (q) RT-PCRs were performed using the PerfeCTa Sybr Green Supermix with ROX (Qanta Biosciences, Gaithersburg, USA) as described previously (Müller et al. 2012). Primers (Table S1) were designed with the Primer3 online tool (Rozen and Skaletsky 2000). A final PCR reaction of 15 µL containing 150 ng cDNA (RNA equivalent), 7.5 µl supermix, and 140 nM of each primer was loaded on an ABI7900HT machine (Applied Biosystems, Foster City, CA USA) and the reactions were run using the following protocol: 3 min at 95 °C and 40 cycles of 15 s at 95 °C/min at 60 °C. A dissociation curve was run after each qPCR to validate that only one product had been amplified.

The ΔΔ-Ct method was used for the calculation of relative expression and the GAPDH (cytosolic NAD-specific glyceraldehyde-3-phosphate dehydrogenase) transcript was used as an internal standard for normalization of expression levels. No-template-controls (NTCs) were also performed for each primer pair to check for contamination of reagents. Only samples whose corresponding NTC showed no amplification signal were used in the analysis.

3.3.6. Protein isolation from tobacco BY2 cells grown on solid media and from BY2 protoplasts

BY2 cells (100 mg) grown on solid media were ground on ice in a 1.5-mL microcentrifuge tube using glass beads in 100 µL GCase extraction buffer (50 mM sodium phosphate pH 6.0, 50 mM NaCl, 0.1% sodium taurocholic acid, 1 mM EDTA, 0.5 mM PMSF). After centrifugation at 14 000 ***g*** for 15 min at 4 °C, the supernatant was removed and transferred to a new microcentrifuge tube. To isolate protein from BY2 protoplasts, protoplasts (2×10^5) were vortexed in the presence of glass beads in 100 µL GCase extraction buffer. Cell debris was removed by centrifugation at 14000 ***g*** for 15 min at 4 °C and the supernatant used for western blot analysis. Protein concentrations

were determined using the Bio-Rad DC™ (Bio-Rad Laboratories, Mississauga, ON CA) protein assay kit with BSA as a standard.

3.3.7. Western blot analyses

Protein extracts from BY2 transgenic lines (20 µg total soluble protein) were separated by 12% SDS-PAGE and transferred onto Amersham Hybond-P (PVDF) membranes. Western blotting was performed using a mouse monoclonal anti-GCase 2E2 (Novus Biologicals, Littleton, CO USA) primary antibody (1: 10,000 dilution) and an anti-mouse HRP (horseradish peroxidase) secondary antibody (1:40,000) as described previously (Babajani et al. 2012). The same blot was then stripped with guanidine hydrochloride stripping buffer (6 M GnHCl, 0.2% Nonidet [NP-40], 0.1 M β-mercaptoethanol, 20 mM TRIS-HCl, pH 7.5) for 10 min (Yeung and Stanley 2009) and re-probed with an anti-GAPDH primary antibody as a loading control.

For western blot analysis of the ER chaperone BiP, and cytosolic HSR markers (HSP70 and HSP90), the antibodies were obtained as a gift from A. Ceriotti, Istituto di Biologia e Biotecnologia Agraria, Milano (rabbit anti-BiP), from Enzo Life Sciences, Brockville, Ontario CA (mouse anti-HSP70), and from Agrisera, Vännäs, Sweden (rabbit anti-HSP90) and were used at dilutions of 1:2,000, 1:5,000, and 1:3,000, respectively. All antibodies had confirmed reactivity to the plant proteins.

3.3.8. Endo H digestion

Cell lysates containing 25 µg of total protein were subjected to overnight incubation with Endo H (New England Biolabs Inc, Mississauga, ON CA) according to the manufacturer's instructions (37 °C; 1 µL of Endo H) upon denaturing the samples in Endo H denaturing buffer (15 min, 100 °C). Digested and undigested samples were then separated on a 10% SDS-PAGE gel and western blot analysis was performed using anti-GCase 2E2 antibodies.

3.3.9. Protoplast isolation from tobacco BY2 solid cultures

Protoplasts were isolated as described previously (Babajani et al. 2012). The freshly isolated protoplasts were collected and re-suspended in protoplast culture medium (4.3 g/L MS salts, 100mg/L myo-inositol, 1 mg/L thiamine, 0.2 mg/L 2,4-dichlorophenoxyacetic acid, 255 mg/L KH_2PO_4, 30 g/L sucrose, pH 5.7). After each protoplast isolation, viability of protoplasts was determined with 0.02% solution of Evans blue (Sigma) prepared in protoplast culture medium.

3.3.10. Detection of secreted GCase protein in the medium of cultured BY2 protoplasts

The culture medium surrounding the protoplasts was isolated by filtration through a Whatman filter paper (Grade 1: 11 µm; medium flow filter paper) (GE Healthcare Life Sciences, Baie d'Urfe, Quebec, CA), and then mixed with chilled acetone in a ratio of 1:5 and incubated at –20 °C for 1 h to precipitate proteins. Centrifugation was then conducted at 10,000 ***g*** for 30 min, and the resulting precipitates were re-suspended in GCase extraction buffer and fractionated by SDS-PAGE, followed by western blot analysis as previously described.

3.4. Results

3.4.1. Steady-state levels of transcripts and protein (L444P GCase) in tobacco BY2 cell lines are variable

Transgenic tobacco BY2 cell lines stably expressing L444P GCase were generated and analyzed by PCR. Northern blot analysis of the PCR-positive transgenic cell lines showed that the steady-state level of transcripts corresponding to L444P GCase was variable among the population of lines in which abundant transcript was detected (**Error! Reference source not found.** A). Western blot analysis of selected high-expressing lines transgenic for L444P GCase detected variable levels of an ~60 kDa protein (**Error! Reference source not found.** B). Interestingly, high levels of the

mutant protein were present in line 39; the other transgenic lines showed low to moderate levels of L444P GCase protein.

Line 36 was selected for further analyses to examine the effects of ERAD inhibitors and proteostasis regulators. It was not feasible to use Line 39 as we were unable to isolate protoplasts from this line, presumably due to the toxicity of the mutant L444P protein.

3.4.2. Endoglycosidase H sensitivity of recombinant L444P GCase

It was important to first assess the intracellular fate of the wild-type and L444P GCase within the tobacco BY2 host cells, i.e., the extent to which the proteins were capable of post-ER trafficking. For this we used an indirect biochemical method to assess the processing of the proteins' N-linked glycans – by monitoring their sensitivity to digestion by *Streptomyces* endoglycosidase H (Endo H). Similar to other eukaryotic cells, in the plant Golgi complex, enzymes convert the original high-mannose N-glycans of proteins (including recombinant GCase) to hybrid and complex N-glycans by a series of sequential reactions; the plant-specific sugars that are associated with these "matured" N-glycans, are β-1,2-xylose and α-1,3-fucose (Gomord and Faye 2004). This modification renders the N-glycan resistant to Endo H; thus this characteristic has been used to follow the transport of secretory proteins through the Golgi complex in eukaryotic cells (Ron and Horowitz 2005; Downing et al. 2006; Mu et al. 2008; Wang et al. 2011). More specifically the N-glycans become resistant to cleavage by Endo H just prior to the addition of xylose and fucose in the Golgi complex, after they have undergone processing by the enzyme Golgi α-mannosidase II, a trimming enzyme that removes 2 mannose residues from the N-glycan $GlcNAcMan_5[GlcNAc]_2$. The wild-type (WT) GCase was largely resistant to Endo H digestion, indicative of its trafficking beyond the medial Golgi complex (Figure 3-2 A). In contrast, the L444P GCase was susceptible to Endo H digestion, generating a product of significantly lower molecular weight (~55 kDa) than that of the untreated L444P GCase (Figure 3-2 A). This suggests that a significant fraction of the L444P GCase variant is not transported out of the ER in the tobacco BY2 cells, presumably due to ER quality control.

3.4.3. Inhibition of the proteasome machinery results in the accumulation of mutant GCase

L444P GCase is degraded by the proteasome machinery in human cells (Ron and Horowitz 2005). We next sought to determine whether the proteasome machinery plays a role in the disposal of mutant GCase in the plant cell environment. We hypothesized that if this is the case, a proteasome inhibitor should stabilize mutant GCase inside the plant cells and increase its intracellular protein levels. Accordingly, we compared the accumulation of wild-type and mutant L444P GCase proteins in BY2 cells treated with or without the proteasome inhibitor MG-132. Relative to treated BY2 cells, in the absence of MG-132 the steady-state level of L444P GCase was reduced, indicating that the mutant protein was subjected to proteasomal degradation (Figure 3-2 B and C, "0"). The 3-d- and 5-d MG-132-treated cells (Figure 3-2 B and C, respectively) showed significantly higher levels of L444P GCase protein compared with untreated controls; a positive effect was observed across the range of concentrations of MG-132 that were tested, and no cytotoxic effect was detected at concentrations up to 30 μM. Treatment with the proteasome inhibitor also had a positive effect on the steady-state intracellular protein levels of wild-type GCase, in particular when applied at concentrations of 10–30 μM (Figure 3-2 B), WT GCase).

3.4.4. Inhibitors of ERAD enhance the accumulation of L444P GCase protein in BY-2 cells, and promote post-ER transport and secretion of the mutant protein in BY-2 protoplasts

Because L444P mutant proteins are retained in the ER and their degradation is at least in part mediated by the proteasome, we predicted that the proteins are eliminated from BY2 cells by the ERAD pathway. In order to investigate the role of the ERAD pathway on the steady-state levels and trafficking of the L444P mutant protein, intact BY2 cells expressing L444P GCase were cultured on solid media containing the ERAD inhibitors Eeyarestatin I (EerI) and Kifunensine (Kif) for 5 d. We hypothesized that EerI- and Kif-mediated ERAD inhibition would increase the ER pool of the mutant GCase, which would be reflected by its increased steady-state levels inside plant cells. Treatment of cells with EerI and Kif (particularly at concentrations of 10 and 5 μM, respectively) increased the levels of L444P GCase as compared with untreated cells

(Figure 3-3, L444P GCase). While EerI had no significant effect on the steady-state intracellular protein levels of the wild-type GCase, Kif was reasonably effective in enhancing the steady-state levels (Figure 3-3), WT GCase).

To determine whether inhibition of ERAD can facilitate the post-ER transport of L444P GCase, the effects of EerI and Kif were examined on the secretion of mutant GCase into the culture medium surrounding the transgenic BY2 protoplasts. In the absence of ERAD inhibitors only a small portion of the L444P mutant was secreted into the culture medium (Figure 3-4 A, "0"). Both ERAD inhibitors promoted the secretion of the L444P mutant protein into the medium surrounding the BY2 protoplasts, particularly at 10 and 20 μM Kif (Figure 3-4 A; concentrations that were also effective in promoting secretion of the wild-type GCase; Figure 3-4 C), and at all concentrations of EerI (Figure 3-4 B). These results suggest that ERAD plays an important role in the stability (steady-state levels) of L444P GCase; with an inhibition of this process, the ER pool of this mutant protein is increased, which further allows for the subsequent post-ER trafficking of mutant L444P GCase. Thus, ERAD inhibition appears to be a viable strategy to facilitate the folding and trafficking of this degradation-prone mutant protein.

3.4.5. ERAD inhibition by EerI and Kif shows selective effects on the expression of genes encoding ER chaperones

It is commonly assumed that accumulation of misfolded proteins in the ER lumen leads to ER stress (Rao and Bredesen 2004). We wanted to investigate if the increase in the secretion of L444P GCase promoted by EerI and Kif could be attributed to the upregulation of genes encoding ER- and cytoplasmic- molecular chaperones, and further if there are changes in the levels of transcripts encoding ER stress-responsive proteins that are typically associated with the accumulation of malfolded proteins in the ER lumen. To determine the extent to which the expression of the various genes was altered by ERAD- inhibitor-treatment of the L444P GCase-trangenic tobacco BY2 cells, quantitative reverse transcription-polymerase chain reaction (RT-PCR) analyses were performed. We were most interested in the transcript levels for representative ER chaperones (BiP1, BiP3, calnexin, calreticulin and protein disulphide isomerase; PDI), ER-stress responsive proteins (Sar1 and Sec61), and representative cytoplasmic HSR-

associated chaperones (HSP40, HSP70, HSP90) (Figure 3-5). The chemical and control treatments were applied to the cells for 5 d. Even though this is a prolonged period of exposure prior to monitoring transcript changes, the reasons for this were related to the fact that the intact BY-2 cells (possessing a cell wall) take considerable time to respond to the inhibitors incorporated into the solid growth medium. The 5 d duration also corresponded to the time at which the accumulation of mutant L444P GCase was most greatly enhanced (Figure 3-3). There were no marked changes in the expression of the target genes induced by either EerI or Kif. EerI treatment induced a slight upregulation of *BiP3* transcripts, and generally did not elicit any changes in the transcription of the other marker genes. Kif induced slight increases in the expression of most of the genes tested, with the most prominent effect on *BiP3,* calnexin and calreticulin transcripts (Figure 3-5). The differential effects of Kif and EerI on ER chaperone gene expression are interesting within the context of their respective mechanisms of ERAD inhibition and the stages at which these chemicals exert their effects, with the former acting earlier on in the pathway (see Discussion). The biological significance of *BIP3* upregulation is difficult to assess because of its generally low level of expression in the BY2 cells.

3.4.6. Celastrol, a proteostasis regulator, rescues post-ER trafficking of L444P GCase: celastrol effects on intact BY-2 cells

The triterpene celastrol has been used as a proteostasis regulator for L444P GCase (Mu et al. 2008). To determine the effect of celastrol on intracellular accumulation and post-ER trafficking of mutant GCase, BY2 cells expressing L444P GCase were grown on solid media containing different concentrations of the proteostasis regulator. Celastrol (0.1 to 5 μM) significantly increased the amount of L444P GCase inside the BY-2 cells as compared with the control (untreated) cells (Figure 3-6 A). The increased steady-state levels of L444P GCase protein in response to celastrol correlated with a partial restoration of L444P GCase folding and post-ER trafficking. Specifically Endo H digestion followed by western blot analyses of the protein showed that the proportion of mutant GCase that was resistant to Endo H (indicative of Golgi transit) was increased with celastrol treatment, in contrast to that within cells not exposed to this proteostasis regulator, in which nearly all of the L444P GCase was Endo H-sensitive

(Figure 3-6 B, C). This became even more pronounced when the celastrol treatment was increased from 5 d to 10 d (Figure 3-6 B, C; 5 d vs.10 d).

3.4.7. Proteostasis regulators promote the secretion of L444P GCase: effects of celastrol and MG-132 on BY-2 protoplasts

We have previously shown that unlike the L444P protein studied here, the wild-type GCase protein is efficiently secreted from tobacco BY2 cells in the absence of any chemical treatment (Babajani et al. 2012). To determine more directly whether proteostasis regulators facilitate the post-ER transport of L444P GCase, we examined the effects of celastrol on the secretion of mutant GCase into the culture medium surrounding the transgenic protoplasts. As shown in Figure 3-7A, in untreated transgenic protoplasts, a small proportion of the L444P GCase was secreted into the culture medium (Figure 3-7A; 0 μM celastrol). Treatment of the protoplasts with celastrol promoted the secretion of L444P mutant protein into the surrounding media over most of the range of concentrations tested (Figure 3-7A), none of which were cytotoxic. Celastrol also increased the steady-state accumulation of mutant L444P GCase inside the BY2 protoplasts (Figure 3-7B). MG-132 at 20 μM enhanced the accumulation of L444P in the BY2 protoplasts and also enhanced the secretion of the mutant protein into the culture medium (data not shown).

Thus, these proteostasis regulators are able to enhance the post-ER trafficking of L444P GCase along the plant secretory pathway. Although indirect evidence, this is indicative that these proteostasis regulators allows some proportion of the mutant protein in the ER lumen to undergo correct folding.

Our results are similar to those reported by Mu et al. 2008, and collectively, indicate that the L444P variant protein can fold and become functional provided that some proportion of the synthesized protein is stabilized in its native state within the ER lumen. This enables the protein to pass ER quality control and traffic out of the ER – enroute to the lysosome in human cells – or, in this case, to the surface of transgenic plant cells for secretion.

3.4.8. Celastrol and MG-132 treatments downregulate HSP90, a cytoplasmic HSR-associated chaperone

Celastrol and MG-132 induce the heat-shock response (HSR) (Bush et al. 1997; Morimoto 1998; Westerheide et al. 2004; Liao et al. 2006; Mu et al. 2008). These regulators may remodel the ER proteostasis environment and secretory apparatus to afford a greater capacity of the ER for protein folding and/or protein export (Schröder and Kaufman 2005; Ron and Walter 2007; Mu et al. 2008). Most of the genes for the ER- proteins (chaperones and ER stress responses) were relatively unaffected by both chemicals (Figure 3-8). However the qRT-PCR analyses revealed that celastrol decreased the transcription of the *Hsp90* gene in the L444P GCase-transgenic cells (Figure 3-8 A). Analysis of the steady-state levels of HSP90 protein by western blot analyses generally supported the gene expression results; as compared with the control, celastrol led to a marked decline in the abundance of HSP90 protein, which was evident even after 24 h, and became more prominent as the time of incubation with the chemical increased (Figure 3-8 C). MG-132 treatment did not induce any appreciable change the steady-state levels of HSP90 protein (Figure 3-8 C). Celastrol and MG-132 had no effects on the steady-state levels of the ER chaperone BiP and cytoplasmic HSP70 proteins (Fig. S1A and B).

3.5. Discussion

Gaucher disease is a prevalent lysosomal storage disease that affects 1 in 60,000 people in the general population. One of the most frequently identified disease-causing mutations in human *GBA1*, the gene encoding lysosomal GCase, is a missense mutation, leading to an L444P substitution in the GCase protein. This mutation severely destabilizes the native structure of GCase and leads to loss of enzyme activity. In general, proteins that are incorrectly folded are recognized by ER chaperones and transported to the cytosol for ubiquitin-dependent proteasomal degradation, a process known as ERAD (Lu et al. 2011; Wang et al. 2011; Wang et al. 2013). L444P GCase undergoes extensive ERAD and is disposed of by the cytoplasmic proteasome machinery (Bendikov-Bar and, Ron 2011). Of interest to potential therapeutic strategies, it has been suggested that GCase variants including the L444P variant are

amenable to restoration of their native conformation (Mu et al. 2008; Wang et al. 2011), and small molecules capable of promoting folding, are thus able to “rescue” the activity of the L444P mutant protein. Proteostasis regulators (Mu et al. 2008) and ERAD inhibitors (Wang et al 2011) are among those able to improve L444P mutant protein functioning, in this case potentially via their ability to remodel the ER proteostasis environment and secretory apparatus to afford a greater capacity for protein folding and/or protein export (Mu et al. 2008; Schröder and Kaufman 2005; Ron and Walter 2007).

In the present study we wanted to investigate whether ERAD inhibitors and proteostasis regulators are able to facilitate post-ER secretion of L444P GCase in a heterologous plant cell system. To achieve this, we generated transgenic tobacco BY2 cells expressing L444P GCase equipped with the signal peptide of the barley cysteine protease proaleurain (Tse et al. 2004) to allow for efficient secretion of mutant recombinant human GCase in the plant host. As no additional targeting information was added, the recombinant protein (if transport-competent) is expected to transit along the secretory pathway to the plant cell surface by a default mechanism. We were more interested in the transgenic lines expressing L444P GCase that exhibited high levels of steady-state GCase transcript. Western blot detection of GCase protein in total soluble protein (TSP) extracts indicated that L444P GCase protein levels were generally low and there was considerable variability across the transgenic BY2 lines in terms of the transcript to protein ratios.

In contrast to the wild-type GCase, which undergoes efficient post-ER transit and secretion in plant cells (Babajani et al. 2012), using biochemical analyses (Endo H sensitivity), our results indicated that the majority of L444P mutant proteins were retained in the ER of the transgenic BY2 cells. Likewise, recombinant L444P GCase variants transiently expressed in HeLa cells are also retained in the ER, similar to the endogenous L444P protein in patient-derived fibroblasts (Ron 2005). Recombinant wild-type GCase expressed in HeLa cells behaves much like the endogenous GCase of human cells, and a large fraction of the synthesized protein is Endo H resistant, indicative of its post-ER transport (Ron 2005).

As in intact BY2 cells, the wild-type GCase is efficiently secreted from transgenic BY2 protoplasts (Babajani et al. 2012). This was of considerable advantage to our study, and allowed us to compare the wild-type GCase to the variant L444P GCase in both intact cells and protoplasts, and to further characterize the effects of pharmacological modulation – application of agents potentially effective in increasing the innate cellular folding capacity. We examined the effects of kifunensine and eeyarestatin I – two small molecules that function as ERAD inhibitors, but block different steps of the ERAD pathway (Wang et al. 2011). Kifunensine (Kif) acts earlier in the ERAD pathway by inhibiting ER mannosidase I, an enzyme which cleaves 3 to 4 mannose residues from the N-glycan(s) of the nascent protein and promotes binding of the misfolded protein with the ER degradation-enhancing-mannosidase-like lectins, EDEMs 1, 2, and 3. Eeyarestatin I (EerI) acts later by inhibiting p97 ATPase activity; this ATPase provides the driving force for "extraction" of the misfolded protein and its "shuffling" to the proteasome (Wang et al. 2011). In the absence of any pharmacological agent, only a small proportion of L444P GCase was secreted by the cultured BY2 protoplasts likely because the misfolded protein undergoes proteasomal degradation. Application of the ERAD inhibitors, Kif and EerI, appeared to facilitate the post-ER trafficking of L444P GCase in transgenic protoplasts, resulting in the enhanced secretion of mutant protein into the culture medium. Additionally, in intact BY2 cells, the same ERAD inhibitors increased the steady-state levels of L444P GCase intracellularly. Inhibition of ERAD likely stabilizes the folded and partly folded forms of the mutant GCase in the ER, allowing a greater proportion of the synthesized protein to accumulate inside plant cells.

In patient-derived fibroblasts, an increase in the activity of mutant GCase correlates with an increase in the transcript levels of BiP (Wang et al. 2011[a,b,c]) and EerI treatment induces increased levels of BiP protein (Wang et al. 2011). In BY2 cells the use of Kif or EerI resulted in only a very slight increase in the low-level expression of the ER stress chaperone *BiP3* gene (Howell 2013), but did not increase the steady-state levels of the BiP protein (Fig. S2). Kif led to modest increases in the steady-state transcripts encoding calnexin and calreticulin. On a cautionary note, the increases in transcripts were not marked, and we were unable to obtain antibodies to the two plant proteins to examine any change in their abundance. Nonetheless, it is likely that the ER molecular chaperones participate in increasing the proportion of the variant protein that

can fold and thus pass the ER quality control mechanisms; as a result the secretion of L444P GCase from the heterologous plant cells is enhanced. Notably, N-glycan trimming processes within the ER lumen allow the calnexin/calreticulin system to both monitor the protein folding status of the nascent protein (a time-sensitive process), and to promote protein folding itself. If still within an early timeframe, the N-glycan status of the nascent protein can signal these two ER molecular chaperones to transiently engage with the nascent protein so as to promote its acquisition of a functional conformation. It is only after having successfully adopted its native conformation that the protein is permitted to exit the ER lumen and undergo further transit along the secretory pathway to its final destination (Roth et al. 2010). If the nascent protein fails to achieve its native conformation within a given time, it will eventually be modified by ER-resident α 1,2-mannosidases, which remove mannose residues from the N-glycan (a process which attenuates N-glycan-association with calnexin/calreticulin); ultimately the misfolded protein is retrotranslocated from the ER to the cytosol for ERAD. Thus, recognition processes surrounding the N-glycan promote protein folding, trafficking and function vs. ERAD via an extrinsic mechanism, i.e., one that depends on intermolecular interactions between the N-glycan and components of the ER proteostasis network (Price et al. 2012).

Interestingly, whereas in plant cells both Eerl and Kif increased the secretion of L444P GCase, in patient-derived fibroblasts only Eerl rescues the folding of mutant L444P protein and induces BiP expression Wang et al. 2011).

Celastrol and MG-132 are proteostasis regulators that appear to mediate the partial restoration of L444P GCase activity and function. One effect of the plant triterpene celastrol[47] is the induction of heat shock protein (HSP) gene expression by activation of a heat shock transcription factor 1 (HSF1) (Westerheide et al. 2004). Direct effects of celastrol on protein conformation can also occur due to its reactivity with the thiol groups of proteins (Trott et al. 2008); its proteostasis effects are at least partly due to an inhibition on the chymotrypsin-like activity of the proteasome (Mu et al. 2008; Yang et al. 2006). MG-132 is a well known inhibitor of 26S proteasome activity (Lee and Goldberg 1998) and induces ER stress (Nakajima et al. 2011).

Here we showed indirectly that celastrol mediated the partial restoration of folding of the L444P GCase protein in BY2 cells, and thus promoted the post-ER trafficking of L444P GCase leading to secretion into the culture medium surrounding the protoplasts. We were interested to see if celastrol also modulated the expression of genes encoding ER molecular chaperones and components of the cytoplasmic HSR (heat shock response). Although located in the cytosol, HSP90 was of considerable interest because it is a chaperone protein that has been implicated in the degradation of misfolded GCase. Among the putative functions of HSP90, some of which are achieved along with its partners (the HSP70-HSP90 system, and/or co-chaperone p23) (Wang et al. 2006), HSP90 may participate in the "extraction" of soluble ERAD substrates from the ER lumen to the cytosol (Taylor et al. 2010). It may further contribute to the assembly and integrity of the 26S proteasome Imai et al. 2003; Yamano et al. 2008) and to directing misfolded proteins to the ubiquitin-proteasome degradation pathway (Lu et al. 2011). One of the known effects of celastrol is an inhibition of the expression of the gene encoding this protein (Hieronymus et al. 2006; Zhang et al. 2008; Sreeramulu et al. 2009; Zhang et al. 2009; Chadli et al. 2010; Calamini et al. 2012). In HeLa cells, recombinant L444P GCase exhibits greater ubiquitination and possesses a greater tenacity for binding to the cytoplasmic HSP90 as compared with the wild-type GCase. With celastrol application, the stability of L444P GCase is enhanced and the subsequent degradation of this mutant protein is reduced (Lu et al. 2011). In the plant BY2 cells celastrol reduced the expression levels of the cytoplasmic HSR-associated gene *Hsp90* and significantly decreased the steady-state levels of the HSP90 protein. Since the chemical ultimately led to a greater proportion of the mutant L444P GCase that underwent post-ER trafficking in the plant cell, it is tempting to speculate that the reduced cytosolic HSP90 (possibly along with compromised proteosome integrity and/or an abrogated capacity of HSP90 to bind to the variant protein), fed back to impede ER-to-cytosol "extraction" of L444P GCase, ultimately reducing the proportion of the GCase variant that is targeted to the proteasome machinery for destruction. Thus, a significant stabilization of the folded L444P protein in BY2 cells would ensue, shifting the cellular machinery toward increased protein accumulation. The greater proportion of synthesized mutant L444P proteins able to pass ER quality control and thus transit along the

secretory pathway is reflected in our finding of the increased L444P GCase protein in the culture medium surrounding the transgenic BY2 protoplasts.

Other proteostasis regulators such as inhibitors of HDAC6 (histone deacetylase 6) appear to curb the rate of degradation of both N370S and L444P GCase by modulating the HSP70–90 system. By reducing the rate of ubiquitination and degradation, but maintaining the rate of transcription, translation and folding, the half-life of the synthesized CGase is increased along with its post-ER trafficking and ultimately steady-state accumulation in the lysosome (Lu et al. 2011).

Yet the underlying mechanisms clearly require additional investigation. Other studies indicate that celastrol enhances the post-ER trafficking of L444P GCase in patient-derived fibroblasts by activating the UPR and, in contrast to this work, by upregulating the expression of genes encoding cytoplasmic HSR chaperones including HSP90 and ER lumenal chaperones (Mu et al. 2008). Perhaps toxicity of celastrol plays a role in inducing the expression of HSR- and UPR- related proteins in patient-derived fibroblasts since mammalian cells may be less resilient to this compound than plant cells.

Using intact BY2 cells we show that the proteasome inhibitor MG-132 significantly increased the steady-state level of mutant L444P protein, indicating that this GCase variant is normally subjected to a proteasome-dependent degradation. Further, treatment of the BY2 protoplasts with MG-132 enhanced post-ER trafficking of the mutant protein along the endomembrane system as reflected by the increased secretion of L444P GCase into the culture media. Similarly, MG-132 stabilizes L444P GCase in patient fibroblasts (Ron and Horowitz 2005; Ron et al. 2010); in the absence of MG132 the steady-state level of L444P GCase variants is reduced (Ron et al. 2010). Partial correction of the folding and lysosomal trafficking of L444P GCase by the proteostasis regulator MG-132 in mutant (L444P) fibroblasts (Mu et al. 2008) is accompanied by enhanced transcript levels of cytoplasmic HSR genes and ER chaperones (Bush et al. 1997; Mu et al. 2008). Although MG-132 caused a slight increase in the low-level expression of the *BiP3* gene in BY2 cells, in contrast to the Mu et al. 2008 study there were no significant changes in the expression the target

cytoplasmic HSR- and ER-related genes. Yet it is still possible that a combination of proteasome inhibition and improved ER-chaperone-functioning is sufficient to stabilize L444P GCase inside the BY2 cells. Post-ER trafficking of mutant GCase ensues as a greater proportion of the mutant GCase proteins becomes competent to pass ER quality control.

In conclusion, our results provide insights into the mechanism by which ERAD inhibitors and proteostasis regulators facilitate the post-ER trafficking of L444P GCase in plant cells.

3.6. Accession Numbers

Sequence data from this article can be found in the GenBank database under accession numbers X60060 (BiP1), X60059 (BiP2), X60061 (BiP3), EB692674 (putative Sec61), AF210431 (Sar1), GQ354817 (PDI), EU984501 (calreticulin), NP_001234129 (putative calnexin), AJ299254 (Putative HSP40), AB689673 (HSP70), and HQ834904 (HSP90).

Acknowledgments

This work was funded by a Michael Smith Foundation for Health Research Senior Scholar Award to A.R.K. (No. CI-SSH-01915[07–1]). We would like to thank Dr Joost Lücker for helpful comments and critical reading of the manuscript.

3.7. References

Avezov E, Frenkel Z, Ehrlich M, Herscovics A, Lederkremer GZ. Endoplasmic reticulum (ER) mannosidase I is compartmentalized and required for N-glycan trimming to Man5-6GlcNAc2 in glycoprotein ER-associated degradation. Mol Biol Cell 2008; 19:216-25.

Babajani G, Tropak MB, Mahuran DJ, Kermode AR. Pharmacological chaperones facilitate the post-ER transport of recombinant N370S mutant β-glucocerebrosidase in plant cells: evidence that N370S is a folding mutant. Mol Genet Metab 2012; 106:323-9.

Balch WE, Morimoto RI, Dillin A, Kelly JW. Adapting proteostasis for disease intervention. Science 2008; 319:916-9.

Ballabio A, Gieselmann V. Lysosomal disorders: from storage to cellular damage. Biochim Biophys Acta 2009; 1793:684-96.

Bendikov-Bar I, Ron I, Filocamo M, Horowitz M. Characterization of the ERAD process of the L444P mutant glucocerebrosidase variant. Blood Cells Mol Dis 2011; 46:4-10.

Bush KT, Goldberg AL, Nigam SK. Proteasome inhibition leads to a heat-shock response, induction of endoplasmic reticulum chaperones, and thermotolerance. J Biol Chem 1997; 272:9086-92.

Calamini B, Morimoto RI. Protein homeostasis as a therapeutic target for diseases of protein conformation. Curr Top Med Chem 2012; 12:2623-40.

Calamini B, Silva MC, Madoux F, Hutt DM, Khanna S, Chalfant MA, Saldanha SA, Hodder P, Tait BD, Garza D, et al. Small-molecule proteostasis regulators for protein conformational diseases. Nat Chem Biol 2012; 8:185-96

Chadli A, Felts SJ, Wang Q, Sullivan WP, Botuyan MV, Fauq A, Ramirez-Alvarado M, Mer G. Celastrol inhibits Hsp90 chaperoning of steroid receptors by inducing fibrillization of the Co-chaperone p23. J Biol Chem 2010; 285:4224-31.

Chakrabarty R, Banerjee R, Chung SM, Farman M, Citovsky V, Hogenhout SA, Tzfira T, Goodin M. PSITE vectors for stable integration or transient expression of autofluorescent protein fusions in plants: probing Nicotiana benthamiana-virus interactions. Mol Plant Microbe Interact 2007; 20:740-50; PMID:17601162.

Downing WL, Galpin JD, Clemens S, Lauzon SM, Samuels AL, Pidkowich MS, Clarke LA, Kermode AR. Synthesis of enzymatically active human alpha-L-iduronidase in Arabidopsis cgl (complex glycan-deficient) seeds. Plant Biotechnol J 2006; 4:169-81.

Fagioli C, Sitia R. Glycoprotein quality control in the endoplasmic reticulum. Mannose trimming by endoplasmic reticulum mannosidase I times the proteasomal degradation of unassembled immunoglobulin subunits. J Biol Chem 2001; 276:12885-92.

Fan JQ. A counterintuitive approach to treat enzyme deficiencies: use of enzyme inhibitors for restoring mutant enzyme activity. Biol Chem 2008; 389:1-11.

Fiebiger E, Hirsch C, Vyas JM, Gordon E, Ploegh HL, Tortorella D. Dissection of the dislocation pathway for type I membrane proteins with a new small molecule inhibitor, eeyarestatin. Mol Biol Cell 2004; 15:1635-46.

Futerman AH, van Meer G. The cell biology of lysosomal storage disorders. Nat Rev Mol Cell Biol 2004; 5:554-65.

Gomord V, Faye L. Posttranslational modification of therapeutic proteins in plants. Curr Opin Plant Biol 2004; 7:171-81.

Grabowski GA. Gaucher disease: gene frequencies and genotype/phenotype correlations. Genet Test 1997; 1:5-12.

Hieronymus H, Lamb J, Ross KN, Peng XP, Clement C, Rodina A, Nieto M, Du J, Stegmaier K, Raj SM, et al. Gene expression signature-based chemical genomic prediction identifies a novel class of HSP90 pathway modulators. Cancer Cell 2006; 10:321-30.

Howell SH. Endoplasmic reticulum stress responses in plants. Annu Rev Plant Biol 2013; 64:477-99.

Hruska KS, LaMarca ME, Scott CR, Sidransky E. Gaucher disease: mutation and polymorphism spectrum in the glucocerebrosidase gene (GBA). Hum Mutat 2008; 29:567-83

Hutt DM, Powers ET, Balch WE. The proteostasis boundary in misfolding diseases of membrane traffic. FEBS Lett 2009; 583:2639-46.

Imai J, Maruya M, Yashiroda H, Yahara I, Tanaka K. The molecular chaperone Hsp90 plays a role in the assembly and maintenance of the 26S proteasome. EMBO J 2003; 22:3557-67.

Khanna R, Benjamin ER, Pellegrino L, Schilling A, Rigat BA, Soska R, Nafar H, Ranes BE, Feng J, Lun Y, et al. The pharmacological chaperone isofagomine increases the activity of the Gaucher disease L444P mutant form of beta-glucosidase. FEBS J 2010; 277:1618-38.

Lee DH, Goldberg AL. Proteasome inhibitors: valuable new tools for cell biologists. Trends Cell Biol 1998; 8:397-403.

Liao W, Li X, Mancini M, Chan L. Proteasome inhibition induces differential heat shock protein response but not unfolded protein response in HepG2 cells. J Cell Biochem 2006; 99:1085-95.

Lu J, Yang C, Chen M, Ye DY, Lonser RR, Brady RO, Zhuang Z. Histone deacetylase inhibitors prevent the degradation and restore the activity of glucocerebrosidase in Gaucher disease. Proc Natl Acad Sci U S A 2011; 108:21200-5.

Mayo KJ, Gonzales BJ, Mason HS. Genetic transformation of tobacco NT1 cells with Agrobacterium tumefaciens. Nat Protoc 2006; 1:1105-11.

Morimoto RI. Regulation of the heat shock transcriptional response: cross talk between a family of heat shock factors, molecular chaperones, and negative regulators. Genes Dev 1998; 12:3788-96.

Mu TW, Ong DS, Wang YJ, Balch WE, Yates JR 3rd, Segatori L, Kelly JW. Chemical and biological approaches synergize to ameliorate protein-folding diseases. Cell 2008; 134:769-81.

Müller K, Bouyer D, Schnittger A, Kermode AR. Evolutionarily conserved histone methylation dynamics during seed life-cycle transitions. PLoS One 2012; 7:e51532.

Nagy JK, Sanders CR. Destabilizing mutations promote membrane protein misfolding. Biochemistry 2004; 43:19-25.

Nakajima S, Kato H, Takahashi S, Johno H, Kitamura M. Inhibition of NF-κB by MG132 through ER stress-mediated induction of LAP and LIP. FEBS Lett 2011; 585:2249-54.

Price JL, Culyba EK, Chen W, Murray AN, Hanson SR, Wong CH, Powers ET, Kelly JW. N-glycosylation of enhanced aromatic sequons to increase glycoprotein stability. Biopolymers 2012; 98:195-211.

Rao RV, Bredesen DE. Misfolded proteins, endoplasmic reticulum stress and neurodegeneration. Curr Opin Cell Biol 2004; 16:653-62.

Ron D, Walter P. Signal integration in the endoplasmic reticulum unfolded protein response. Nat Rev Mol Cell Biol 2007; 8:519-29.

Ron I, Horowitz M. ER retention and degradation as the molecular basis underlying Gaucher disease heterogeneity. Hum Mol Genet 2005; 14:2387-98.

Ron I, Rapaport D, Horowitz M. Interaction between parkin and mutant glucocerebrosidase variants: a possible link between Parkinson disease and Gaucher disease. Hum Mol Genet 2010; 19:3771-81.

Rozen S, Skaletsky H. Primer3 on the WWW for general users and for biologist programmers. Methods Mol Biol 2000; 132:365-86.

Sawkar AR, Adamski-Werner SL, Cheng WC, Wong CH, Beutler E, Zimmer KP, Kelly JW. Gaucher disease-associated glucocerebrosidases show mutation-dependent chemical chaperoning profiles. Chem Biol 2005; 12:1235-44.

Sawkar AR, Cheng WC, Beutler E, Wong CH, Balch WE, Kelly JW. Chemical chaperones increase the cellular activity of N370S beta -glucosidase: a therapeutic strategy for Gaucher disease. Proc Natl Acad Sci U S A 2002; 99:15428-33; PMID:12434014.

Schmitz M, Alfalah M, Aerts JM, Naim HY, Zimmer KP. Impaired trafficking of mutants of lysosomal glucocerebrosidase in Gaucher's disease. Int J Biochem Cell Biol 2005; 37:2310-20.

Schröder M, Kaufman RJ. The mammalian unfolded protein response. Annu Rev Biochem 2005; 74:739-89.

Sethi G, Ahn KS, Pandey MK, Aggarwal BB. Celastrol, a novel triterpene, potentiates TNF-induced apoptosis and suppresses invasion of tumor cells by inhibiting NF-kappaB-regulated gene products and TAK1-mediated NF-kappaB activation. Blood 2007; 109:2727-35.

Sreeramulu S, Gande SL, Göbel M, Schwalbe H. Molecular mechanism of inhibition of the human protein complex Hsp90-Cdc37, a kinome chaperone-cochaperone, by triterpene celastrol. Angew Chem Int Ed Engl 2009; 48:5853-5.

Taylor M, Navarro-Garcia F, Huerta J, Burress H, Massey S, Ireton K, Teter K. Hsp90 is required for transfer of the cholera toxin A1 subunit from the endoplasmic reticulum to the cytosol. J Biol Chem 2010; 285:31261-7.

Tropak MB, Kornhaber GJ, Rigat BA, Maegawa GH, Buttner JD, Blanchard JE, Murphy C, Tuske SJ, Coales SJ, Hamuro Y, et al. Identification of pharmacological chaperones for Gaucher disease and characterization of their effects on beta-glucocerebrosidase by hydrogen/deuterium exchange mass spectrometry. Chembiochem 2008; 9:2650-62.

Trott A, West JD, Klaić L, Westerheide SD, Silverman RB, Morimoto RI, Morano KA. Activation of heat shock and antioxidant responses by the natural product celastrol: transcriptional signatures of a thiol-targeted molecule. Mol Biol Cell 2008; 19:1104-12.

Tse YC, Mo B, Hillmer S, Zhao M, Lo SW, Robinson DG, Jiang L. Identification of multivesicular bodies as prevacuolar compartments in Nicotiana tabacum BY-2 cells. Plant Cell 2004; 16:672-93.

Roth J, Zuber C, Park S, Jang I, Lee Y, Kysela KG, Le Fourn V, Santimaria R, Guhl B, Cho JW. Protein N-glycosylation, protein folding, and protein quality control. Mol Cells 2010; 30:497-506.

Wang F, Agnello G, Sotolongo N, Segatori L. Ca2+ homeostasis modulation enhances the amenability of L444P glucosylcerebrosidase to proteostasis regulation in patient-derived fibroblasts. ACS Chem Biol 2011a; 6:158-68.

Wang F, Segatori L. Remodeling the proteostasis network to rescue glucocerebrosidase variants by inhibiting ER-associated degradation and enhancing ER folding. PLoS One 2013; 8:e61418.

Wang F, Song W, Brancati G, Segatori L. Inhibition of endoplasmic reticulum-associated degradation rescues native folding in loss of function protein misfolding diseases. J Biol Chem 2011b; 286:43454-64.

Wang Q, Shinkre BA, Lee JG, Weniger MA, Liu Y, Chen W, Wiestner A, Trenkle WC, Ye Y. The ERAD inhibitor Eeyarestatin I is a bifunctional compound with a membrane-binding domain and a p97/VCP inhibitory group. PLoS One 2010; 5:e15479.

Wang F, Chou A, Segatori L. Lacidipine remodels protein folding and Ca 2+ homeostasis in Gaucher's disease fibroblasts: a mechanism to rescue mutant glucocerebrosidase. Chem Biol 2011c; 18:766-76.

Wang X, Venable J, LaPointe P, Hutt DM, Koulov AV, Coppinger J, Gurkan C, Kellner W, Matteson J, Plutner H, et al. Hsp90 cochaperone Aha1 downregulation rescues misfolding of CFTR in cystic fibrosis. Cell 2006; 127:803-15.

Westerheide SD, Bosman JD, Mbadugha BN, Kawahara TL, Matsumoto G, Kim S, Gu W, Devlin JP, Silverman RB, Morimoto RI. Celastrols as inducers of the heat shock response and cytoprotection. J Biol Chem 2004; 279:56053-60.

Yamano T, Mizukami S, Murata S, Chiba T, Tanaka K, Udono H. Hsp90-mediated assembly of the 26 S proteasome is involved in major histocompatibility complex class I antigen processing. J Biol Chem 2008; 283:28060-5.

Yang H, Chen D, Cui QC, Yuan X, Dou QP. Celastrol, a triterpene extracted from the Chinese "Thunder of God Vine," is a potent proteasome inhibitor and suppresses human prostate cancer growth in nude mice. Cancer Res 2006; 66:4758-65; PMID:16651429.

Yeung YG, Stanley ER. A solution for stripping antibodies from polyvinylidene fluoride immunoblots for multiple reprobing. Anal Biochem 2009; 389:89-91.

Zhang T, Hamza A, Cao X, Wang B, Yu S, Zhan CG, Sun D. A novel Hsp90 inhibitor to disrupt Hsp90/Cdc37 complex against pancreatic cancer cells. Mol Cancer Ther 2008; 7:162-70.

Zhang T, Li Y, Yu Y, Zou P, Jiang Y, Sun D. Characterization of celastrol to inhibit hsp90 and cdc37 interaction. J Biol Chem 2009; 284:35381-9.

3.8. Figures

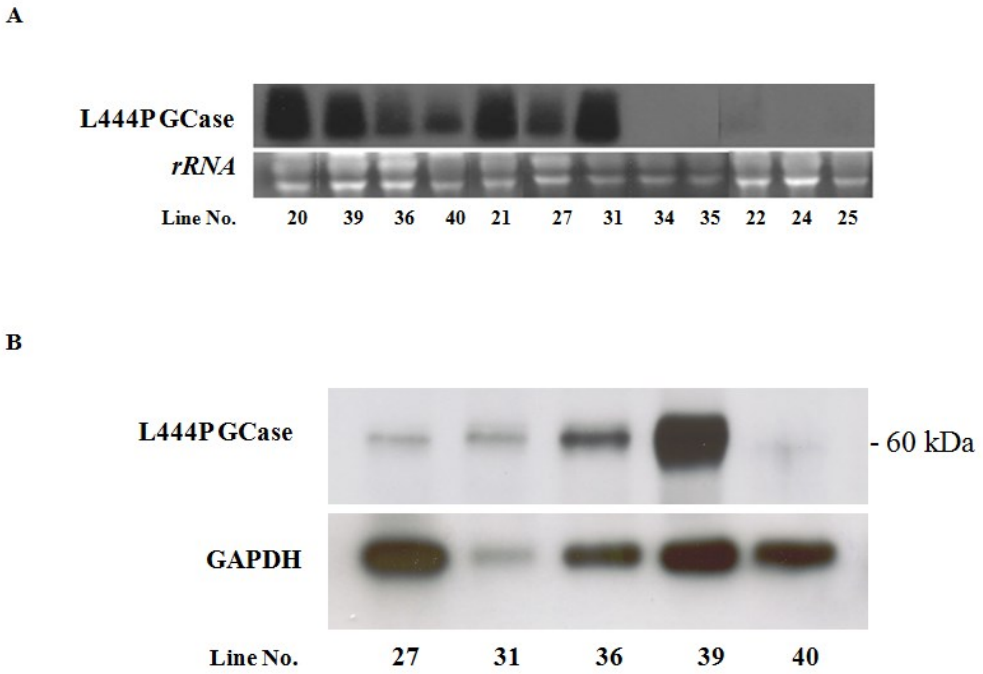


Figure 3-1: Steady-state GCase transcripts (A) and protein levels (B) associated with independent transgenic BY2 tobacco lines expressing L444P GCase.

(**A**) northern blot analyses were based on total RNA (15 µg) isolated from individual transgenic lines in which the BY2 cells had been grown on fresh solid medium for 10 d. A radiolabeled full-length cDNA probe was used to detect the GCase transcripts. Ethidium bromide staining of ribosomal RNA is shown at the bottom. (**B**) For western blot analysis, total soluble protein was extracted from 5 selected lines in which the BY2 cells had been grown on fresh solid medium for 10 d. Soluble protein (20 µg) was fractionated by 12% SDS-PAGE, prior to analysis using a monoclonal anti-GCase antibody (2E2). The marker protein GAPDH (NAD-specific glyceraldehyde-3-phosphate dehydrogenase) was used as a control for loading on the same membranes following stripping of membranes and incubation with GAPDH antibodies.

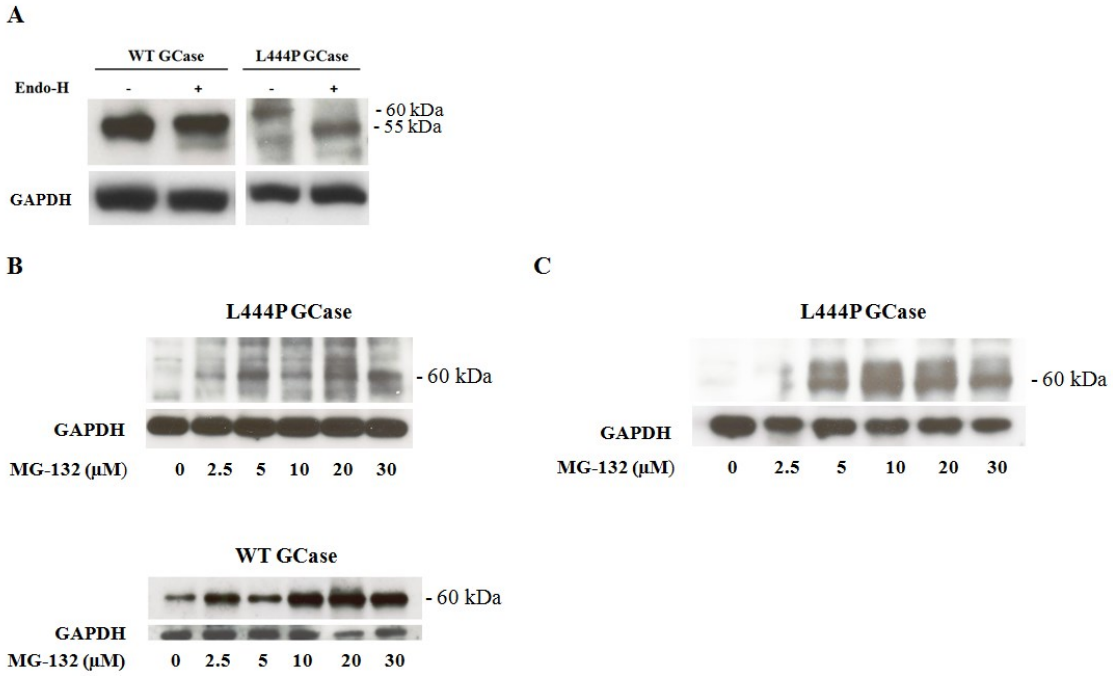


Figure 3-2: Endo H sensitivity of WT- and L444P- GCase (A) and western blot analyses to determine the effect of the proteasome inhibitor MG-132 on the accumulation of WT- and L444P- GCase in BY2 transgenic lines (B, C).

For Endo H analyses (**A**), lysates (25 µg) of BY2 cells expressing WT- or L444P- GCase were subjected to overnight digestion with Endo H prior to western blotting. To examine the effects of MG-132 on the steady-state levels of GCase protein (L444P and WT), the transgenic BY2 cells were transferred to solid fresh media containing different concentrations of MG-132 (as indicated) and grown for 3 d (**B**) and 5 d (**C**). WT GCase line 60 described in Babajani et al. (2012) was used in this study. Protein extracts from BY2 cells (20 µg total soluble protein) were subjected to western blot analysis. GAPDH was used as a loading control. Note that the multiple bands (~60 kDa) evident in figure (**C**) likely represent various glycoforms of the GCase enzyme expressed in the plant cells.

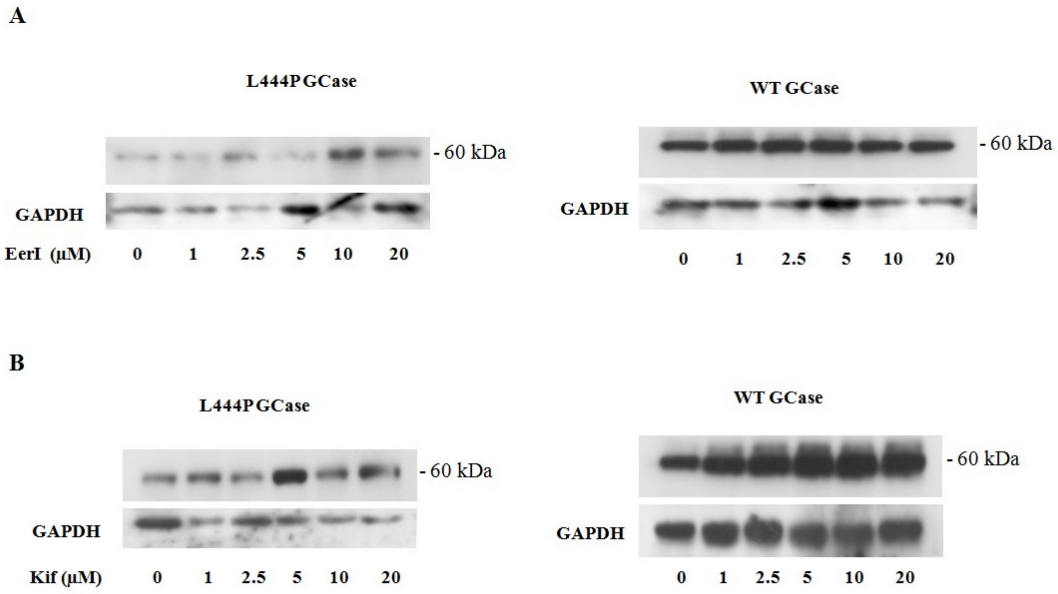


Figure 3-3: Effect of EerI (A) and Kif (B) on the steady-state intracellular levels of WT- and L444P- GCase proteins of BY2 cells as determined by western blot analyses.

Cells were treated with different concentrations of EerI or Kif as indicated for 5 d. GAPDH was used as a loading control.

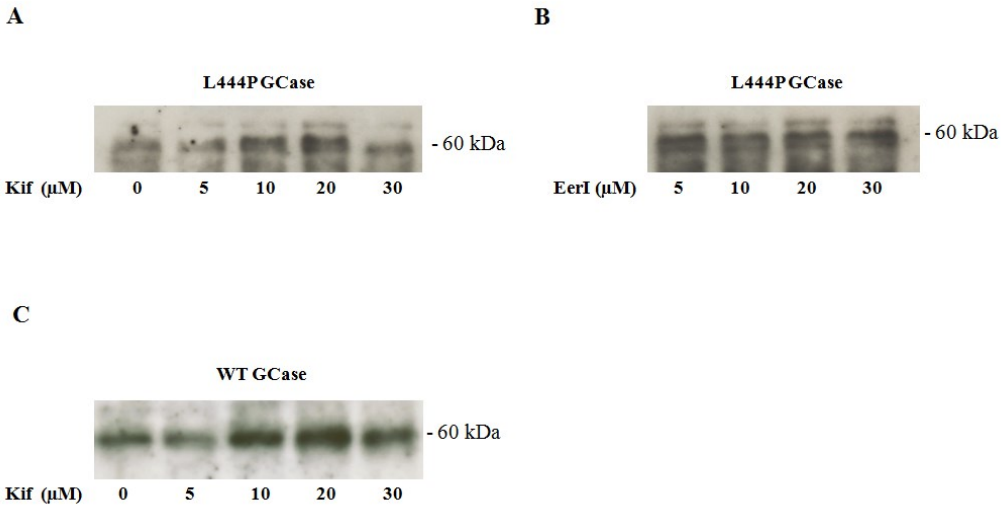


Figure 3-4: Effect of Kif (A, C) and EerI (B) on GCase secretion from transgenic protoplasts expressing WT GCase (C) or L444P GCase (A, B).

Protoplasts (200,000 per mL) were incubated in control culture medium (no inhibitor; "0") or in culture medium containing different concentrations of Kif or EerI (as indicated) for 3 d. For (A) and (B) (L444P), one lane was used as the control (0 µM; shown in (A) only) for the Kif and EerI inhibitor treatments. Acetone-precipitated proteins from 0.5 mL media were subjected to western blot analyses. Evans blue tests of protoplast viability during a 3-d period are shown in Figure S3.

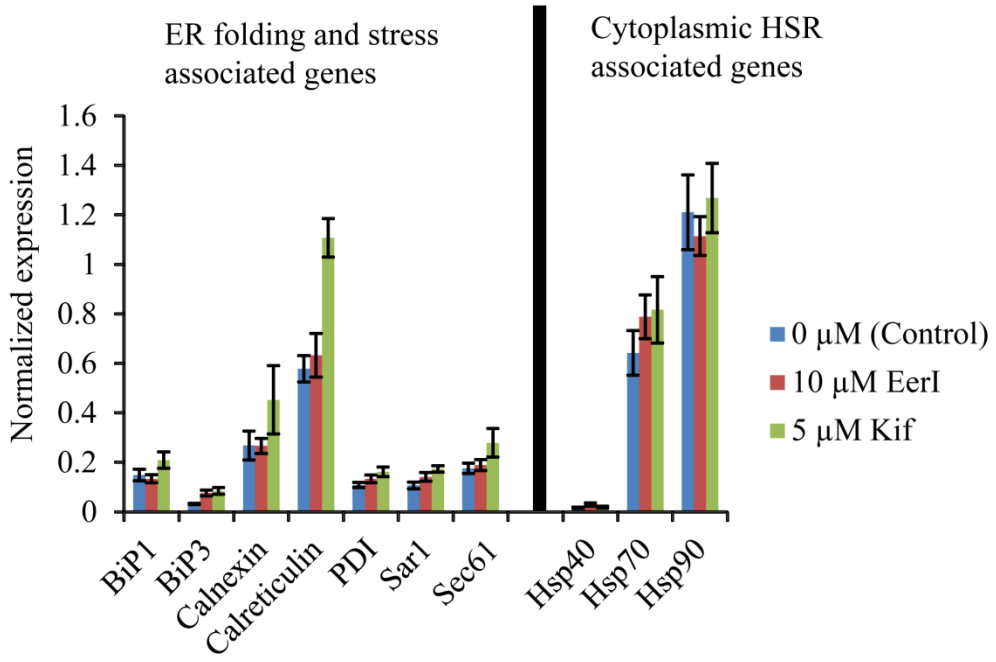


Figure 3-5: Expression analyses of ER chaperone-, ER-stress-response- and cytoplasmic heat shock response (HSR)- genes after treatment with ERAD inhibitors EerI and Kif.

BY2 cells expressing L444P GCase were grown in solid culture medium in the absence or presence of ERAD inhibitors for 5 d prior to quantitative (q)RT-PCR. GAPDH was used as a housekeeping gene to normalize the expression. Averages of 3 biological replicates are shown +/– SE.

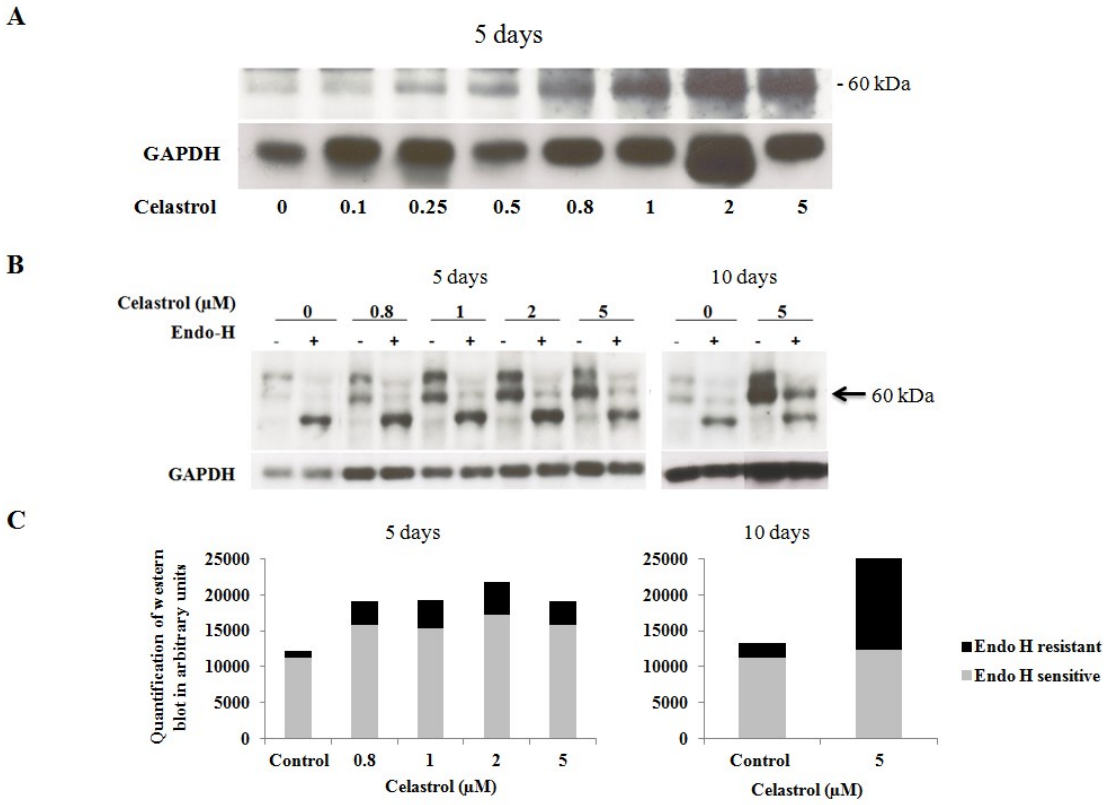


Figure 3-6: Effect of celastrol on intracellular levels (A) and Endo H sensitivity (B,C) of L444P GCase in BY2 cells. Seven-d-old BY2 cells were transferred to solid media containing different concentrations of celastrol (as indicated).

(**A**) Effect of celastrol on the intracellular levels of L444P GCase as determined by western blot analysis. (**B**) Effect of celastrol (5- and 10-d exposure) on L444P GCase trafficking within BY2 cells as determined indirectly by Endo H analyses. Arrow head indicates Endo H-resistant forms of L444P GCase. GAPDH was used as a loading control. (**C**) Quantification of Endo H results using National Institutes of Health ImageJ analysis software. The gray portion of each bar represents Endo H-sensitive bands (lower molecular weight) corresponding to ER-retained L444P GCase and the black portion of the bar represents Endo H-resistant bands (higher molecular weight) corresponding to the post-ER trafficked L444P GCase (Golgi transit).

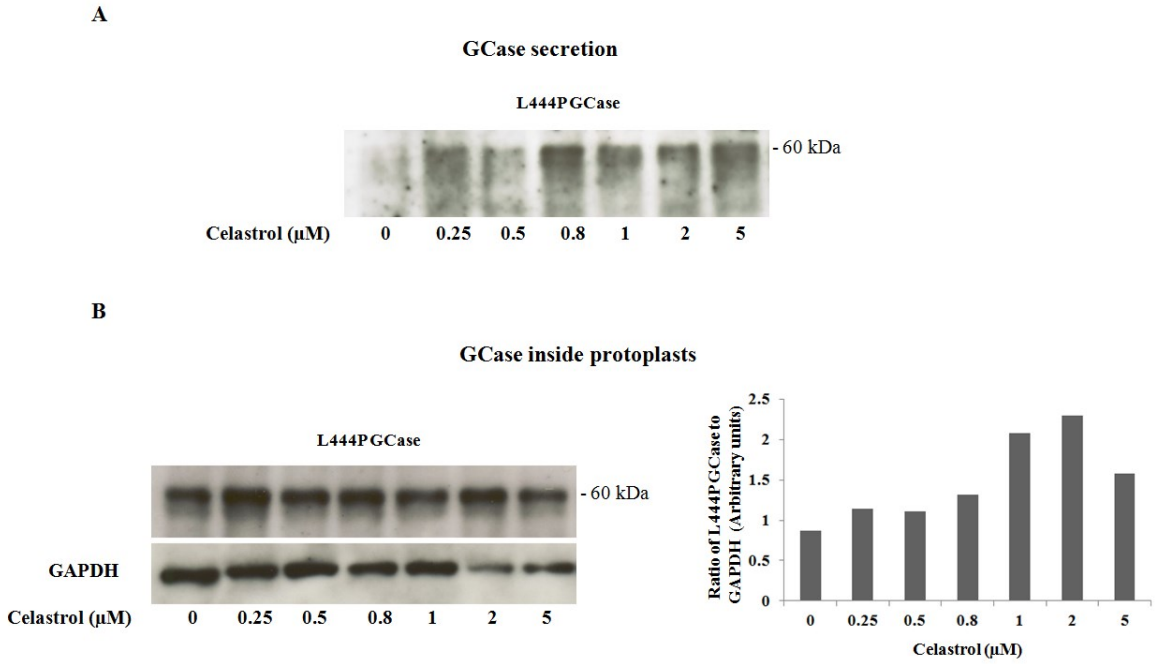


Figure 3-7: Effect of celastrol on L444P GCase secretion from transgenic protoplasts (A) and on L444P GCase accumulation levels inside the protoplasts (B).

Protoplasts (200,000 per mL) were incubated in culture medium with no celastrol or in culture medium containing different concentrations of celastrol (as indicated) for 3 d. Acetone-precipitated proteins from 0.5 mL media were subjected to western blot analyses in (A) Extracts from the transgenic protoplasts were used to determine the intracellular levels of L444P GCase in (B). The marker protein GAPDH was used as control for loading on the same membranes following stripping of membranes and incubation with GAPDH antibodies. Band intensities were quantified with NIH ImageJ software. The ratio of L444P GCase to GAPDH is shown ("B" right panel).

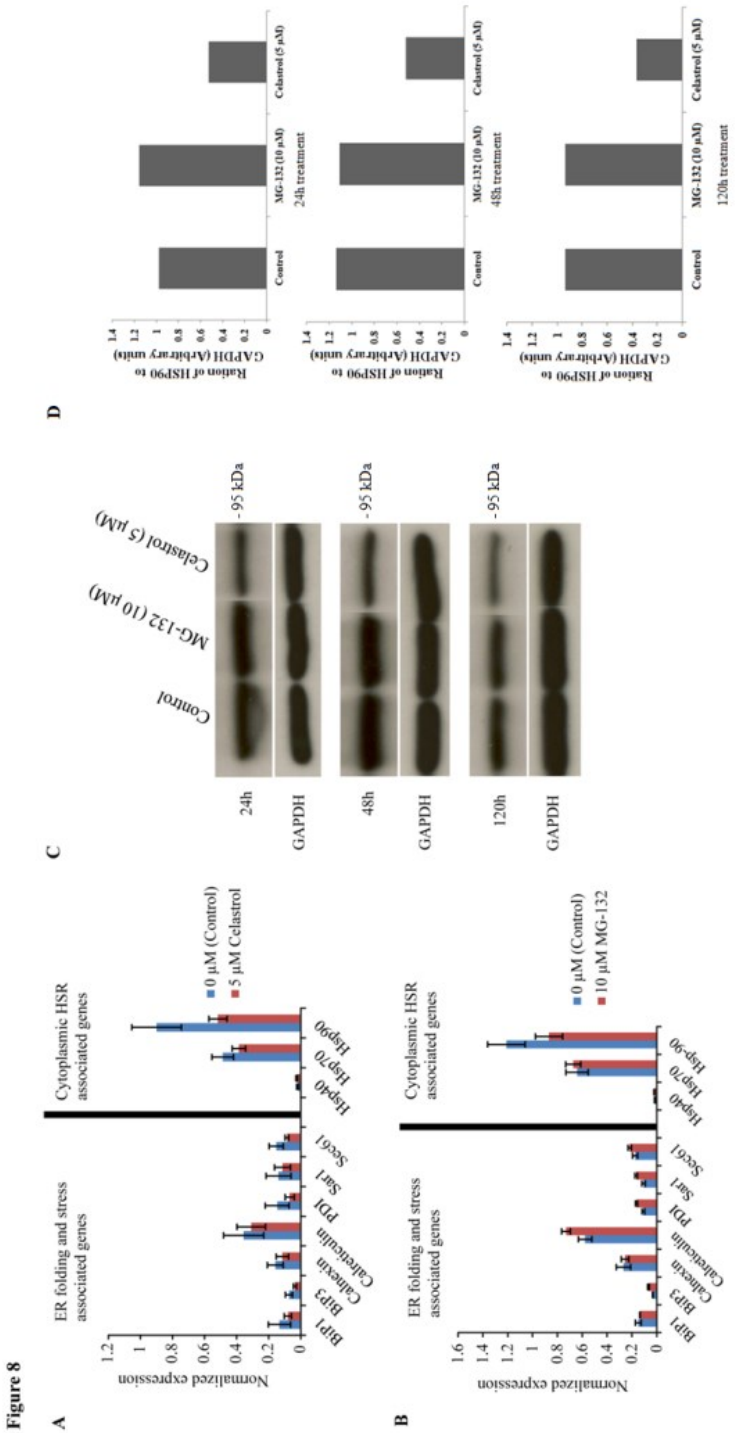


Figure 3-8: Expression analyses of ER chaperone-, ER-stress-response- and cytoplasmic heat shock response (HSR)- genes in response to MG-132 (A) or celastrol (B) treatments.

Transgenic cells expressing L444P GCase were grown in solid culture medium in the absence or presence of MG-132 (10 µM) or celastrol (5 µM) for 5 d, in (**A**) and (**B**) respectively, prior to qRT-PCR analyses. GAPDH was used as a housekeeping gene. Values are the mean ± standard error of three or four biological replicates for MG-132 or celastrol treatments, respectively. (**C**) HSP90 abundance in BY2 cells treated with celastrol or MG-132 for different durations as determined by western blot analyses. GAPDH is shown as a loading control. (**D**) The band intensities were quantified using the ImageJ software and presented as the ratio of HSP90 to GAPDH loading control.

3.9. Supplementary Data

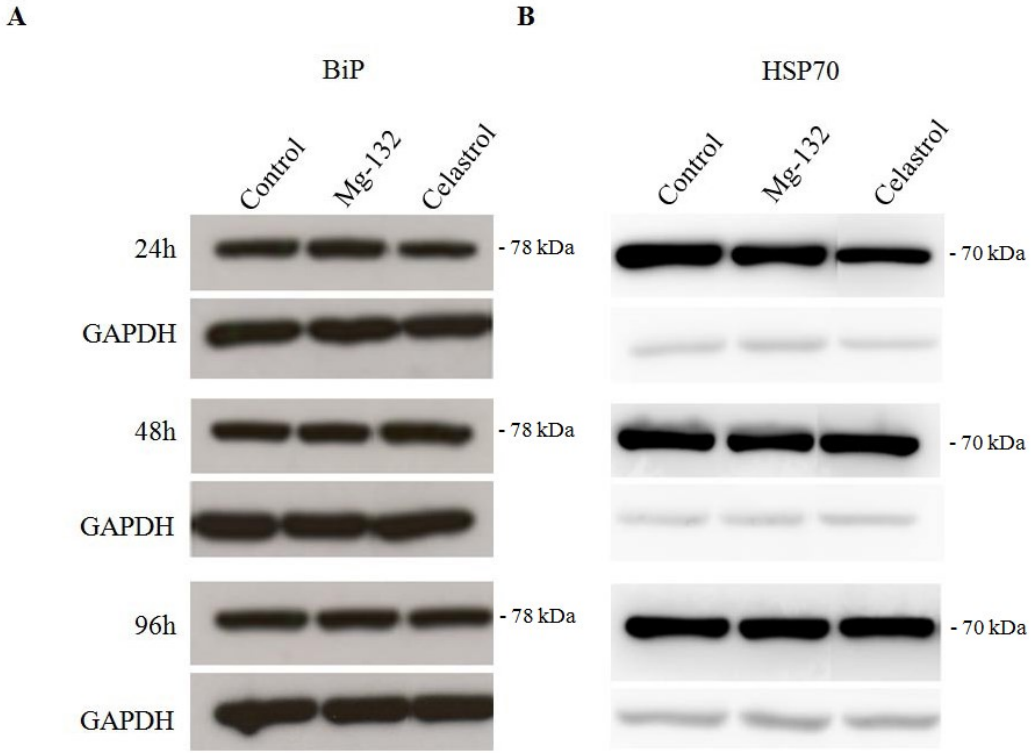


Figure 3-S 1: Western blot analyses of BiP (A) and HSP70 (B) in BY2 cells treated with MG-132 or Celastrol.

BY2 cells expressing L444P GCase were grown on solid control culture medium (no inhibitor) or on culture medium containing MG-132 (10 µM) or Celastrol (5 µM) for 24, 48 and 96 h prior to western blot analyses. GAPDH was used as a loading control.

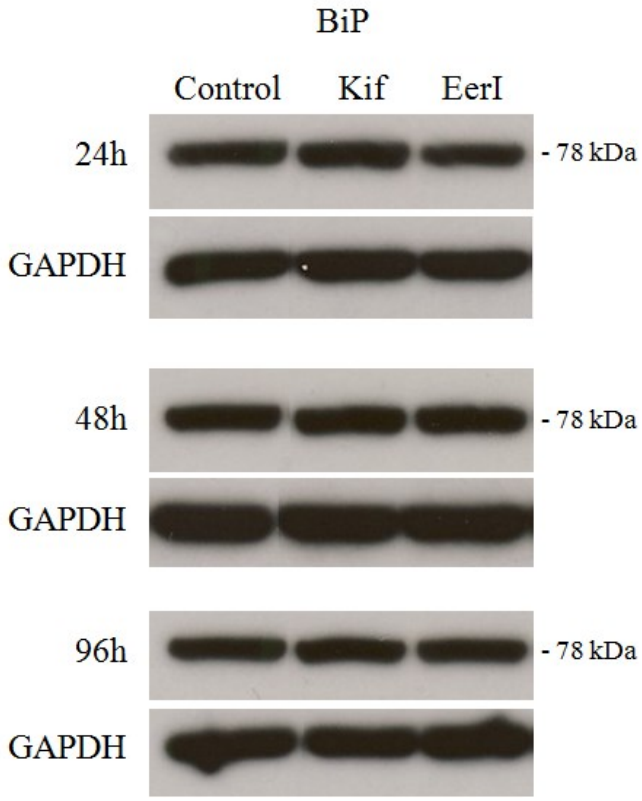


Figure 3-S 2: Western blot analyses of BiP in BY2 cells treated with Kifunensine or Eeyarestatin I.

BY2 cells expressing L444P GCase were grown on solid control culture medium (no inhibitor) or on culture medium containing Kif (5 µM) or EerI (10 µM) for 24, 48 and 96 h prior to western blot analyses. GAPDH is shown as a loading control.

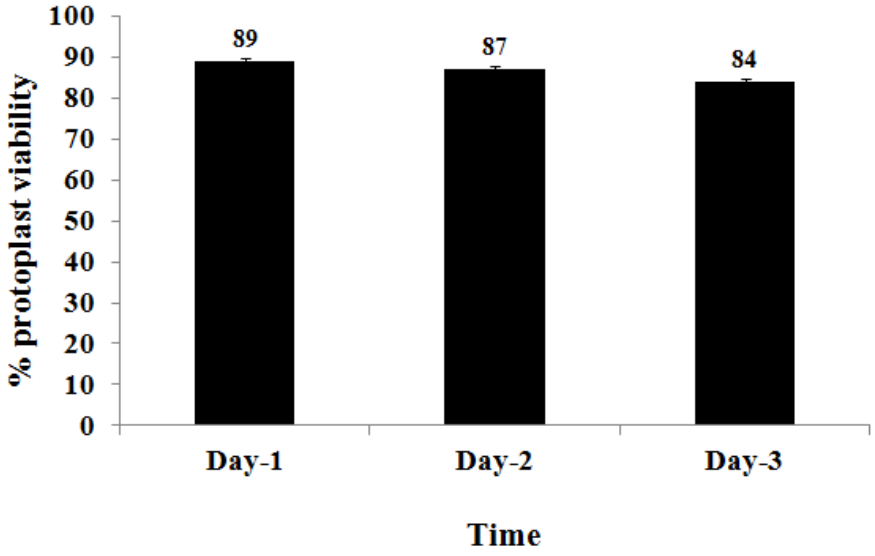


Figure 3-S 3: Viability of the population of protoplasts (200,000 per sample) over a 3-d period (representative of the treatment/incubation duration).

Protoplasts were isolated from transgenic BY2 cells expressing mutant GCase and the viability at d 1, 2 and 3 determined using Evans Blue. Percent viability (as compared to freshly harvested [d=0] protoplasts) was calculated daily. Bars are based on three biological replicates $\pm$ S.E.

Table 3-S 1: Primers used to analyse gene expression

Nicotiana tabacum (tobacco) ER chaperone- and ER-stress responsive genes

Sar1-F	TGGGATTATGGCAGAAGGAG
Sar1-R	TTGGGTATTGTGTTGGCTGA
Putative Sec61-F	GAGATTTCGCCAAAGACAGC
Putative Sec61-R	ACCAATCGCCGTACGAGTAG
BiP3-F	AAGCTCGAGTCCGATGAGAA
BiP3-R	CAGCTGTGATGATTGGGTTG
PDI-F	AACGCGGGTATTGTTGAGTC
PDI-R	TCAGCTTCCCAGCTTCATCT
Calreticulin-F	CCTCGAGGAGAGTTTCAACG
Calreticulin-R	CTCCATTCCACTTGCCAGAT
Putative Calnexin-F	GTGGAGCCACAAACAAGGTT
Putative Calnexin-R	CTCATTGTTCGGTCTCAGCA
BiP1-F	CGTCATCCCAACCAAGAAGT
BiP1-R	TCCAAGCAGCCTACAGTCCT

***Nicotiana tabacum* cytoplasmic heat shock response genes**

Putative Hsp40-F	ACACACTTGGATGGCAGACA
Putative Hsp40-R	CCCTCATAAATGGCCTCTGA
Hsp70-F	TGCTGAAGCTTTCCTTGGTT
Hsp70-R	AAGACCGGCAATAACACCAG
Hsp90-F	TGCTCAACCTGAGCTCTTCA
Hsp90-R	CTCGCAATTGTACCCAGGTT

***Nicotiana tabacum* qRT-PCR reference gene**

GAPDH-F	GGTGCCAAGAAGGTTGTGAT
GAPDH-R	CAAGGCAGTTGGTAGTGCAA

Chapter 4.

An *in vivo* plant-cell-based screening system for identifying putative pharmacological chaperones for treatment of the human lysosomal storage disease Mucopolysaccharidosis type I (MPS I).

The above-titled work was prepared a manuscript for submission (still under preparation):

Gholamreza Babajani[1], Xu He[1], Sheetal Raithatha[2], Tom Pfeifer[2], Lorne A. Clarke[3] and Allison R. Kermode[1]

[1]Department of Biological Sciences, Simon Fraser University, 8888 University Dr., Burnaby, BC, V5A 1S6, Canada

[2]The Center for Drug Research and Development, Pharmaceutical Sciences Building, University of British Columbia, 2405 Wesbrook Mall, Vancouver, BC V6T 1Z3 Canada

[3]Department of Medical Genetics, University of British Columbia, The Child and Family Research Institute, 950 W 28th Avenue, Vancouver, British Columbia, Canada V5Z 4H4

Keywords: α-L-Iduronidase, missense mutations, mucopolysaccharidosis I, high-throughput screening (HTS), cell-based assay, tobacco BY2 cells, protoplasts, post-ER transport, secretion

Abbreviations: IDUA: α-L-iduronidase; ERT: enzyme replacement therapy; PC: pharmacological chaperone; ERAD: ER-associated degradation

4.1. Abstract

Small-molecule- enzyme enhancement therapy has emerged as an attractive approach for the treatment of lysosomal storage diseases (LSDs), a broad group of genetic diseases caused by mutations in genes encoding lysosomal enzymes or proteins required for lysosomal function. Missense mutant lysosomal enzymes normally subjected to rapid disposal by ER-associated degradation can be stabilized by small molecule chaperones that increase residual enzyme activity largely by increasing the transport and maturation of the mutant enzyme. We developed a plant-cell-based screening system to identify putative small molecule therapeutics for the LSD mucopolysaccharidosis I (MPS I), by selecting for library molecules capable of enhancing the post-ER transport of two missense mutant α-L-iduronidase (IDUA) proteins, P533R and R383H. Since the recombinant IDUA variants were equipped with a signal peptide, and the expression cells - transgenic tobacco BY2 cells - possess no lysosomes, the assay was based on increased IDUA activity in the secretion media. The plant-cell-based assay was developed to screen a library of 1,040 Food and Drug Administration-approved drugs. Downstream validation of the hits identified in the primary screening resulted in the identification of a potential candidate molecule for P533R IDUA. The candidate compound (X-372) significantly increased the amount of active P533R-IDUA and wildtype-IDUA in the medium and further enhanced the secretion of WT and mutant IDUA proteins into the culture medium surrounding the transgenic BY2 protoplasts. Further development of this small molecule may yield a potential therapeutic for attenuated MPS I patients for which there is no impairment or only slight impairment of the functional domains of the mutant IDUA protein.

4.2. Introduction

Lysosomal storage disorders (LSDs) are a group of over 70 diverse genetic diseases that result from mutations in genes encoding lysosomal enzymes or proteins required for lysosomal function (Shultz et al. 2011). For many LSDs the underlying etiology involves the absence or deficiency of a single lysosomal hydrolase. The intralysosomal accumulation of the enzyme's substrate(s) and disruption of other cellular

processes lead to disease via primary and secondary mechanisms (Futerman and van Meer 2004; Shultz et al. 2011; Ballabio et al. 2011). Mucopolysaccharidosis I (MPS I), an autosomal recessive LSD characterized by progressive dysfunction of multiple organ systems (Clarke 2008), is caused by a deficiency of α-L-iduronidase (IDUA), a soluble lysosomal hydrolase that mediates an early step in the degradation of the glycosaminoglycans (GAGs), heparan sulphate and dermatan sulphate. More than 100 mutations in the IDUA gene underlie MPS I diseases (Terlato and Cox 2003), and three clinical phenotypes of varying severity are used to describe MPS I patients: Hurler, Hurler-Scheie and Scheie for the severe, intermediate and attenuated forms of the disease, respectively (Roubicek et al. 1985; Clarke 2011). With the recent elucidation of the three-dimensional structure of IDUA (Bie et al. 2013; Maita et al. 2013), it is now possible for the first time to link a given missense mutation (and corresponding defect in the IDUA enzyme) to the clinical phenotype, provided that the relevant biochemical and cell biology studies on protein function/stability, and cellular homeostasis are also undertaken.

The present approved treatments for LSDs are enzyme replacement therapies (ERT) involving the intravenous administration of a purified recombinant human lysosomal enzyme via weekly infusions into patients – a process that takes advantage of plasma membrane receptor-mediated cellular uptake of the deficient enzyme (Grabowski and Hopkin 2004, Desnick and Schuchman 2012). ERT is available for only 7 of the LSDs. ERT for MPS I has altered the natural history of this rare disease but the treatment does not significantly impact the most devastating disease complications of progressive bone, joint and cardiac involvement (Clarke 2008). In addition, the recombinant enzyme is unable to pass through the blood-brain barrier (BBB) and thus CNS pathology in patients affected by the more severe 'neuronopathic' forms of MPS I is entirely recalcitrant to treatment (Desnick and Schuchman 2012). Another challenge is associated with the relatively high cost of ERT for LSDs; the average annual drug cost of ERT for a 12 year old child with MPS I is over $400,000 USD. Some of these drawbacks have led to the pursuit of small molecule 'pharmacological chaperones' (PCs) as potential alternative or adjunctive therapeutics. These small molecules have the potential advantages of being less costly, amenable to oral delivery, and allowing for passage through the blood-brain-barrier; they are attractive for treatment of LSDs as many of the

underlying disease-causing mutations are missense mutations or small deletions that lead to the deficiency of a hydrolytic enzyme because of a defect in its folding and/or maturation (Sawkar et al. 2005; Yu et al. 2007; Fan 2008; Valenzano et al. 2011).

Various library screens have identified pharmacological chaperones, many of which are active-site-specific inhibitors or 'mixed' inhibitors. Lead pharmacological chaperones and other small molecules are being investigated for a number of LSDs including Gaucher, Fabry, and Tay-Sachs/Sandhoff diseases (Sawkar et al. 2005; Steet et al. 2006; Yu et al. 2007; Tropak et al. 2007, 2008; Fan 2008; Maegawa et al. 2009; Khanna et al. 2010; Goddard-Borger et al. 2012; Zimran et al. 2013). The small molecule appears to act as a folding template to assist the mutant protein in achieving a native-like conformation in the endoplasmic reticulum (ER). Because of this, a greater proportion of synthesized variant proteins are able to achieve their correctly folded conformation and thus undergo post-ER transport. It is also possible that the pharmacological chaperone stabilizes the correctly folded proteins due to their improved thermodynamic stability. Folded lysosomal enzymes can now pass the quality control mechanisms of the ER and ultimately traffic to the lysosome; in essence, the equilibrium is shifted toward folding at the expense of ER-associated degradation (ERAD) and other disposal pathways. As such, the small molecule, when used at sub-inhibitory concentrations, would ultimately be effective in restoring residual activity of the enzyme in the lysosome (*E*nzyme *E*nhancement *T*herapy; EET).

In order to identify putative pharmacological chaperones, a number of different high-throughput screening methods have been utilized (Fan 2008, Beck 2010), including the use of purified enzyme to identify inhibitors *in vitro* (Tropak 2007), and thermodenaturation assays (Maegawa et al. 2009). Patient-derived cell lines have also been used in throughput screens (Geng et al. 2011), as they have the added advantage of potentially providing access to targets associated with biochemical changes or signalling pathways that are secondary to the disease process (Geng et al. 2011). However, the inadvertent elimination of potential leads may occur in the primary screening due to extremely low residual enzyme activities in the cells, particularly if lines are used that carry mutation(s) that severely destabilize the lysosomal protein, leading to its rapid disposal by ERAD.

In the presents study, we describe a plant-cell-based system for library screening that proved to be efficacious in respect of identifying putative small molecule chaperones. This was achieved by selecting for library molecules that enhanced the post-ER transport of two missense mutant IDUA proteins (P533R- and R383H- IDUA), expressed transgenically in tobacco Bright-yellow2 (BY2) cultured protoplasts. The screening of a library of 1,040 Food and Drug Administration-approved drugs (the National Institute of Neurological Disorders and Stroke collection) uncovered several candidate molecules that increased IDUA activity in the secretion media. Downstream validation assays identified one molecule in particular that is now under intensive study as a putative MPS I therapeutic.

4.3. Materials and Methods

4.3.1. Generation of transgenic tobacco BY2 lines expressing WT and mutant IDUA

The P533R and R383H mutations were engineered within the human wildtype (WT)-IDUA cDNA as described in Bie et al. (2013) for the P533R-IDUA using the Quick Change site-directed mutagenesis kit (Stratagene, Santa Clara, CA, USA). To allow for secretion of the mutant-variant- and WT- IDUA into the culture medium, a signal-peptide-encoding sequence from the barley proaleurain gene (Tse et al. 2004) (MAHARVLLLALAVLATAAVAVA) was fused upstream of the respective coding sequences using the following pair of primers: 5'-CTCGCCGTCCTGGCCACGGCCGCCGTCGCCGTCGCCGAGGCCCCGCACCTGGTG CAGGTG-3' (SPp-IDUA-F1), 5'-CACCATGGCCCACGCCCGCGTCC TCCTCCTGGCGCTCGCCGTCCTGGCCACGGCCG-3' (SPp-IDUA-F2), TCAT GGATTGCCCGGGGATGG (IDUA-R) Re-amplified cDNA products were cloned into a Gateway entry vector pENTR/D-TOPO (Invitrogen, Burlington, ON, Canada). The nucleotide sequences were then determined using the M13 reverse and forward universal primers. The resulting constructs containing sequences encoding WT, P533R-IDUA and R383H mutants were sub-cloned into a Gateway expression vector (pSITE-0B) (Chakrabarty et al. 2007), which carries the neomycin phosphotransferase II gene (for kanamycin resistance) and the cauliflower mosaic virus (CaMV) 35S promoter (with

a duplicated enhancer element; 2X35S) to drive expression of the WT and mutant genes.

Vectors containing the IDUA expression constructs were introduced into *Agrobacterium tumefaciens* (GV 3101) by electroporation, and the transformed *Agrobacterium* suspension cultured cells were used to generate transgenic tobacco BY-2 cells as described previously (Mayo et al. 2006; Fu et al. 2009). Transgenic BY2 cell lines were maintained on solid Murashige and Skoog (MS) culture medium (PhytoTechnology Laboratories, Overland Park, KS, USA) and sub-cultured twice a month.

4.3.2. Northern blot analyses to detect IDUA transcripts of transgenic BY-2 lines

Northern hybridization analysis to detect steady-state levels of transcripts for WT and mutant IDUA in transgenic BY2 cells was performed using radiolabelled full-length cDNA probe for the human *IDUA* gene and 15 µg of total RNA extracted with Trizol reagent (Sigma-Aldrich, St. Louis, MO, USA) as outlined in Babajani et al. (2012).

4.3.3. Protoplast isolation from tobacco BY2 cells maintained on solid cultures

Protoplasts were isolated as described previously (Lee et al. 2008, Babajani et al. 2012). The freshly isolated protoplasts were collected and re-suspended in protoplast culture medium (4.3 g/L MS salts, 100mg/L myo-inositol, 1 mg/L thiamine, 0.2 mg/L 2,4-dichlorophenoxyacetic acid, 255 mg/L KH2PO4, 30 g/L sucrose, pH 5.7). After each protoplast isolation, viability of protoplasts was determined with 0.02% solution of Evans blue (Sigma-Aldrich, St. Louis, MO, USA) prepared in protoplast culture medium. The number of protoplasts was estimated by counting with a haemocytometer (Hausser Scientific, Horsham, USA).

4.3.4. Determination of IDUA activities in cellular protein extracts and secretion media

To assess the population of BY2 transgenic lines for IDUA activities, assays were conducted using extracts derived from intact BY2 cells cultured on solid medium. To assess IDUA secretion, western blot analyses and activity assays were performed directly on the liquid culture media surrounding transgenic BY2 protoplasts (derived from selected lines as indicated in Figure legends). All of the IDUA activity assays used the fluorogenic substrate sodium 4-methylumbelliferyl α-L-idopyranosiduronic acid (4MUI) (Toronto Research Chemicals Inc., Toronto, ON, Canada) (Hopwood et al. 1979; Clements et al. 1985; Downing et al, 2006). Twenty five µL assay buffer (0.89 mM 4-MUI, 0.1 M dimethylglutarate buffer, pH 4.5, 2 mM sodium metabisulfite and 0.7% bovine serum albumin) were added to 25 µL of soluble extract (10-20 µg total protein) or liquid culture media as appropriate and the mixture incubated at 37 °C. The reaction was terminated by addition of 200 µL stop buffer (2-amino-2-methyl-1-propanol, 0.1M). Fluorescence of the reaction product, 4MU, was determined by using a microplate fluorometer (excitation, 365 nm; emission, 450 nm).

4.3.5. Enzyme kinetic parameters of purified R383H- and wild-type-IDUA

After screening the population of transgenic BY2 lines by IDUA activity assays and western blot analyses, two stable high-expressing BY2 lines for WT- and R383H-IDUA (lines 9 and 42 were identified for IDUA protein purification. IDUA was purified to homogeneity as indicated by SDS-PAGE analysis using methods described in Bie et al. (2013). Michaelis-Menten kinetics for plant-derived IDUA were determined at 37 °C and pH 4.5 using the fluorometric assay described in He et al. (2012). Reactions were performed in a total volume of 100 µL, with ~35 ng of purified plant-derived IDUA (BY-2-derived WT IDUA and R383H-IDUA) in a buffer containing 0.1 M dimethylglutarate (pH 4.5), 2 mM sodium metabisulfite and 0.35% bovine serum albumin and the 4-MUI substrate (at concentrations of 1-750 µM) (Bie et al. 2013). All measurements were made in triplicate. Rates of reaction were determined by dividing by the reaction time and concentration of the enzyme, and fit to a Michaelis-Menten curve using GraphPad

Prism version 6.01 for Windows (GraphPad Software, La Jolla, California, USA, www.graphpad.com).

4.3.6. Primary Screening of NINDS Library

The National Institute of Neurological Disorders and Stroke (NINDS) collection of 1,040 compounds was used to perform the screening, after first diluting the library in 10% dimethyl sulfoxide (DMSO). Protoplast culture media (45 µL) was first transferred to each well of clear 96-well flat-bottom plates (Corning) using Matrix Wellmate microplate dispenser (Thermo Scientific, Waltham, MA, USA) followed by automated transfer of 5 µL of NINDS library compounds using the Biomek FX (Beckman Coulter) (20 µM final concentration) to each well. Finally, 20,000 protoplasts were dispensed per well using the Matrix Wellmate microplate dispenser (total final volume 100 µl) and then incubated with NINDS compounds (one compound/well) for 3 d at 27 °C. After the 3 d, twenty five µl of media was transferred to a black 96 well standard assay plate (Corning) using the Biomek FX and analyzed for IDUA activity by adding 25 µL of assay buffer containing 4-MUI substrate for 5 h. The assay was terminated by increasing the pH to 10.5 by adding 2-amino-2-methyl-1-propanol (0.1M, 200 µL). The fluorescent product generated was measured using a microplate fluorometer (excitation, 365 nm; emission, 450 nm) (Varioskan Flash Microplate Reader, Thermo Scientific, Waltham, MA, USA). The statistical Z' factor test was used to measure the quality of the assay. Assays with a Z' factor of $\geqq$ 0.5 indicate that the assay is robust enough to identify enhancers/inhibitors of enzyme activity reliably (Zhang et al. 1999). The percent activation in response to NINDS compounds was calculated according to the equation: %Activation = (X-C-)/(C+-C-)*100, where X is the reading per well, C- is the negative control average (untransformed cells in the presence of DMSO) and C+ is the positive control average (activity of secreted mutant IDUA in the presence of DMSO). Compounds of interest demonstrating statistical significance (i.e. hits from the primary screening effort) were defined as those that decreased or increased the amount of secreted IDUA activity by <30% or >160%, respectively

4.3.7. Reconfirmation Stage of the Primary Screening Process

Following the primary screening, the hit compounds as described above were re-tested in screening reconfirmation assays. Single-point reconfirmation assays were conducted first using identical conditions to those of the primary screening. Those compounds that returned significant activity were then subjected to concentration-response evaluation. Within the parameters defined above, there were 4 compounds that enhanced and 5 compounds that decreased the amount of secreted IDUA activity. The dose-response curves of the four candidate compounds selected from the reconfirmation screening were determined by the endpoint IDUA assay, in the presence of seven concentrations (0.02–120 μM) of candidate compounds.

4.3.8. Heat inactivation assay

Heat inactivation experiments were performed using P533R and WT- IDUA proteins purified from tobacco BY2 transgenic lines (lines 9 and 20, respectively). As another control, the purified wild-type IDUA from seeds of *cgl* (complex-*gl*ycan-deficient) Arabidopsis plants was also used (He et al., 2013; Bie et al., 2013). The WT- and mutant- IDUA proteins were purified using concanavalin A-Sepharose and anti-IDUA affinity chromatography as described previously (He et al. 2012). Equal amounts of purified enzymes (~ 0.05 μg) were diluted in CP buffer (pH 4.2), containing 0.5% BSA. Diluted samples of purified enzymes containing different concentrations of the test compound (300 μM, 600 μM and 1200 μM) or DMSO were split into two aliquots; one was left on ice, and the other heat-treated at 50 °C for 15, 30, 45, 60, 75 and 90 min. The heat-treated enzymes were kept on ice until completion of the time series. For assaying enzyme activity, samples were pre-equilibrated at room temperature for 10 min, followed by addition of IDUA assay buffer, and incubation at 37 °C for a further 30 min. Remaining activity was calculated as a ratio of IDUA activity in the presence of the test compound during heat incubation versus activity of the corresponding aliquot that was kept on ice.

4.3.9. Western blot analyses of IDUA protein in cellular protein extracts and secretion media

Western blot analyses were conducted on extracts derived from intact BY2 cells cultured on solid medium (e.g. for screening the population of the transgenic BY2 lines) as well as on proteins of the liquid culture media surrounding transgenic BY2 protoplasts after their precipitation (see below) and solubilization in 100 uL of IDUA extraction buffer (20 mM Tris, 0.5 M NaCl, 0.02% sodium azide, pH 7.0, protease inhibitor cocktail (Sigma)). Protein extracts from BY2 transgenic lines (20 µg total soluble protein) were separated by 12% SDS-PAGE and transferred onto Amersham Hybond-P (PVDF) membranes. Western blotting was performed using primary antibodies (rabbit anti-IDUA 1A (1: 500) (Fu et al. 2009), followed by secondary antibody (HRP-conjugated goat anti rabbit (Millipore) (1:5000) as described previously (Babajani et al. 2012). The same blot was then stripped with guanidine hydrochloride stripping buffer (6 M GnHCl, 0.2% Nonidet [NP-40], 0.1 M β-mercaptoethanol, 20 mM Tris-HCl, pH 7.5) for 10 min (Yeung and Stanley 2009) and re-probed with an anti-GAPDH primary antibody (1:10000 dilution) as a loading control.

4.3.10. Precipitation of secreted protein from the liquid medium of cultured BY2 protoplasts

For protein precipitation from the cultured media of protoplasts, the culture medium was mixed with chilled acetone in a ratio of 1:5 with gentle shaking and incubated at -20 °C for 1 h, followed by centrifugation at 10,000 ***g*** for 30 min. The resulting precipitates were re-suspended in IDUA extraction buffer and mixed with SDS-PAGE running buffer prior to separation by SDS-PAGE, followed by western blot analysis as previously described.

4.3.11. Preparation of total soluble protein extracts from tobacco BY2 cells grown on solid media and from BY2 protoplasts

BY2 cells (100 mg) grown on solid media or protoplasts (200,000) were cooled on ice in a 1.5-mL microcentrifuge tube in 100 µL IDUA extraction buffer along with glass beads (3mm, Fisher scientific, Ottawa, ON, Canada) to facilitate grinding by vortexing.

Cell debris was removed by centrifugation at 14000 g for 15 min at 4 °C and the supernatant used for western blot analysis. Protein concentrations were determined using the Bio-Rad DCTM (Bio-Rad Laboratories, Mississauga, ON, Canada) protein assay kit with BSA as a standard.

4.4. Results

4.4.1. Characterization of tobacco BY2 cells expressing recombinant wild-type- and mutant α-L-iduronidase (IDUA)

Tobacco BY2 cells were stably transformed with the IDUA gene constructs and regenerated lines were screened by PCR. Northern blot analysis of the PCR-positive transgenic cell lines showed differences in steady-state levels of the transcripts amongst the population of lines studied (wild-type (WT)-IDUA, P533R-IDUA and R383H-IDUA) and some high expression lines were identified (line 9 for P533R IDUA and line 42 for R383H IDUA) (Figure 4-1 A). Western blot analysis of selected high-expressing lines transgenic for WT-IDUA and mutant IDUA (two lines per construct) detected variable levels of the ~ 78 kDa IDUA protein (Figure 4-1 B). In general, the protein levels and activities associated with the BY2 lines expressing the mutant IDUAs were lower than those associated with the transgenic lines expressing the WT-IDUA (Figure 4-1 B and C). However, R383H-IDUA protein and activity were the lowest detected by western blotting and enzyme assay (Figure 4-1 B and C).

4.4.2. Establishment of the screen: secretion characteristics of the recombinant proteins *in vivo*

To establish the basis for our in vivo BY2 screening system, we first monitored the secretion characteristics of the recombinant WT- and mutant-IDUA proteins from BY2 protoplasts (BY2 cells without their surrounding cell walls) cultured in liquid media. For this we used the BY2 cell lines that were determined to be high-expressing lines as noted above (Lines 19 and 20 for WT IDUA, line 9 for P533R IDUA and line 42 for R383H IDUA). Our premise was that WT-IDUA would be relatively efficiently secreted, while the two mutant IDUA proteins would be less efficiently secreted from the BY2

protoplasts due to their reduced ability to undergo post-ER transport. Using a standardized number of protoplasts (2 x 105) we determined whether the WT- and mutant- IDUA enzymes were secreted from the transgenic BY2 protoplasts, and further whether activity assays of the media represented a sensitive means of determining protein secretion. As noted subsequently (Figure 4-7, “0”), as compared to the WT-IDUA protein, the mutant proteins were secreted to a lesser extent, as expected. Enzymatic assays on 25 µL culture media surrounding the transgenic protoplasts were conducted at daily intervals during a three-day culture period; these assays showed that the small amounts of proteins that were secreted from transgenic P533R- and R383H-IDUA protoplasts were nonetheless sufficient to be detected with the sensitive activity assay. As expected, the absolute levels of activity secreted from the BY2 protoplasts transgenic for the two mutant proteins were less than those from the protoplasts transgenic for the WT IDUA (Figure 4-1 D). The western blot analyses presented subsequently (Figure 4-7, “0”) indicated that the R383H-IDUA protein was less capable of secretion from protoplasts over a 3-day period than was the P533R protein. This was further evident from the enzyme activity monitoring (Figure 4-1 D). No IDUA activity was detected in the media of untransformed BY-2 cells (Figure 4-1 D).

4.4.3. Kinetic parameters of the purified mutant and wildtype IDUA enzymes

To examine the effects of the mutations on IDUA activity, in vitro assays were performed on the purified recombinant enzymes. The K_m of the R383H IDUA mutant for the artificial (4MUI) substrate was relatively unaffected, whereas the V_{max} decreased by 1.7-fold as compared to those kinetic parameters of the wild type enzyme (Figure 4-2 A). Interestingly, the V_{max} of the R383H IDUA mutant was ~13% higher than that of P533R IDUA mutant. As was the case with the P533R mutant variant (Bie et al. 2013), the K_m of the R383H IDUA was similar to the WT-IDUA (Figure 4-2 B), indicating that both mutations do not affect substrate binding affinity.

4.4.4. Primary screen against NINDS library: Identification of candidate enhancers of IDUA secretion

To identify the enhancers of IDUA secretion, the activities of P533R- or R383H-IDUA were evaluated in the presence of each of the 1,040 compounds from the NINDS small-molecule library. The design and time-line of the HTS assay is depicted in Figure 4-3. Screening was carried out separately for each mutation. In each 96-well plate, untreated P533R- or R383H-IDUA protoplasts were seeded in column 1 as a control, as no pharmacological chaperone exists for MPS I to be used as a positive control for the HTS assay. Another control consisted of untransformed protoplasts that were seeded in column 12 of each plate. Both controls were treated with DMSO only. Protoplasts expressing mutant IDUA were treated with NINDS compounds (20 μM) in columns 2 to 11 and incubated for 3 days prior to performing the activity assay. The scatter plot results from each screening are shown in Figure 4-4. The average Z' statistic (Zhang et al. 1999) of 0.55 or 0.67 calculated for P533R- or R383H-IDUA, respectively, were well within the acceptable range for a robust and interpretable screening (Zhang et al. 1999). All compounds that enhanced the IDUA activity >160% were considered as primary hits and subjected to a secondary screening. A total of 29 or 25 hits were identified for P533R-IDUA or R383H-IDUA respectively.

4.4.5. Validation of candidate compounds from primary screen

A reconfirmation screening of candidate compounds identified in the primary screen was then performed to evaluate the effect of potential hits on the activity of mutant IDUA and to eliminate the false positives. The secondary screening was carried out using identical conditions to those of the primary screening (Figure 4-5). Four compounds including X-373, benzylpenicillin potassium, tamoxifen citrate, and tulobuterol were confirmed for P533R-IDUA to increase the amount of IDUA activity in the secretion media (Table 4-1). Subsequent screening of R383H-IDUA resulted in identification of only one compound (methimazole) capable of enhancing IDUA activity in the protoplasts secretion media. The compounds verified in the secondary screen were considered as "hits" and subjected to dose-response curve studies to identify the specific range of concentrations over which these compounds enhanced the activity of the mutant IDUA proteins in the plant culture medium. The compounds of interest were used

at 8 different concentrations (0.02–120 μM) in dose-response curve studies. The candidate compound identified for R383H-IDUA did not show an effect in the range of concentrations tested (data not shown). From the four compounds obtained in the secondary screening for P533R-IDUA, X-372 showed the best dose-response curve and was therefore identified as the strongest candidate enhancer molecule (Figure 4-6 A). X-372 significantly increased the amount of active P533R-IDUA in the media at concentrations of 120 μM. The effect of X-372 on the increase of P533R-IDUA activity in the secretion medium was subsequently examined at a wider range of concentrations. This showed that the optimum concentration of X-372 was close to 120 μM; treatment with 240 μM or higher lowered the amount of activity of the P533R mutant associated with the secretion medium possibly due to cytotoxic effects (data not shown). X-372 also enhanced the activity of WT-IDUA in the culture medium at concentrations of 120 μM (Figure 4-6 B), whereas such an effect was not observed for cells expressing R383H IDUA (Figure 4-6 C).

4.4.6. The lead small molecule acts to enhance the secretion of the mutant IDUA protein into the culture medium.

The positive effect of the small molecule "enhancer" of IDUA could either be effected by a capacity to enhance IDUA enzyme activity *per se*, or by improving the proportion of synthesized protein that is able to undergo post-ER transport as a result of passing ER quality control. To demonstrate if the hit compound identified by the HTS acts in part by facilitating the post-ER transport of mutant IDUA protein, western blot analysis was used to examine the effect of X-372 on secretion of WT and mutant IDUA proteins into the culture medium surrounding the transgenic BY2 protoplasts. In the absence of X-372 only a small portion of the P533R mutant was secreted in the culture medium (Figure 4-7 A, "0"). X-372 clearly promoted the secretion of the P533R mutant protein into the medium surrounding the BY2 protoplasts, particularly at 120 and 140 μM X-372 (Figure 4-7 A). In the absence of X-372, R383H IDUA was not detectable in the media (Figure 4-7 B, "0"). Interestingly, treatment with X-372 increased the secretion of this mutant protein into the protoplast culture medium (Figure 4-7 B). The lead compound also strongly promoted the secretion of WT-IDUA into the protoplast culture media (Figure 4-7 C). These results suggest that X-372 may mediate its effects

intracellularly either by directly interacting with the IDUA protein in the ER to facilitate folding, or by regulating other cellular mechanisms such as the proteostasis network, which further allows for the subsequent post-ER trafficking of mutant IDUA.

4.4.7. Thermostability experiment did not show a direct interaction between lead small molecule and IDUA protein

To determine whether X-372 is capable of increasing the stability of the mutant enzyme, the effect of the compound on thermal denaturation was analyzed. Purified P533R protein was incubated at 50 °C in the presence of either DMSO or different concentrations of X-372 for 15, 30, 45, 60, 75, or 90 min and the fraction remaining activity was calculated. The same experiment was performed using purified *cgl*-IDUA (WT-IDUA generated in seeds of a *cgl* Arabidopsis background; see methods). X-372 showed no significant increase on the stability of cgl-(WT)-IDUA, P533R-IDUA and BY2-(WT)-IDUA proteins during thermal denaturation (Figure 4-8 A, B and C).

4.5. Discussion

Most of high throughput screening (HTS) assays have been developed to specifically identify PCs that bind tightly to the active site of the lysosomal enzyme under study (Tropak et al. 2007, Maegawa et al. 2009). However, pharmacological chaperones that act as *enzyme enhancement'* agents need not function as active site inhibitors as recent reports show that small molecules such as proteostasis regulators and Ca^{2+} channel blockers can enhance the lysosomal trafficking of the severely destabilized glucocerebrosidase (GCase) variant, L444P GCase (Mu et al. 2008, Wang and Segatori, 2013). Therefore, some important small molecules may be missed using single-target biochemical assays involving purified proteins in the present screening systems. Based on these observations, developing cell-based assays provides the opportunity to identify small molecules that function equivalently to pharmacological chaperones (i.e. promote the post-ER trafficking of the mutant lysosomal protein), but act via a different molecular mechanism (Mu et al. 2008; Geng et al. 2011; Wang el al., 2011). Cell-based assays conveniently allow for selection of membrane permeable compounds and can provide information about their cytotoxicity (An and Tolliday 2010).

We have noted the advantages and disadvantages of using patient-derived cell lines in an HTS campaign (Geng et al. 2011) in the Introduction (see earlier), particularly the low residual enzyme activity associated with the rapid clearance of mutant misfolded proteins by ERAD, making it difficult to detect potential leads in the primary screening. For instance, in patient cell lines the IDUA protein is severely destabilized by the P533R mutation in patient-derived fibroblasts (Scott et al. 1992, Matte et al. 2003), while in plant cells the recombinant P533R protein is less susceptible to disrupted function and homeostasis (Bie et al. 2013).

The use of plant cells can overcome some of the limitations associated with more conventional HTS. Tobacco BY2 cells have been previously used to successfully express WT IDUA; in this system, active IDUA is synthesized and is efficiently secreted into the plant culture media (Fu et al. 2009). We show that in the case of our cells expressing mutant IDUA that the enzyme activity and detectable protein levels are considerably higher than what is feasible from patient derived cells, and thus plant cells prove to be a suitable alternative in a HTS platform. BY2 cells expressing WT-IDUA maintained high levels of IDUA protein, whereas, not surprisingly the two mutant proteins P533R- and R383H-IDUA were comparatively reduced in steady-state amounts. The R383H BY2 cells demonstrated an unexpected low level of mutant IDUA protein, given that this mutation leads to a less severe MPS I phenotype than does the P533R mutation. Perhaps if more R383H-IDUA lines were screened for expression and secretion we might have identified a line with a higher amount of protein. A comparative analysis of the activities of secreted WT, P533R and R383H mutant IDUA in protoplasts culture media, revealed that both mutant proteins are catalytically active in the media. Kinetic studies on BY2 cells expressing R383H IDUA demonstrated a normal K_m but a reduced V_{max} for R383H. Similarly MPS I patient fibroblasts with the R383H mutation have a reduced Vmax for R383H while the K_m reported is normal (Matte et al. 2003). In transfected CHO-K1 cells, protein levels corresponding to P533R-IDUA are detectable at small amounts by immunoblot analysis and their abundance is lower than R383H-IDUA of CHO-K1 (Matte et al. 2003). Unlike in the plant system, the P533R-IDUA of CHO-K1 cells was not secreted into the cell culture medium possibly due to its impaired intracellular processing and produced a very low percentage of activity compared to the wild-type control (Matte et al. 2003).

Based on this background characterization of the mutant and wildtype IDUA proteins in our tobacco BY2 transgenic system, we were able to develop a plant-based assay for HTS. This screen was based on the principle that a small molecule that has efficacy as a pharmacological chaperone will promote the folding of a mutant protein in the ER. Normally the missense mutant protein is impaired in its ability to obtain a functional conformation, which is a requirement for exiting the lumen of the ER due to quality control mechanisms (Sawkar et al. 2002, Sawkar et al. 2005, Fan 2008). In the presence of a pharmacological chaperone, the equilibrium is shifted toward folding at the expense of ER-associated degradation (ERAD), enabling the folded lysosomal enzyme to engage the trafficking machinery that transports it to the lysosome of mammalian cells. Such a molecule would ultimately be effective in restoring residual activity of the enzyme in the lysosome. The assay that we have set up is based on the ability of the compound to facilitate post-ER transport competence of the IDUA enzyme within the secretory pathway of plant cells. This functions selectively and specifically for screening as library molecules are selected based on their capability to enhance the post-ER transport of the two missense mutant IDUA proteins, P533R and R383H. Since the recombinant IDUA variants were equipped with a signal peptide, and the expression cells - transgenic tobacco BY2 cells - possess no lysosomes, post-ER transport, in turn is readily measured by assessing secretion of active enzyme into the surrounding medium.

Therefore, the readout in the HTS assay was based on detectable IDUA activity in the protoplasts culture media. Since the assay is cell-based, various classes of small molecules including proteostasis regulators can be identified besides pharmacological chaperones; the requirement for selection encompasses *enzyme enhancement* by orchestrating different elements of molecular pathways that can remediate the folding, maturation/trafficking, or proteostasis of the normally degradation-prone IDUA mutant.

The screening of the NINDS library has been previously resulted in the identification of potential small-molecules that function as *enzyme enhancement* therapy (EET)-agents for different LSDs such as late-onset GM2 gangliosidosis (Maegawa et al. 2007), as well as potential pharmacological agents for neurodegenerative diseases (Boston-Howes et al. 2008, Masuda et al. 2008, Wang et al. 2007). Using a thermal

denaturation assay, screening of the NINDS library led to the identification of ambroxol (ABX) as a pharmacological chaperone for mutant GCase (Maegawa et al. 2009). A screening based on the ability of each compound to promote the secretion of mutant IDUA from transgenic BY2 protoplasts identified X-372 as the major hit. To our knowledge X-372 is the first pharmacological chaperone identified for MPS I. Our findings showing the ability of the lead compound to strongly increase secretion of IDUA protein and activity (for WT- and P533R-IDUA) showing its specific ability to enhance the post-ER trafficking of mutant IDUA. In the absence of X-372, only a small proportion of both mutant proteins was secreted by the cultured protoplasts.

We further used a thermal-denaturation assay to determine whether X-372 can interact allosterically with the IDUA enzyme. Currently all pharmacological chaperones identified for LSDs can stabilize the enzyme against thermal-denaturation, and most are inhibitors. However, only a small number of these molecules function as pharmacological chaperones in patient cells (Tropak et al. 2008). X-372 did not show a direct interaction between with the IDUA protein. This may suggest that X-372 is involved via an indirect mechanism leading to the observed enhanced secretion of IDUA protein. The general effect of X-373 on secretion of WT and mutant IDUA might suggest a role for X-372 as a proteostasis regulator. It has previously been shown that proteostasis regulators such as celastrol or MG-132 can assist mutant lysosomal proteins to avoid ERAD and pass ER quality control (Mu et al. 2008). However, to show the role of X-372 as a proteostasis regulator further investigation will be required which involves conducting molecular and biochemical analyses to determine marker genes/proteins that mediate the protein folding pathway, ER-stress responses and cellular homeostasis responses.

Another advantage of X-372 as an EET-agent is that, it has been identified from a FDA-approved library and thus testing this compound in clinical trials to evaluate it as an EET-agent for MPS I might easily be expedited. In addition, other medical properties of this drug may also be beneficial in improving other aspects of the pathogenesis of MPS I.

In summary, we have demonstrated that BY2 cells can be used in a cell-based HTS assay against small molecule libraries. Mutant IDUA proteins secreted into the culture media preserved their residual enzyme activity. Taking advantage of partial secretion of mutant IDUA from transgenic protoplasts, a screening based on the IDUA enzyme assay was developed. The cellular nature of our plant-based system facilitates the elimination of compounds with cytotoxic effects in the primary screening or during dose-response studies. The ease of scale-up allows implementation of this plant-based system in screening campaigns against larger and more diverse libraries of small molecules to identify novel potential drugs for MPSI disease. Furthermore, plant-based HTS system brings a novel opportunity to identify small molecules for severely mutated proteins where their enzymatic activity is completely lost in patient-derived cells which is evident from our results in the discovery of one very promising PC compound. The system proposed in this study can be used for other LSDs and may have efficacy for protein conformation diseases in general.

Acknowledgments

We thank Dr. Don Mahuran and Dr. Michael Tropak (SickKids hospital, Toronto, Canada) for generously providing the NINDS library. We thank Dr. Liwen Jiang for kindly providing anti-IDUA (1A) antibody. We would also like to thank Dr. Joost Lücker for helpful comments and critical reading of the manuscript. This work was supported by Natural Sciences and Engineering Research Council of Canada (NSERC) Strategic, the Canadian MPS Society and Michael Smith Foundation for Health Research grants awarded to A.R.K.

4.6. References

Alif N, Hess K, Straczek J, Sebbar S, N'Bou A, Nabet P, Dousset B. Mucopolysaccharidosis type I: Characterization of a common mutation that causes hurler syndrome in moroccan subjects. Ann Hum Genet 1999; 63:9-16.

An WF, Tolliday N. Cell-based assays for high-throughput screening. Mol Biotechnol 2010; 45:180-186.

Babajani G, Tropak MB, Mahuran DJ, Kermode AR. Pharmacological chaperones facilitate the post-ER transport of recombinant N370S mutant beta-glucocerebrosidase in plant cells: Evidence that N370S is a folding mutant. Mol Genet Metab 2012; 106:323-329.

Beck M. Emerging drugs for lysosomal storage diseases. Expert Opin Emerg Drugs 2010; 15:495-507.

Bie H, Yin J, He X, Kermode AR, Goddard-Borger ED, Withers SG, James MN. Insights into mucopolysaccharidosis I from the structure and action of alpha-L-iduronidase. Nat Chem Biol 2013; 9:739-745.

Boston-Howes W, Williams EO, Bogush A, Scolere M, Pasinelli P, Trotti D. Nordihydroguaiaretic acid increases glutamate uptake in vitro and in vivo: Therapeutic implications for amyotrophic lateral sclerosis. Exp Neurol 2008; 213:229-237.

Bunge S, Clements PR, Byers S, Kleijer WJ, Brooks DA, Hopwood JJ. Genotype-phenotype correlations in mucopolysaccharidosis type I using enzyme kinetics, immunoquantification and in vitro turnover studies. Biochim Biophys Acta 1998; 1407:249-256.

Chakrabarty R, Banerjee R, Chung SM, Farman M, Citovsky V, Hogenhout SA, Tzfira T, Goodin M. PSITE vectors for stable integration or transient expression of autofluorescent protein fusions in plants: Probing nicotiana benthamiana-virus interactions. Mol Plant Microbe Interact 2007; 20:740-750.

Clarke LA. Pathogenesis of skeletal and connective tissue involvement in the mucopolysaccharidoses: Glycosaminoglycan storage is merely the instigator. Rheumatology (Oxford) 2011; 50 Suppl 5:v13-8.

Clarke LA. The mucopolysaccharidoses: A success of molecular medicine. Expert Rev Mol Med 2008; 10:e1.

Clements PR, Brooks DA, Saccone GT, Hopwood JJ. Human alpha-L-iduronidase. 1. purification, monoclonal antibody production, native and subunit molecular mass. Eur J Biochem 1985; 152:21-28.

Desnick RJ, Schuchman EH. Enzyme replacement therapy for lysosomal diseases: Lessons from 20 years of experience and remaining challenges. Annu Rev Genomics Hum Genet 2012; 13:307-335.

Downing WL, Galpin JD, Clemens S, Lauzon SM, Samuels AL, Pidkowich MS, Clarke LA, Kermode AR. Synthesis of enzymatically active human alpha-L-iduronidase in arabidopsis cgl (complex glycan-deficient) seeds. Plant Biotechnol J 2006; 4:169-181.

Fan JQ. A counterintuitive approach to treat enzyme deficiencies: Use of enzyme inhibitors for restoring mutant enzyme activity. Biol Chem 2008; 389:1-11.

Fu L H, Miao Y, Lo SW, Seto TC, Sun S, Xu Z, Clemens S, Clarke L, Kermode AR, Production and characterization of soluble human lysosomal enzyme α-iduronidase with high activity from culture media of transgenic tobacco BY-2 cells Original Research. Plant Science 2009; 177: 668-675.

Futerman AH, van Meer G. The cell biology of lysosomal storage disorders. Nat Rev Mol Cell Biol 2004; 5:554-565.

Gatti R, DiNatale P, Villani GR, Filocamo M, Muller V, Guo XH, Nelson PV, Scott HS, Hopwood JJ. Mutations among italian mucopolysaccharidosis type I patients. J Inherit Metab Dis 1997; 20:803-806.

Geng H, Whiteley G, Ribbens J, Zheng W, Southall N, Hu X, Marugan JJ, Ferrer M, Maegawa GH. Novel patient cell-based HTS assay for identification of small molecules for a lysosomal storage disease. PLoS One 2011; 6:e29504.

Gillece P, Luz JM, Lennarz WJ, de La Cruz FJ, Romisch K. Export of a cysteine-free misfolded secretory protein from the endoplasmic reticulum for degradation requires interaction with protein disulfide isomerase. J Cell Biol 1999; 147:1443-1456.

Grabowski GA, Hopkin RJ. Enzyme therapy for lysosomal storage disease: Principles, practice, and prospects. Annu Rev Genomics Hum Genet 2003; 4:403-436.

Grubb S, Guo L, Fisher EA, Brodsky JL. Protein disulfide isomerases contribute differentially to the endoplasmic reticulum-associated degradation of apolipoprotein B and other substrates. Mol Biol Cell 2012; 23:520-532.

He X, Haselhorst T, von Itzstein M, Kolarich D, Packer NH, Gloster TM, Vocadlo DJ, Clarke LA, Qian Y, Kermode AR. Production of alpha-L-iduronidase in maize for the potential treatment of a human lysosomal storage disease. Nat Commun 2012; 3:1062.

He X, Haselhorst T, von Itzstein M, Kolarich D, Packer NH, Kermode AR. Influence of an ER-retention signal on the N-glycosylation of recombinant human alpha-L-iduronidase generated in seeds of arabidopsis. Plant Mol Biol 2012; 79:157-169.

Hopwood JJ, Muller V, Smithson A, Baggett N. A fluorometric assay using 4-methylumbelliferyl alpha-L-iduronide for the estimation of alpha-L-iduronidase activity and the detection of hurler and scheie syndromes. Clin Chim Acta 1979; 92:257-265.

Hosokawa N, Wada I, Hasegawa K, Yorihuzi T, Tremblay LO, Herscovics A, Nagata K. A novel ER alpha-mannosidase-like protein accelerates ER-associated degradation. EMBO Rep 2001; 2:415-422.

Jakob CA, Bodmer D, Spirig U, Battig P, Marcil A, Dignard D, Bergeron JJ, Thomas DY, Aebi M. Htm1p, a mannosidase-like protein, is involved in glycoprotein degradation in yeast. EMBO Rep 2001; 2:423-430.

Laradi S, Tukel T, Erazo M, Shabbeer J, Chkioua L, Khedhiri S, Ferchichi S, Chaabouni M, Miled A, Desnick RJ. Mucopolysaccharidosis I: Alpha-L-iduronidase mutations in three tunisian families. J Inherit Metab Dis 2005; 28:1019-1026.

Lee LY, Fang MJ, Kuang LY, Gelvin SB. Vectors for multi-color bimolecular fluorescence complementation to investigate protein-protein interactions in living plant cells. Plant Methods 2008; 4:24-4811-4-24.

Maegawa GH, Tropak MB, Buttner JD, Rigat BA, Fuller M, Pandit D, Tang L, Kornhaber GJ, Hamuro Y, Clarke JT, Mahuran DJ. Identification and characterization of ambroxol as an enzyme enhancement agent for gaucher disease. J Biol Chem 2009; 284:23502-23516.

Maita N, Tsukimura T, Taniguchi T, Saito S, Ohno K, Taniguchi H, Sakuraba H. Human alpha-L-iduronidase uses its own N-glycan as a substrate-binding and catalytic module. Proc Natl Acad Sci U S A 2013; 110:14628-14633.

Masuda N, Peng Q, Li Q, Jiang M, Liang Y, Wang X, Zhao M, Wang W, Ross CA, Duan W. Tiagabine is neuroprotective in the N171-82Q and R6/2 mouse models of huntington's disease. Neurobiol Dis 2008; 30:293-302.

Matte U, Yogalingam G, Brooks D, Leistner S, Schwartz I, Lima L, Norato DY, Brum JM, Beesley C, Winchester B, Giugliani R, Hopwood JJ. Identification and characterization of 13 new mutations in mucopolysaccharidosis type I patients. Mol Genet Metab 2003; 78:37-43.

Mayo KJ, Gonzales BJ, Mason HS. Genetic transformation of tobacco NT1 cells with agrobacterium tumefaciens. Nat Protoc 2006; 1:1105-1111.

Mu TW, Ong DS, Wang YJ, Balch WE, Yates JR 3rd, Segatori L, Kelly JW. Chemical and biological approaches synergize to ameliorate protein-folding diseases. Cell 2008; 134:769-81.

Nakatsukasa K, Nishikawa S, Hosokawa N, Nagata K, Endo T. Mnl1p, an alpha - mannosidase-like protein in yeast saccharomyces cerevisiae, is required for endoplasmic reticulum-associated degradation of glycoproteins. J Biol Chem 2001; 276:8635-8638.

Roubicek M, Gehler J, Spranger J. The clinical spectrum of alpha-L-iduronidase deficiency. Am J Med Genet 1985; 20:471-481.

Sawkar AR, Adamski-Werner SL, Cheng WC, Wong CH, Beutler E, Zimmer KP, Kelly JW. Gaucher disease-associated glucocerebrosidases show mutation-dependent chemical chaperoning profiles. Chem Biol 2005; 12:1235-1244.

Sawkar AR, Cheng WC, Beutler E, Wong CH, Balch WE, Kelly JW. Chemical chaperones increase the cellular activity of N370S beta -glucosidase: A therapeutic strategy for gaucher disease. Proc Natl Acad Sci U S A 2002; 99:15428-15433.

Scott HS, Litjens T, Nelson PV, Brooks DA, Hopwood JJ, Morris CP. Alpha-L-iduronidase mutations (Q70X and P533R) associate with a severe hurler phenotype. Hum Mutat 1992; 1:333-339.

Terlato NJ, Cox GF. Can mucopolysaccharidosis type I disease severity be predicted based on a patient's genotype? A comprehensive review of the literature. Genet Med 2003; 5:286-294.

Tropak MB, Blanchard JE, Withers SG, Brown ED, Mahuran D. High-throughput screening for human lysosomal beta-N-acetyl hexosaminidase inhibitors acting as pharmacological chaperones. Chem Biol 2007; 14:153-164.

Tse YC, Mo B, Hillmer S, Zhao M, Lo SW, Robinson DG, Jiang L. Identification of multivesicular bodies as prevacuolar compartments in nicotiana tabacum BY-2 cells. Plant Cell 2004; 16:672-693.

Valenzano KJ, Khanna R, Powe AC, Boyd R, Lee G, Flanagan JJ, Benjamin ER. Identification and characterization of pharmacological chaperones to correct enzyme deficiencies in lysosomal storage disorders. Assay Drug Dev Technol 2011; 9:213-235.

Wang F, Segatori L. Remodeling the proteostasis network to rescue glucocerebrosidase variants by inhibiting ER-associated degradation and enhancing ER folding. PLoS One 2013; 8:e61418.

Wang F, Song W, Brancati G, Segatori L. Inhibition of endoplasmic reticulum-associated degradation rescues native folding in loss of function protein misfolding diseases. J Biol Chem 2011; 286:43454-43464.

Wang H, Guan Y, Wang X, Smith K, Cormier K, Zhu S, Stavrovskaya IG, Huo C, Ferrante RJ, Kristal BS, Friedlander RM. Nortriptyline delays disease onset in models of chronic neurodegeneration. Eur J Neurosci 2007; 26:633-641.

Yeung YG, Stanley ER. A solution for stripping antibodies from polyvinylidene fluoride immunoblots for multiple reprobing. Anal Biochem 2009; 389:89-91.

Yu Z, Sawkar AR, Kelly JW. Pharmacologic chaperoning as a strategy to treat gaucher disease. FEBS J 2007; 274:4944-4950.

Zhang JH, Chung TD, Oldenburg KR. A simple statistical parameter for use in evaluation and validation of high throughput screening assays. J Biomol Screen 1999; 4:67-73.

4.7. Figures

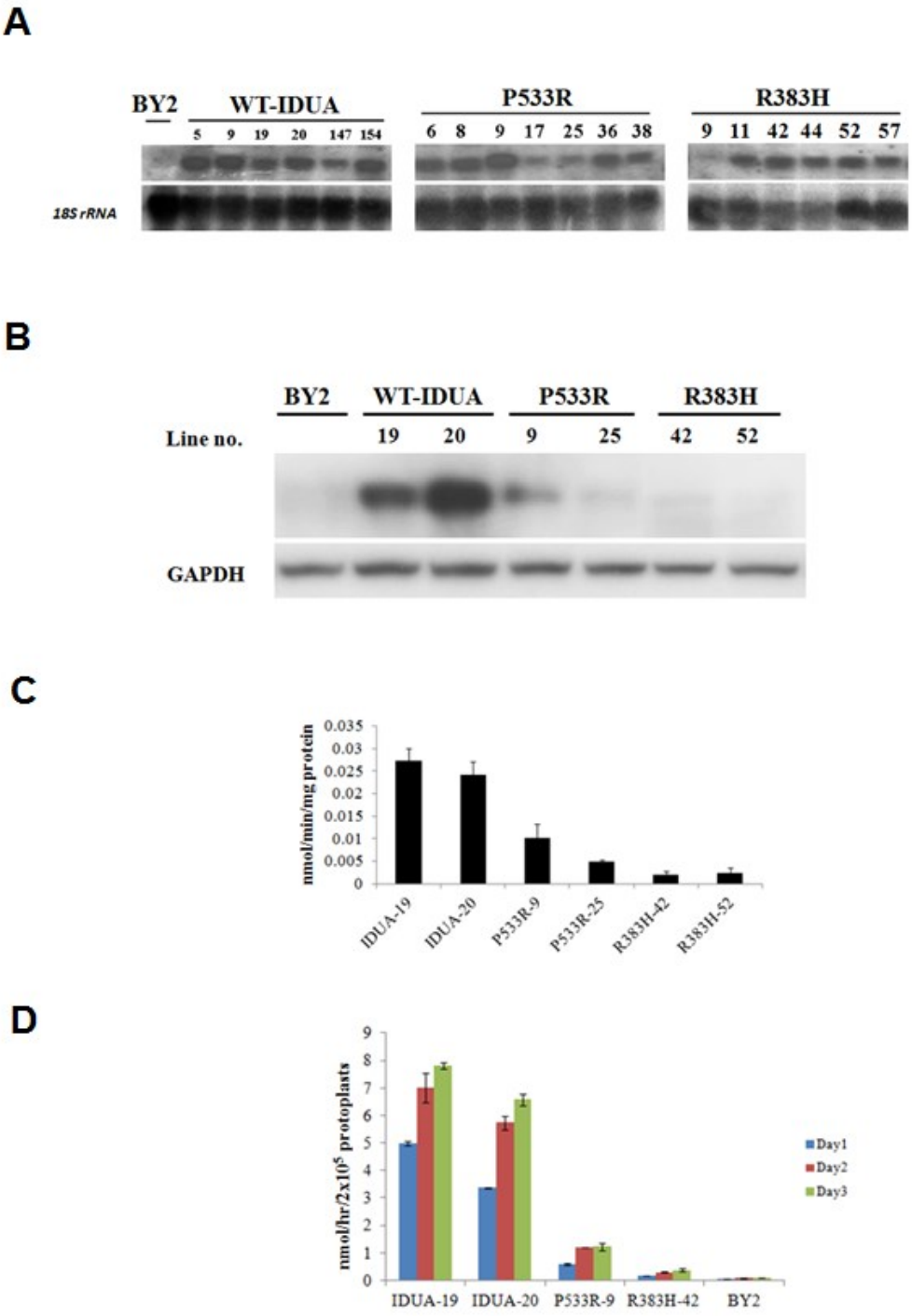
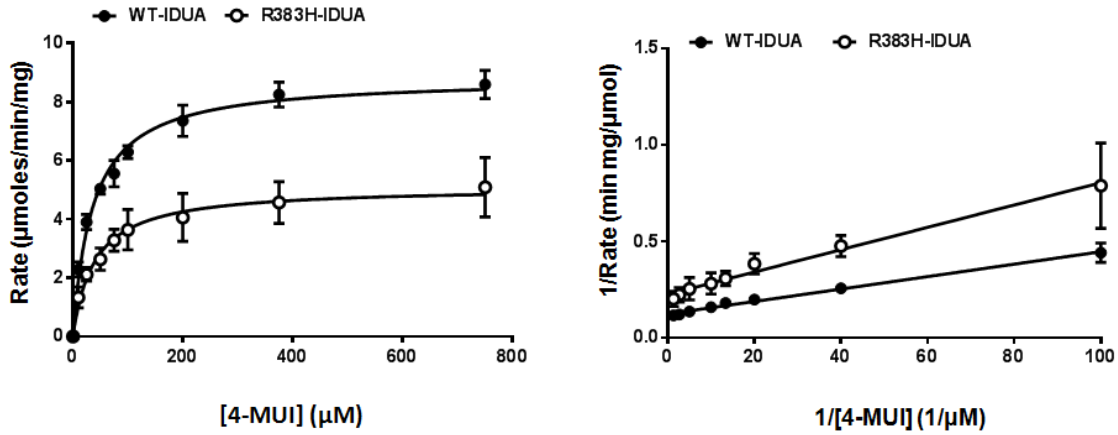


Figure 4-1: Steady-state IDUA transcripts and protein levels associated with independent transgenic tobacco BY2 lines expressing WT-, P533R-, and R383H-IDUA.

(A) Northern blot analyses were based on total RNA (15 µg) isolated from 10-d-old individual transgenic lines. A radiolabeled full-length cDNA probe was used to detect the IDUA transcripts. Ethidium bromide staining of ribosomal RNA is shown at the bottom. (**B**) For western blot analysis, total soluble protein (20 µg) from selected lines was fractionated by 12% SDS-PAGE, prior to analysis using a polyclonal anti-IDUA antibody (1A). The marker protein GAPDH (NAD-specific glyceraldehyde-3-phosphate dehydrogenase) was used as a control for loading on the same membranes following stripping of membranes and incubation with GAPDH antibodies. **(C)** The activity of WT and mutant IDUA in selected high-expressing lines. Ten or 20 µg of total protein extracted from selected lines expressing WT-, P533R-, and R383H-IDUA was used to determine the activity. (**D**) The activity secreted IDUA was assessed in a time course manner. After each time point cultured media of untransformed BY2 cells and selected transgenic lines (as indicated by numbers) expressing WT and mutant IDUA were collected and used for the IDUA activity assay. IDUA activity was measured in triplicate and expressed as nmol/hr units with error bars showing SE.

A



B

	WT-IDUA	R383H-IDUA	P533R-IDUA
V_{max}[a]	8.8 ± 0.18	5.1 ± 0.25	4.5 ± 0.15
K_m[b]	36.2 ± 2.9	39.5 ± 7.49	35.3 ± 3.66

[a]µmoles MU/min/mg protein
[b]µM

Figure 4-2: Determination of enzyme kinetic parameters of BY2-synthesized α-L-iduronidase.

IDUA was purified to homogeneity as indicated by SDS-PAGE analysis. **(A)** Michaelis Menten kinetics for plant-derived α-L-iduronidase were determined at 37 °C and pH 4.5 using a fluorometric assay as described by He et al. (2012) (left panel). Rates of catalysis were determined by dividing by the reaction time and concentration of the enzyme, and fit to a Michaelis Menten curve using GraphPad Prism (left panel). All measurements were made in triplicate. **(B)** Comparison between the kinetics properties of WT-, R383H- and P533R IDUA.

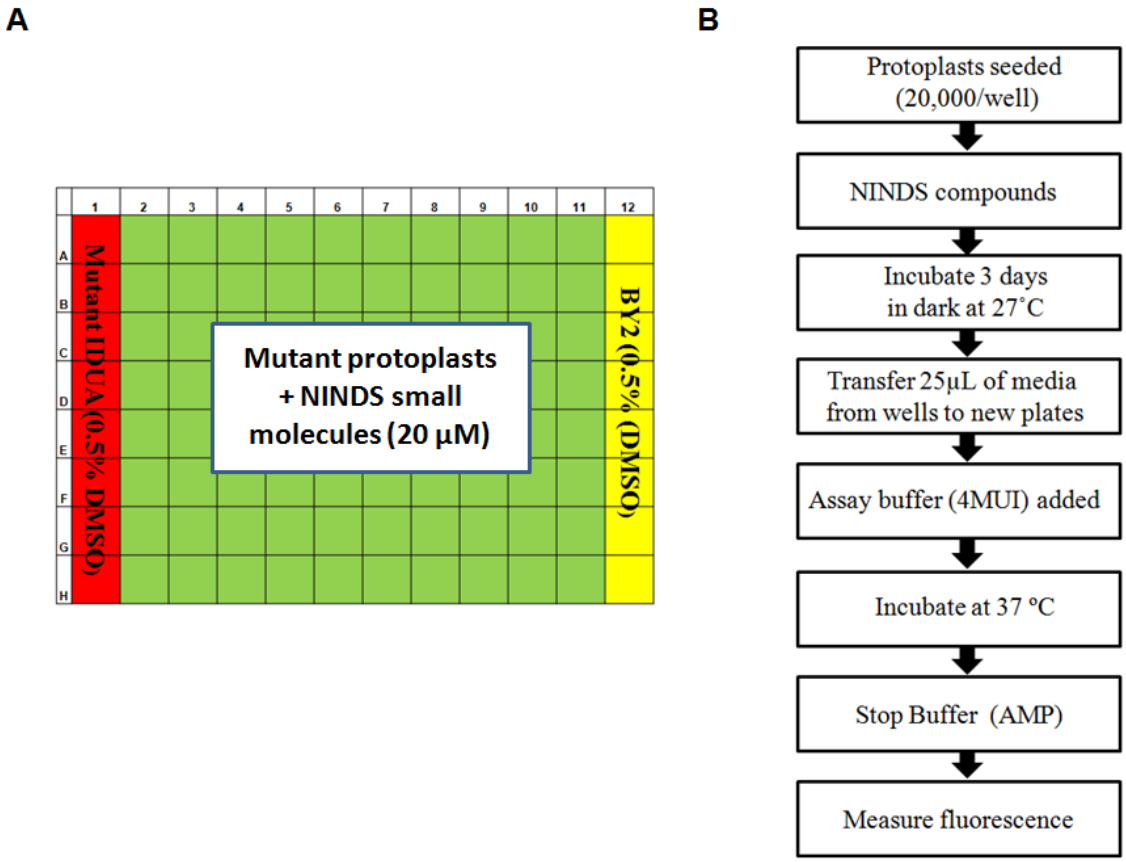


Figure 4-3: The design of plant-based HTS assay for mutant IDUA in 96-well plates.

(**A**) The disposition of mutant IDUA treated with library compounds (green), mutant IDUA treated with 0.5% DMSO (red) and BY2 protoplasts (yellow) in a 96-well plate. (**B**) The time-line of events and steps involved in the plant-based HTS assay for IDUA. NINDS library of 1040 small molecules at 20 µM concentration was used to perform the HTS for P533R- and R383H-IDUA.

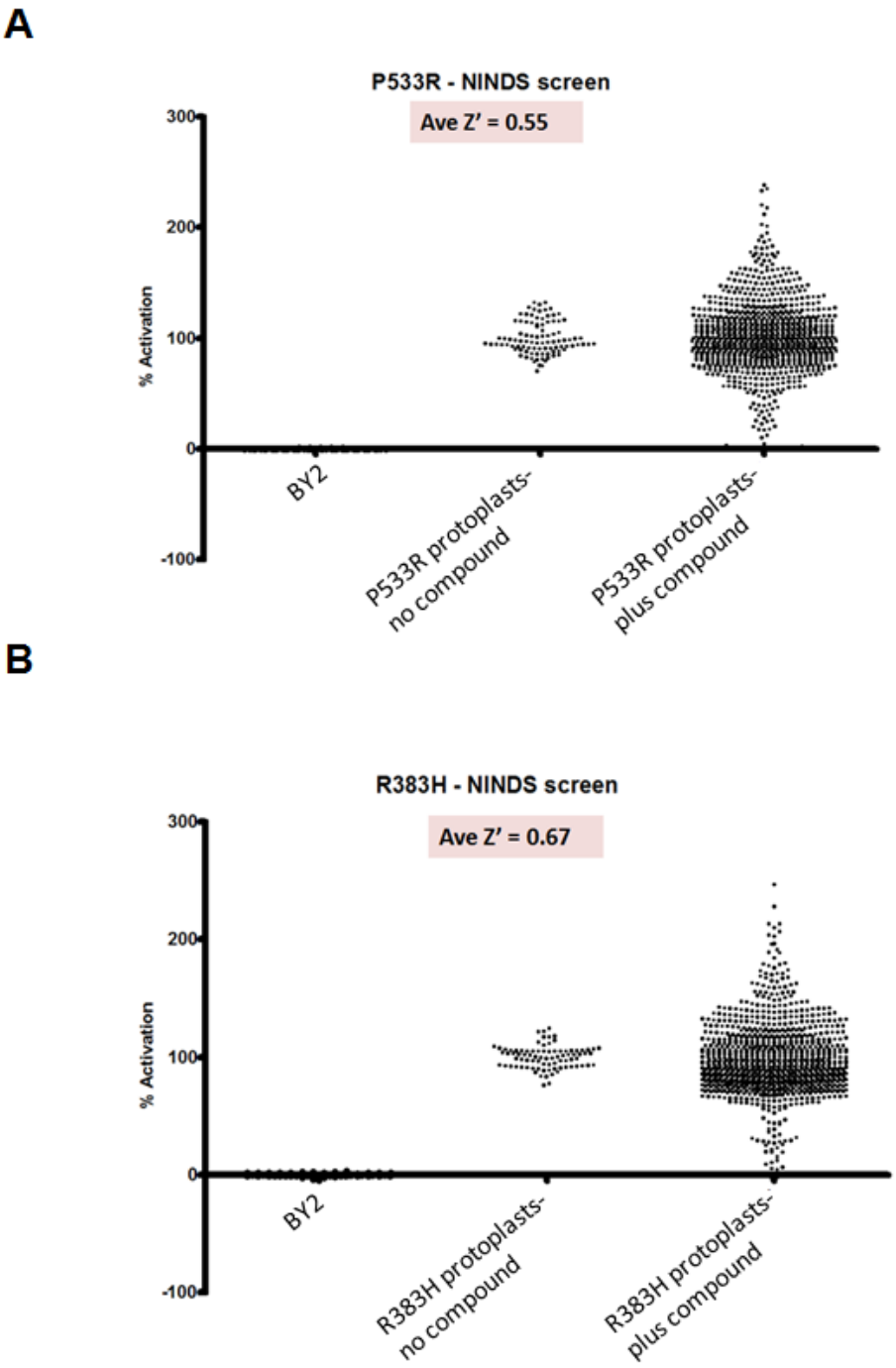


Figure 4-4: Primary screen of a 1040 compound NINDS library for P533R and R383H.

Plots show the activity of P533R **(A)** or R383H **(B)** mutant proteins in the presence of individual compounds from the library. Percent activation was calculated using the formula: %Activation= $(X-C^-)/(C^+-C^-)$ *100, where X is reading per well, C^- is negative control average (untransformed cells in the presence of DMSO) and C^+ is positive control average (activity of secreted mutant IDUA in the presence of DMSO).

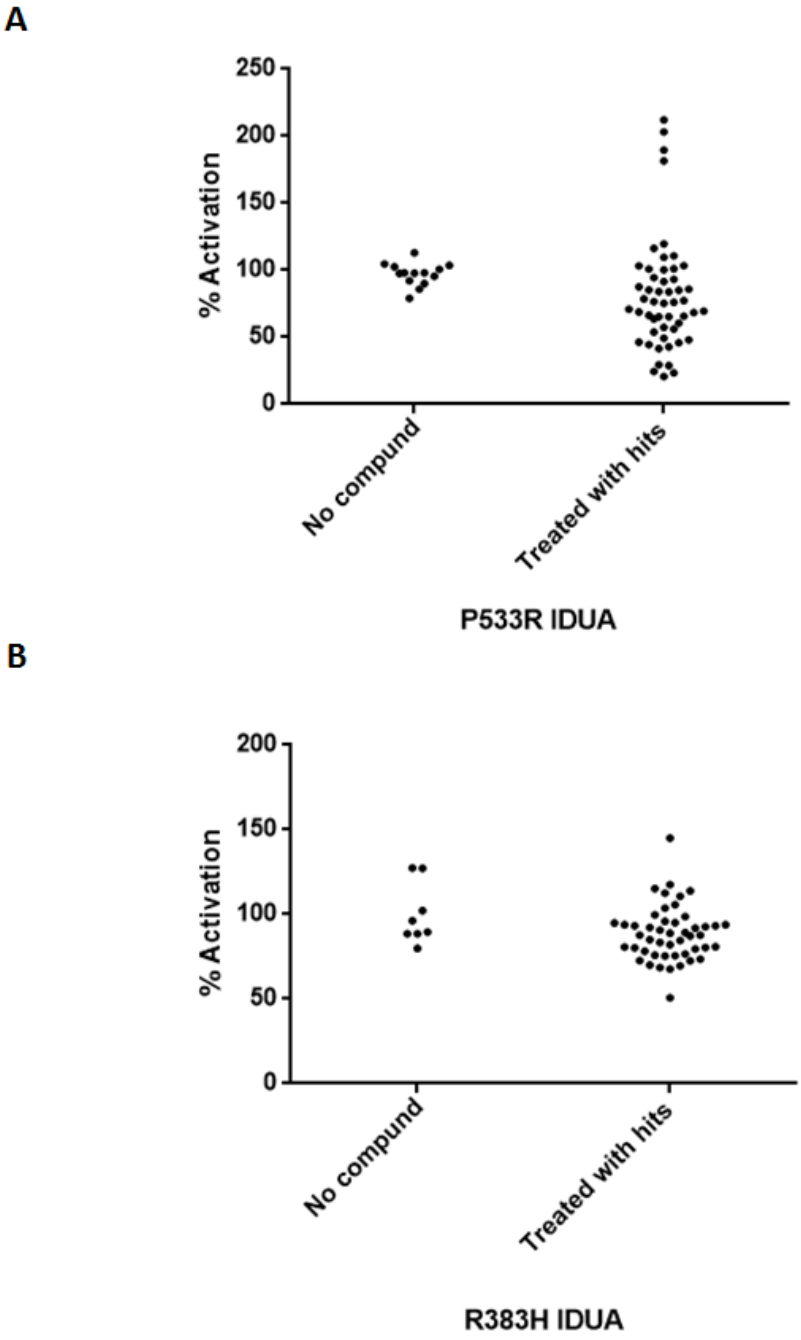


Figure 4-5: Reconfirmation screen of hits identified in the primary screen.
Target compounds were picked from NINDS library and single-point reconfirmation assays were conducted using identical conditions to those of the primary screening. Percent activation was calculated as described in figure 4.

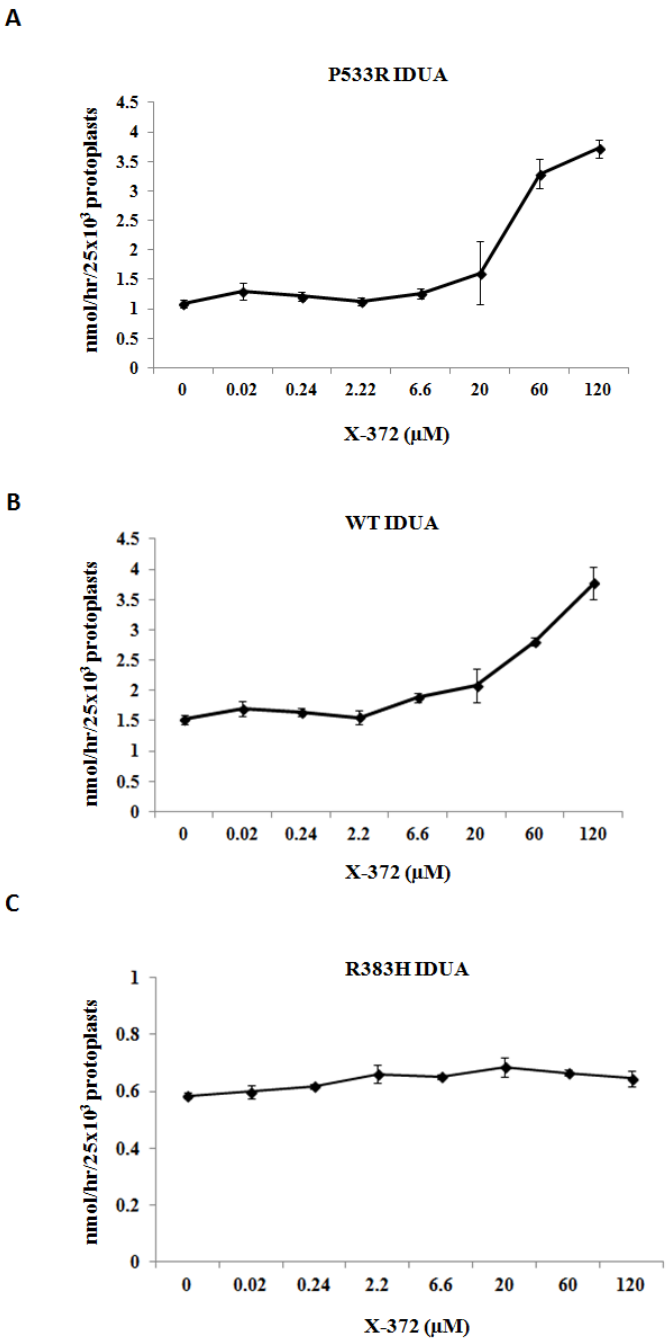


Figure 4-6: Dose-response curves for X-372.
Protoplasts expressing P533R- **(A)**, R383H- **(B)** and WT- IDUA **(C)** were treated with different concentrations of X-372 (as indicated) for 3 days. Twenty five microliter of media was used to assess the activity of WT and mutant in response to X372. Bars represent the SE.

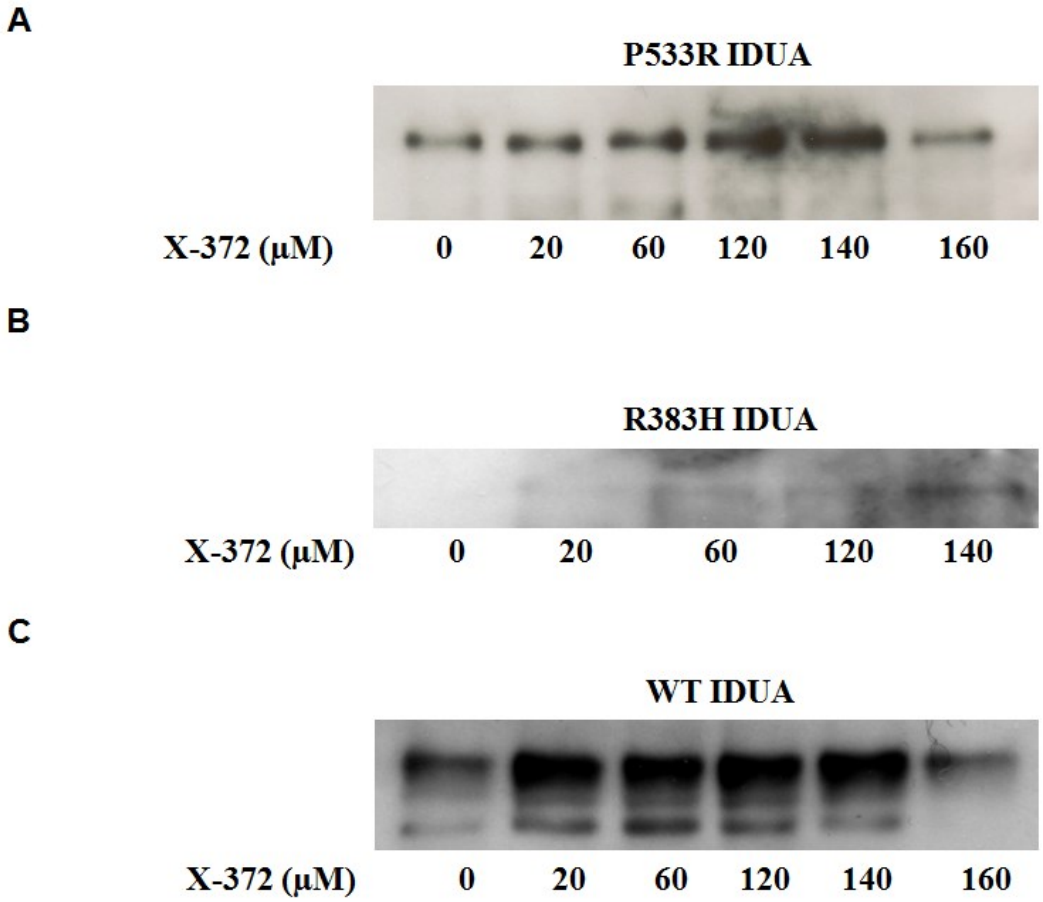


Figure 4-7: Effect of X-372 on IDUA secretion from transgenic protoplasts. Protoplasts (300,000 per mL) isolated from BY2 cells expressing P533R IDUA **(A)**, R383H IDUA **(B)** or WT IDUA **(C)** were incubated in culture medium with no compound or in culture medium containing different concentrations of X-372 (as indicated) for 3 days. Acetone-precipitated proteins from media were subjected to western blot analyses. A rabbit polyclonal anti-IDUA (1A) antibody was used to detect secreted IDUA protein.

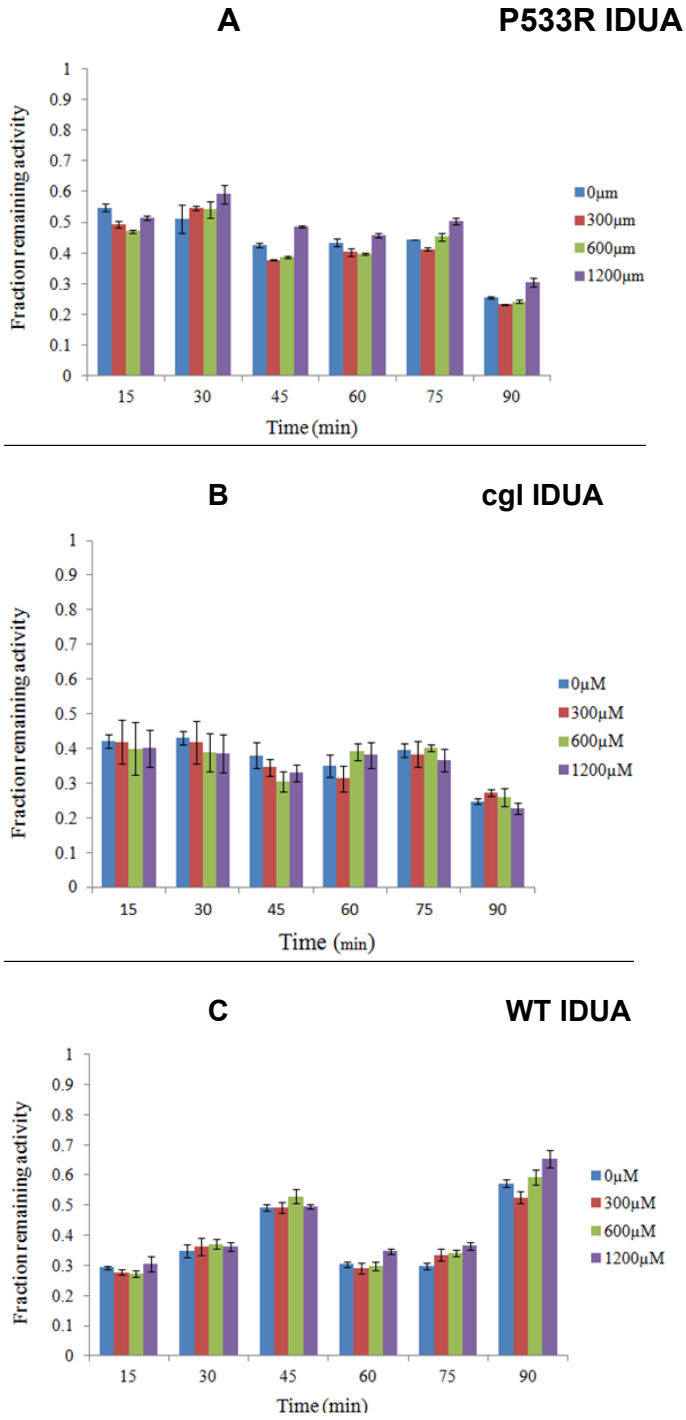
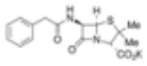
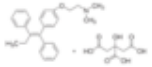
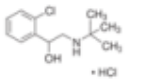


Figure 4-8: Thermal-denaturation assay to assess IDUA-X372 interactions on purified proteins.

Purified P533R-IDUA **(A)**, cgl-IDUA **(B)** and WT-IDUA **(C)** proteins were incubated at 50°C in the presence of DMSO or different concentrations of X-372 (as indicated) for 15, 30, 45, 60, 75, or 90 min. ‘‘Fraction remaining MUGS activity,’’ was calculated using the formula: (MU fluorescence of compound-treated sample incubated at 50°C for a given time)/(MU fluorescence of compound-treated sample left at 4 °C).

Table 4-1: List of compounds that increased the amount of P533R IDUA activity in the secretion media verified in the reconfirmation screening

Drug name	Chemical formula	Therapy	Structure	Secreted IDUA increased by %	Response to dose concentrations
X-372	X	X	X	181	+
BENZYL PENICILLIN POTASSIUM	C16H17KN2O4S	Antibacterial		212	-
TAMOXIFEN CITRATE	C32H37NO8	Estrogen antagonist, Antineoplastic		189	-
TULOBUTEROL	C12H19Cl2NO	Bronchodilator, Beta adrenergic agonist		203	-

Chapter 5.

Conclusions, significance of present studies, and future directions

This research targeted a new endeavour in the field of molecular pharming and introduced a novel plant-based system to identify potential therapeutics for LSDs – namely small molecules that are able to 'rescue' mutant lysosomal enzymes (so-called 'pharmacological chaperones'). Proof-of-concept studies showed that pharmacological chaperones such as *N*-(*n*-nonyl) deoxynojirimycin and ambroxol can promote the post-ER trafficking and secretion of N370S protein, a missense mutant variant that underlies the LSD known as Gaucher disease. One of our major findings in this study contradicts an assertion in the literature that small molecule chaperones increase N370S GCase activity (as assayed in treated patient cell lysates) by stabilizing the enzyme in the lysosome, and suggest that the mutant protein is impaired in its ability to obtain its functional folded conformation, which is a requirement for exiting the lumen of the ER. We assert that N370S GCase mutant is a chaperonable mutant, and further that the protein variant is significantly impaired in its catalytic activity.

We used our novel system to study the mechanisms by which inhibitors of ER-associated degradation (ERAD) (kifunensine and eeyarestatin I), and the proteostasis regulators (celastrol and MG-132) stabilize and promote the post-ER trafficking of mutant L444P GCase, a mutant variant that can underlie severe Gaucher disease. Transcriptional profiling and western blot analyses were used to follow marker genes/proteins that mediate the protein folding pathway, ER-stress responses and cellular homeostasis responses. Notably the proteostasis regulator celastrol stabilized the L444P GCase potentially through down-modulation of HSP90, a chaperone that plays a key role in the ERAD process, in contrast to what has been reported in the literature.

We further developed a plant-based system expressing mutant α-L-iduronidase (IDUA) for use in a high-throughput setting to identify putative pharmacological chaperones for treatment of another LSD, mucopolysaccharidosis I (MPS I). The screening of a library of FDA-approved drugs - the NINDS library- provided a feasible and practical approach for our purpose. The drugs available in such libraries are already in current use for various medical purposes and thus have already passed extensive investigations for safety in different clinical trials. Screening of the NINDS library using our plant-based system resulted in the identification of X-372 as a potential pharmacological chaperone for mutant IDUA. This compound was shown to function as an enzyme enhancement agent of mutant IDUA in BY cells. X-372 significantly enhanced the post-ER trafficking and activity of both mutant and wild-type IDUA in BY2 protoplasts.

This research established for the first time the proof-of-principle that plant cells represent an appropriate and valuable *in vivo* system for small molecule drug discovery.

5.1. Future questions and directions:

1. Does the pharmacological chaperone for MPS I function to enhance IDUA activity in human cultured patient deficient cell lines? Investigate the effect of X-372 on IDUA trafficking and activity in homozygous and heterozygous mutant fibroblast cell lines.

2. What is the mechanism by which X-372 facilitates post-ER protein transport of mutant IDUA? For instance, does X-372 act as a proteostasis regulator, and/or alleviate ER stress? Conduct molecular and biochemical analyses to follow marker genes/proteins that mediate the protein folding pathway, ER-stress responses and cellular homeostasis responses.

3. Can the plant-based system be used for screening additional small molecule libraries? Optimize the plant-based system to conduct large-scale screening to identify additional pharmacological chaperones for MPS I.

4. Can the plant-based screen identify pharmacological chaperones for other LSDs? Develop a facile high-throughput screen for identifying putative pharmacological chaperones for Gaucher disease. One of the limitations we faced in this research was due to the presence of endogenous glucosidases which recognize the same substrates as human GCase, thus contributing too much background 'noise' in screens based on GCase enzyme assays. As a future approach, it will be interesting to investigate various tags which facilitate purification of secreted mutant GCase in the plant culture media. This approach will overcome the limitation of activity-based screens that are presently fraught by background generated by endogenous plant glucosidases. Using a plant system to screen for small molecule chaperones capable of stabilizing mutant L444P GCase and other GCase variants could uncover leads for Gaucher disease treatments, and possibly other diseases such as Parkinson's disease, which has been linked to GCase mutations.