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Modulating the Lysosome-Autophagy System to Restore Homeostasis in in vitro Model Systems of Lysosomal Storage Disorders

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ABSTRACT

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The protein quality control system is a complex network that promotes the folding and trafficking of newly synthesized proteins and regulates the degradation of misfolded proteins and protein aggregates. Failure of the quality control system to maintain protein homeostasis (or proteostasis) characterizes the cellular pathogenesis of a number of human diseases. This study aims to develop cell engineering strategies to manipulate the lysosome-autophagy system to enhance folding and processing of lysosomal enzymes as well as to enhance the cellular clearance capacity. Specifically, I investigated the role of transcription factor EB (TFEB), a master regulator of lysosomal biogenesis and function, in regulating lysosomal proteostasis and autophagic clearance. Chemical and genetic modulation of TFEB was found to enhance folding, trafficking and activity of unstable, degradation-prone lysosomal enzymes in *in vitro* models of lysosomal storage disorders. Moreover, TFEB was found to mediate autophagy activation and autophagic clearance of storage material observed upon 2-hydroxypropyl-β-cyclodextrin administration. To further investigate the design of nanoparticles that activate autophagy, I tested the impact of nanoparticle size and surface charge on the lysosome-autophagy system. Autophagic clearance was found to depend highly
on surface charge. Specifically, cell exposure to polystyrene nanoparticles presenting neutral or anionic surface results in activation of autophagic clearance, whereas cell exposure to polystyrene nanoparticles presenting cationic surface results in impairment of lysosomal function and blockage of autophagic flux. Ceria nanoparticles (or nanoceria) are widely used in a variety of applications including as UV blockers and catalysts in industrial processes. Recent studies also revealed that ceria nanoparticles present antioxidant properties, suggesting a potential role of nanoceria in a variety of biomedical applications. In this study, ceria nanoparticles stabilized by organic surface coatings were found to activate the lysosome-autophagy system and enhance autophagic clearance.

In summary, this work provides proof-of-principle demonstration of chemical and biological strategies to activate the lysosome-autophagy system for restoring lysosomal proteostasis and enhancing autophagic clearance in model systems of diseases characterized by deficiencies in lysosomal enzymes activities and aberrant accumulation of lysosomal substrates. These findings lay the foundation for the development of nanotherapeutics for the treatment of diseases associated with inefficient autophagic clearance.
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<tr>
<td>LSDs</td>
<td>lysosomal storage disorders</td>
</tr>
<tr>
<td>GD</td>
<td>Gaucher’s disease</td>
</tr>
<tr>
<td>ER</td>
<td>endoplasmic reticulum</td>
</tr>
<tr>
<td>ERAD</td>
<td>ER-associated degradation</td>
</tr>
<tr>
<td>GC</td>
<td>β-glucocerebrosidase</td>
</tr>
<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>ERT</td>
<td>enzyme replacement therapy</td>
</tr>
<tr>
<td>SRT</td>
<td>substrate reduction therapy</td>
</tr>
<tr>
<td>CCT</td>
<td>chemical chaperone therapy</td>
</tr>
<tr>
<td>HexA</td>
<td>β-hexosaminidase A</td>
</tr>
<tr>
<td>NCL</td>
<td>neuronal ceroid lipofuscinosis</td>
</tr>
<tr>
<td>LINCL</td>
<td>late infantile neuronal ceroid lipofuscinosis</td>
</tr>
<tr>
<td>TPP1</td>
<td>tripeptidyl peptidase</td>
</tr>
<tr>
<td>UPS</td>
<td>ubiquitin-proteasome system</td>
</tr>
<tr>
<td>CMA</td>
<td>chaperone-mediated autophagy</td>
</tr>
<tr>
<td>TFEB</td>
<td>transcription factor EB</td>
</tr>
<tr>
<td>CLEAR</td>
<td>Coordinated Lysosomal Expression and Regulation</td>
</tr>
<tr>
<td>mTOR</td>
<td>mammalian target of rapamycin</td>
</tr>
<tr>
<td>qRT-PCR</td>
<td>quantitative RT-PCR</td>
</tr>
<tr>
<td>PI</td>
<td>propidium iodide</td>
</tr>
<tr>
<td>siRNA</td>
<td>small interfering RNA</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>HPβCD</td>
<td>2-hydroxypropyl-β-cyclodextrin</td>
</tr>
<tr>
<td>LC3</td>
<td>microtubule-associated light chain protein 3</td>
</tr>
<tr>
<td>GFP</td>
<td>green fluorescent protein</td>
</tr>
<tr>
<td>GlcNAc</td>
<td>N-Acetylglucosamine</td>
</tr>
<tr>
<td>PEG</td>
<td>polyethylene glycol</td>
</tr>
<tr>
<td>PVP</td>
<td>polyvinylpyrrolidone</td>
</tr>
<tr>
<td>TEM</td>
<td>transmission electron microscopy</td>
</tr>
<tr>
<td>XRD</td>
<td>x-ray diffraction</td>
</tr>
<tr>
<td>DLS</td>
<td>dynamic light scattering</td>
</tr>
<tr>
<td>ICP-MS</td>
<td>inductively coupled plasma mass spectrometry</td>
</tr>
<tr>
<td>ICP-AES</td>
<td>inductively coupled plasma atomic emission spectroscopy</td>
</tr>
<tr>
<td>PS</td>
<td>non functionalized polystyrene</td>
</tr>
<tr>
<td>PS-COOH</td>
<td>carboxyl-functionalized polystyrene</td>
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<tr>
<td>PS-NH$_2$</td>
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1.1. The Cellular Quality Control System

The cellular quality control system is a sophisticated network of pathways that assist folding of newly synthesized proteins, promote native folding of misfolded proteins, and eliminate aberrant proteins (1). The quality control system is composed of i) molecular chaperones that facilitate the folding of proteins into their native 3D structures and stabilize the structure of folding intermediates to prevent misfolding and limit aggregation of misfolded intermediates, and ii) the degradation machinery, which consists of the ubiquitin-proteasome system and the lysosome-autophagy system that mediate disposal of misfolded and aggregated proteins, respectively (2) (Figure 1.1).
Figure 1.1. Schematic illustration of the quality control system. The quality control system is composed of molecular chaperones and degradation systems. Molecular chaperones facilitate folding of newly synthesized proteins, promote refolding of misfolded proteins, and prevent aggregation of misfolded proteins. Irremediably misfolded proteins and protein aggregates are targeted to degradation machines, which consists of the ubiquitin-proteasome system and the lysosome-autophagy system that mediate disposal of misfolded and aggregated proteins, respectively.
Because native folding is essential for biological activity, failure of a protein to reach or maintain its native conformation may result in loss of protein function, which often leads to deleterious consequences for the cell’s physiology (2). Not surprisingly, failure of the quality control system to maintain protein homeostasis (or proteostasis) is associated with the cellular pathogenesis of a number of diseases characterized by imbalance between folded and misfolded states of a disease specific protein, and collectively referred to as protein misfolding diseases. Failure of the quality control system to maintain protein homeostasis can be due to impairment of components of the quality control system (3) or aberrant accumulation of misfolded proteins that exceed the capacity of this system (4). Understanding the molecular mechanisms that regulate protein quality control is thus important for the design of cell engineering strategies to ameliorate the phenotypes associated with the development of protein misfolding diseases. The overall goal of this study was to understand and modulate cellular quality control mechanisms that control protein folding, processing, and degradation and restore cellular homeostasis under conditions of proteotoxic stress.

As mentioned before, the quality control system relies on molecular chaperones to assist folding of newly synthesized proteins and target non-native proteins for degradation (1,5). Molecular chaperones recognize and bind to hydrophobic patches on the surface of newly synthesized polypeptides, folding intermediates, and misfolded conformations, thereby reducing the risk of aggregation resulting either from misfolded protein self-association or association
with other cellular components within the crowded cellular milieu (6). Upon reaching native folding, substrates are released from the chaperone machinery and can be processed through post-translational modifications and trafficked to their final cellular localization.

The degradation machinery eliminates aberrant or otherwise unstable protein conformations to recycle building blocks for protein synthesis and prevent undesired interactions with other cellular components. The two main pathways that mediate protein degradation are the ubiquitin-proteasome system (UPS) and the lysosome-autophagy system (7).

The UPS provides the primary route for the degradation of soluble misfolded proteins, thereby preventing disruptive consequences that might arise from the accumulation of misfolded proteins and maintaining a physiologic balance between folded and misfolded proteins (8). The UPS comprises a series of complex pathways for the recognition, targeting, and degradation of misfolded proteins (8). It mainly involves two steps: i) labeling of target proteins with ubiquitin molecules catalyzed by a cascade of enzymatic reactions involving ubiquitin-activating enzymes (E1 enzymes), ubiquitin-conjugating enzymes (E2 enzymes) and ubiquitin ligases (E3 enzymes); and ii) degradation of polyubiquitinated proteins by the proteasome (8,9).

Cytoplasmic proteins are directly targeted to the UPS for degradation. Extracytosolic proteins, which are folded in the endoplasmic reticulum (ER) and
processed through the secretory pathway, are retrotranslocated to the cytosol and subsequently degraded by the proteasome (10) through a process called ER-associated degradation (ERAD) (11).

The UPS is not only involved in the degradation of misfolded proteins, but also regulates the half-life of a number of cellular proteins, thus playing an important role in DNA transcription (12), cell cycle control (13), and immune response (14).

The UPS is mainly involved in the degradation of soluble, short-lived proteins, whereas the lysosome-autophagy system is responsible for long-lived proteins (15). Specifically, the lysosome-autophagy system mediates the degradation of aberrant proteins and protein aggregates (16). The lysosome-autophagy system is also involved in the elimination of other cellular waste materials such as damaged organelles (17). Thus, the lysosome-autophagy system provides an important survival mechanism to supply energy resources under nutrient deprivation through recycling cytoplasmic material (18-20). A number of studies have also described the role of autophagy in the disposal of foreign material, such as viruses and parasites (21,22), and in the defense against bacterial infections (23-25).

Three different forms of autophagy have been described: macroautophagy, microautophagy, and chaperone-mediated autophagy (26). Macroautophagy is the most common route of autophagic degradation, and it involves degradation of
cytoplasmic material compartmentalized into double-membrane vesicles (autophagosomes) by the lysosomes, which are organelles containing a battery of hydrolytic enzymes capable of degrading any type of biomolecule. This complex cellular pathway involves three steps: i) compartmentalization of material to be degraded into autophagosomes, ii) fusion of autophagosomes with lysosomes to form autophagolysosomes, and iii) degradation of autophagic cargo (27-29).

Microautophagy involves engulfment of cytoplasmic material directly into the lysosome (30). Macroautophagy and microautophagy participate in the degradation of both proteins and organelles (31). Chaperone-mediated autophagy (CMA), on the other hand, mediates only the degradation of soluble proteins (32): cytoplasmic proteins that present a KFERQ motif in their amino acid sequence are selectively translocated into the lysosomes in a process mediated by the chaperone Hsc70 (32).

As mentioned above, the lysosome-autophagy system plays an important role in many cellular functions, including quality control, cellular energy generation, cell and tissue remodeling, and cellular defense (16). Not surprisingly, inefficient autophagic activity is associated with development of a number of human diseases characterized by aberrant accumulation of intracellular substrates (33-35). Alterations of autophagy have been specifically observed in association with development of neurodegenerative diseases, such as Huntington's (36), Parkinson's (37), and Alzheimer's (38). Autophagy deficiency has also been observed in a number of lysosomal storage disorders (39), such as neuronal ceroid lipofuscinoses (40), mucopolysaccharidosis (35), and Pompe disease (41).
Macroautophagy (hereafter referred to as autophagy) is the most common route of autophagic clearance. Effective autophagic activity relies upon the coordinated regulation of autophagosome and lysosome biogenesis and function (17). Recent studies suggest that a number of lysosomal genes and autophagy genes exhibit coordinated transcriptional behavior and are regulated by the transcription factor EB (TFEB) (42), which belongs to the MiT/TFE subfamily of helix-loop-helix (bHLH) transcription factors (43). TFEB targets share a common regulatory motif in their promoter regions, namely the Coordinated Lysosomal Expression and Regulation (CLEAR) element (GTCACGTGAC) (42). TFEB targets have been identified by a combination of genomic and transcriptomic approaches and include lysosomal hydrolases, lysosomal membrane proteins and proteins associated with lysosomal biogenesis and autophagy (44). TFEB modulates lysosomal clearance by i) regulating the expression of lysosomal enzymes that are part of the CLEAR network (42); ii) regulating the expression of proteins involved in lysosomal protein trafficking that mediate lysosome biogenesis (45); and iii) regulating the expression of genes involved in different steps of the autophagy pathway, from cargo recognition and autophagosome biogenesis to lysosome-autophagosome fusion and substrate degradation (46).

Taken together, these studies point to the role of TFEB at the crossroad of the regulatory mechanisms that coordinate the autophagy and lysosomal pathways and, importantly, to the function of TFEB as a regulator of lysosomal proteostasis and autophagic clearance (47,48) (Figure 1.2).
Figure 1.2. TFEB regulates the lysosome-autophagy system. TFEB modulates the lysosome-autophagy system by i) regulating the expression of lysosomal enzymes; ii) regulating the expression of proteins involved in lysosome and autophagosome biogenesis; and iii) promoting lysosome-autophagosome fusion and substrate degradation.
TFEB activation is part of the cellular response to lysosomal stress and plays a key role in the regulating enhancement of lysosomal degradation. Under basal conditions, TFEB is mainly localized in the cytoplasm. Upon accumulation of lysosomal material, which induces lysosomal stress, TFEB translocates from the cytoplasm to the nucleus, resulting in the activation of genes encoding proteins involved in the lysosome-autophagy system (42). The status of lysosomes is communicated to TFEB via mTOR (mammalian target of rapamycin), a kinase that localizes on the lysosomal membrane as part of mTOR complex 1 (mTORC1). mTORC1 senses and transduces the signals arising from the changes in lysosomal status, and controls TFEB phosphorylation and localization. Specifically, under basal conditions and in the absence of lysosomal stress, TFEB is recruited to the lysosomal membrane by mTORC1 and phosphorylated. Phosphorylation by mTORC1 maintains TFEB in the cytoplasm and prevents its nuclear translocation. Starvation or lysosomal stress leads to mTORC1 dissociation from Rag GTPases on the lysosomal membrane resulting in mTORC1 inactivation. Under these conditions, TFEB is not maintained in a phosphorylated state by mTORC1 (49,50), and it translocates into the nucleus where it upregulates the expression of its target genes (42,51). To study the role of TFEB as a potential target for upregulating the lysosome-autophagy system and restore homeostasis in cells presenting accumulation of waste material, I selected a series of in vitro model systems of diseases characterized by inefficient or impaired activity of the lysosome-autophagy
system and cellular accumulation of undigested macromolecules, as described in detail in the next section.

1.2. Lysosomal Storage Disorders

Lysosomal storage disorders (LSDs) are a group of inherited metabolic diseases characterized by deficiencies in specific lysosomal hydrolytic activities that result from mutations in genes encoding lysosomal proteins, including hydrolytic enzymes, membrane proteins, and transporters responsible for trafficking of lysosomal proteins (52). The hallmark of LSDs is the accumulation of lysosomal storage material such as lipids and mucopolysaccharides (53). To date, approximately 50 LSDs have been identified (54). Despite manifesting with a diverse range of clinical symptoms, LSDs present similar cellular pathogenesis, as they are all characterized by loss of specific lysosomal hydrolytic functions and accumulation of undegraded lysosomal substrates. Many LSDs are individually rare and for this reason have been considered orphan diseases; however, they collectively represent a rather large family of diseases with a significant patient population (55).

1.2.1. Gaucher's Disease

Gaucher’s disease (GD) is the most common among LSDs with an overall prevalence of 1 in 60,000 people worldwide and 1 in 1,000 people in the Ashkenazi
Jewish population (56,57). GD is an autosomal recessive disorder mainly caused by mutations in the gene encoding the lysosomal hydrolase β-glucocerebrosidase (GC). As all lysosomal proteins, folding of newly synthesized GC occurs in the ER. Once the protein reaches its native three-dimensional conformation, it bypasses the ER quality control system and proceeds through the Golgi to the lysosome where it hydrolyzes glucosylceramide into glucose and ceramide. Mutated GC variants that fail to fold into their native structure are retained in the ER until native folding is reached or the substrate is recognized as irremediably misfolded and retrotranslocated to the cytosol for ERAD (11,58) (Figure 1.3).

![Figure 1.3. Glucocerebrosidase (GC) folding and trafficking through the secretory pathway.](image)

Newly synthesized GC progresses through a series of folding intermediates in the ER until it reaches native folding, and proceeds through the Golgi to the lysosome, where it hydrolyses glucosylceramide to glucose and ceramide. Misfolded GC variants are retrotranslocated to cytoplasm for ERAD.
Deficiency in GC results in diminished enzymatic activity and excessive buildup of glucosylceramide in the lysosomes. Affected organs include the spleen, liver, kidneys, lungs, bone marrow, and, in some patients, even the brain. Patients show varied degrees of hepatosplenomegaly, liver malfunction, hypersplenism, anemia, thrombocytopenia, skeletal diseases, and neurodegeneration (59). GD has been traditionally classified into three major subtypes based on the clinical symptoms. Type 1 (non neuronopathic) is the most common type of GD, especially in the Ashkenazi Jewish population. Type 1 GD is typically a macrophage disorder and does not affect the central nervous system. Patients with type 1 GD manifest a variety of symptoms arising from early childhood to adulthood. Type 2 (acute neuronopathic) and type 3 (chronic neuronopathic) are rarer than type 1, but involve neurologic symptoms, including eye movement and central auditory disorders. This classification has been recently revisited as clinical studies revealed that patients with different types of GD may share similar phenotypes, while patients with the same genotypes can present different clinical manifestations, suggesting a continuum in the spectrum of GD manifestations (60,61).

More than 200 missense or nonsense mutations in β-glucocerebrosidase gene (GBA, NM_000157) that result in GD have been identified so far (http://www.hgmd.cf.ac.uk/) (62). Most mutations do not directly impair the protein's enzymatic activity, but rather destabilize its native structure, thereby compromising its folding. Interestingly, these GC variants presenting non-
inactivating mutations that impact folding retain catalytic activity if forced to fold into their native 3D structures (63-65).

The most common mutation is the N370S substitution (66,67), which accounts for 70% of mutant alleles in Ashkenazi Jews and 25% in non-Jewish patients, is mostly associated with development of type 1 GD (68). N370S GC retains about 10% specific activity of the wild type enzyme (66). Although the link between genotype and phenotype remains unclear, clinical evidence suggests that the N370S GC variant displays sufficient residual activity to prevent the development of severe manifestations of the disease, such as neurodegeneration (66). The second most common mutation identified is the L444P substitution, which disrupts the protein hydrophobic core, resulting in complete loss of enzymatic activity (69). Not surprisingly, L444P GC is always associated with the development of neuronopathic symptoms in homozygous patients. Although it is generally speculated that the level of residual enzymatic activity is the major determinant of the severity of the disease, the relationship between the destabilizing effect of GC mutations, the residual enzymatic activity, and the severity of the disease, has not been established.

As mentioned above, both the N370S and L444P substitutions are misfolding, non-inactivating mutations. Unstable N370S and L444P GC variants retain catalytic activity if forced to fold into their native 3D structure (63,64,70). Hence, efforts have been devoted to the development of strategies to rescue folding, trafficking, and activity of the unstable enzyme variants (70-72). Specifically, the enzymatic activity of these variants was restored by enhancing the cellular folding capacity of cells
derived from GD patients through the use of small molecule proteostasis modulators that influence general cellular folding pathways that maintain protein homeostasis, such as chaperones and the UPS (70,73), Ca\textsuperscript{2+} homeostasis (71,72) or ERAD (74). In this study, cells derived from GD patients with GC misfolding mutations were used as a model system to investigate the role of TFEB in regulating lysosomal proteostasis, specifically in controlling folding, processing, and activity of unstable lysosomal enzymes.

1.2.2. Tay-Sachs Disease

Tay-Sachs disease is caused by deficient activity of the lysosomal enzyme β-hexosaminidase A (HexA) and aberrant storage of gangliosides GM2 (N-AcGalβ1,4(NeuAcα2,3)Gal β1,4Glc-cermide) (75,76). The most common mutations in the gene encoding HexA do not impair HexA catalytic activity but rather destabilize its folding. As a result, HexA variants are targeted to ERAD, which leads to loss of HexA activity in the lysosome (75). The G269S substitution, one of the most prevalent HexA mutations, is a misfolding, non-inactivation mutation (77). Forcing native folding of G269S HexA leads to an increase in lysosomal HexA activity (70,76), similar to what was observed in cells derived from GD harboring GC variants containing misfolding, non-inactivating mutations. In this study, cells derived from patient with Tay-Sachs disease are used as a model system to investigate the role of
TFEB in regulating lysosomal proteostasis and demonstrate that proof-of-principle demonstrations conducted using GD cells may be applicable to multiple LSDs.

1.2.3. Neuronal Ceroid Lipofuscinosis

Neuronal ceroid lipofuscinoses (NCLs) are a group of more than twelve genetically distinct neurodegenerative LSDs affecting children and young adults (78,79). The hallmark of NCLs is the aberrant intracellular accumulation of autofluorescent ceroid lipopigment due to mutations in genes encoding proteins involved in lysosomal biogenesis and function (80). NCLs are classified based on the defective gene as well as the age of disease onset (81). For instance, late infantile neuronal ceroid lipofuscinosis (LINCL) is associated with deficiency of Tripeptidyl peptidase (TPP1) activity, which is caused by mutations in CLN2 gene. In this study, cells derived from patient with LINCL were used as a model system to test cell engineering strategies to enhance autophagic clearance of ceroid lipopigment.

1.2.4. Therapeutic options for LSDs

Therapeutic options for LSDs include enzyme replacement therapy (ERT), substrate reduction therapy (SRT), chemical chaperone therapy (CCT), and gene-therapy (82). The following section summarizes the available treatment modalities for LSDs and the remaining challenges associated with clinical applications.
The most commonly used approach to treat LSDs is enzyme replacement therapy (ERT), which involves intravenous injection of the wild type, functional enzyme to replace the deficient lysosomal enzyme with the ultimate goal to reduce the burden imposed by the accumulation of lysosomal material (83). The effectiveness of ERT was first demonstrated for the treatment of GD. ERT is now approved for other six LSDs and is being evaluated in clinical trials for several other LSDs (83). However, due to the enzyme’s short half-life and poor delivery to certain affected areas (particularly bones and lungs), ERT requires weekly injections and involves significant investment of money and resources per patient for life (84). Moreover, ERT fails to address neuronopathic symptoms due to the inability of the injected enzyme to cross the blood-brain barrier, and it is therefore currently only used to treat non-neuronopathic forms of LSDs (85).

Substrate reduction therapy (SRT) has been proposed as an alternative to ERT. SRT is based on inhibition of the synthesis of macromolecules that accumulate in the lysosomes of tissues and organs of patients with LSDs, with the ultimate goal to restore the ratio between storage material and enzymatic function. For instance, N-butyldeoxynojirimycin (NB-DNJ, Zavesca®) is an FDA approved small molecule for the treatment of non-neuronopathic forms of GD (86) and neurological manifestations of Niemann-Pick type C (87). NB-DNJ prevents the synthesis of glucosylceramide by inhibiting glucosyltransferase, the enzyme responsible for catalyzing the biosynthesis of glycosphingolipids in the Golgi (88,89). In GD patients, glucosyltransferase inhibition allows restoring the cellular physiologic ratio of
glucosylceramide concentration to GC hydrolytic activity, thereby reducing glucosylceramide buildup in the lysosome. In patients with Niemann-Pick type C, which is characterized by accumulation of glycosphingolipids, inhibition of glucosyltransferase by NB-DNJ reduces glycosphingolipid synthesis (90). However, because NB-DNJ treatment reduces synthesis of all gluco-based glycolipids, considerable side effects typically occur (91,92). Thus, to date, SRT it is only used to treat LSD patients when ERT cannot be considered an option (93).

A new therapeutic approach to prevent lysosomal storage was recently developed based on the use of small molecules that structurally resemble the enzyme inhibitor called “pharmacologic” or “chemical” chaperones and bind to enzyme variants harboring non-inactivating mutations and stabilize their native structure. The small molecule serves to chaperone the natively folded mutated enzyme variant through the secretory pathway to the lysosome, where the enzyme variant is typically more stable due to the acidic pH for which these enzymes have been evolutionarily selected. Chemical chaperones are easily displaced from the enzyme active site by the substrate, which is present at high concentration, and substrate hydrolysis occurs. In summary, chemical chaperones rescue native folding of enzyme variants that contain misfolding, non-inactivating mutations, thereby restoring enzymatic activity in the lysosomes (94,95). Chemical chaperone therapy (CCT) has the potential to treat patients with neuronopathic symptoms because small molecules are typically able to cross the blood brain barrier. In addition, oral bioavailability and low cost of small molecules make CCT a promising therapeutic
option for patients with LSDs. However, only a limited set of enzymes variants associated with LSDs can be rescued using chemical chaperones. This barrier is likely due to the intrinsic mechanism of chemical chaperones that stabilize the protein native structure by mimicking the binding mechanism of the enzyme inhibitor and thus interacting specifically with the enzyme active site. For instance, N-(n-nonyl)deoxynojirimycin (NN-DNJ) functions as a chemical chaperone by rescuing folding and increasing the activity of different GC variants, including N370S GC (63,64). However, the L444P GC variant is not amenable to CCT, most likely because the mutation is located in a domain structurally distinct from the active-site domain (96). Moreover, CCT cannot rescue the activity of enzyme variants with inactivating mutations, such as deletions, truncations, and substitutions in the active site.

Treatment of LSDs through gene therapy has also been explored (97). A number of features of the diseases, including modest requirement for control overexpression level (as only 5-10% of wild type enzyme activity is required to alleviate symptoms and overexpression of lysosomal enzymes does not seem to have detrimental effects), single-gene defects, and ubiquitous expression of lysosomal enzymes, make LSDs good targets for gene therapy. Over the years, many gene therapy approaches have been tested in animal models of lysosomal storage diseases, and two main approaches have emerged as the most promising for translation into human clinical trials: in vivo gene transfer using viral vector delivery systems to deliver therapeutic genes; or ex vivo modification of bone marrow stem
cells in vitro using viral vectors encoding native enzymes followed by transplantation (98). Compared to ERT, gene therapy has the potential for long-term expression of the therapeutic protein (99). However, although multiple peripheral organ systems can be efficiently treated, systemic delivery of viral gene transfer vectors has only a limited effect on the central nervous system (CNS), and direct delivery to the CNS involves invasive procedures and limited diffusion of the vector (97,100). Moreover, safety issues associated with the use of viral vehicles also need to be evaluated.

In summary, the treatment of most LSDs is currently an unmet medical need, as available treatment options are limited to specific LSDs and typically fail to address the symptoms associated with neuropathic forms of the disease. In an attempt to provide treatment options that can overcome the blood brain barrier challenge and restore CNS functionality, efforts have been recently focused on the development of small molecule-based strategies aimed at rescuing folding, lysosomal trafficking, and activity of endogenous lysosomal enzyme variants. A variety of small molecule proteostasis modulators, including proteasome inhibitors (70,101), Ca^{2+} blockers (71,102), and ERAD inhibitors (74), were found to enhance folding and activity of L444P GC variant, which is the most prevalent mutation in GD patients with CNS symptoms. However, given the mechanism of action of proteostasis modulators that affect general cellular pathways and do not specifically target lysosomal proteins, unwanted consequences on other essential cellular functions are likely to occur. Possible side effects triggered particularly by the
induction of ER stress and activation of the unfolded protein response could be detrimental to cell function or viability and ultimately jeopardize effective rescue of lysosomal proteostasis (71,74).

In an attempt to identify modulators specific to lysosomal proteostasis, I focused on TFEB, a recently identified master regulator of the lysosome-autophagy pathways (42). As explained in details in Section 1.1, TFEB modulates lysosomal pathways by controlling the expression of lysosomal enzymes involved in the degradation of proteins, glycosaminoglycans, sphingolipids and glycogen (42,44). TFEB also modulates cellular clearance by controlling autophagy (46) and lysosomal exocytosis (45) and is thus a promising target for the development of therapeutic approaches for the treatment of diseases in which impaired autophagy or the storage of undegraded material plays a role in the pathogenic cascade (45,103,104). These diseases encompass LSDs (45), but also include protein deposition diseases, such as Huntington’s (42,105) and Alzheimer’s (106). Therefore, in this study, I aimed to identify TFEB modulators that enhance the capacity of the lysosome-autophagy system, thereby rescuing the folding of mutated, unstable proteins or promoting clearance of unwanted cellular material.
1.3. Nanoparticles as Autophagy Activators

β-Cyclodextrins (βCD), a family of cyclic oligosaccharides known to deplete cholesterol from biological membranes (107-109), were reported to induce activation of autophagy upon cellular uptake (110) (Figure 1.4). Particularly, 2-hydroxypropyl-β-cyclodextrin (HPβCD) is an FDA-approved drug delivery vehicle used in a variety of pharmaceutical applications (111). The recent serendipitous finding that HPβCD alone could increase the life span of a mouse model of the cholesterol storage disorder (112,113), Niemann-Pick type C (NPC) disease (114,115), raised significant questions regarding the inert nature of HPβCD. It was previously demonstrated that HPβCD can extract excess cholesterol from biological membranes by trapping it into its hydrophobic core (107,108). This interaction provided a mechanistic hypothesis for the observed HPβCD-mediated clearance of cholesterol in the brain of NPC mice and in fibroblasts derived from NPC patients (112,116-118). Not surprisingly, these findings have paved the way for a new therapeutic avenue for NPC, culminating in the recently approved clinical trial in which HPβCD is the active agent (http://clinicaltrials.gov/show/NCT01747135). The dissection of the cellular pathways impacted by the administration of HPβCD, however, is still in its early infancy. Recent findings demonstrated that depletion of cholesterol using HPβCD triggers activation of autophagy (110). However, the molecular mechanisms that regulate activation of autophagy observed upon HPβCD treatment remain unclear. Moreover, whether HPβCD treatment may lead to
depletion of additional cellular substrates that are normally cleared by autophagy is not known.

**Figure 1.4. Structure of β-cyclodextrin.** β-cyclodextrin is a cyclic glucose oligosaccharide with seven D-glucopyranonsyl residues. The diameter of the cavity and the outer rim are 0.70 nm and 1.66 nm, respectively.

A number of synthetic nanoparticles were also found to interact with the lysosome-autophagy system when internalized into cells and to enhance formation of autophagosomes (119-123). These findings suggest that nanoparticles may activate the autophagy pathway. As described in Section 1.1, autophagic clearance is mediated by sequestration of intracellular cargo into double-membrane vesicles called autophagosomes (124). Fusion of autophagosomes with lysosomes results in the formation of autophagolysosomes where degradation takes place. Increasing evidence suggests that nanoparticles of different size and composition are sequestered into autophagic vesicles, such as autophagosomes and autophagolysosomes, when internalized into cells (119-123). This sequestration
may occur because when nanoparticles are internalized into cells, like any type of nanosized material perceived by the cell as foreign or toxic (such as viruses and pathogens), they may induce activation of cellular clearance mechanisms. However, the molecular mechanism of nanoparticle-induced autophagy and the consequent impact on cell physiology are still unclear. Therefore, it is of critical importance to investigate the impacts of nanoparticles on the autophagy system and to determine whether nanoparticle-induced activation of autophagy results in enhanced clearance of storage material.

Interestingly, compartmentalization of nanoparticles into autophagosomes is not necessarily always followed by clearance of autophagosomes by the lysosomes and may result instead in autophagy dysfunction and accumulation of autophagosomes. Gold nanoparticles, for instance, may impair lysosomal function, leading to blockage of the autophagic flux (125). Cationic nanoparticles were also reported to induce autophagy, but also to disrupt lysosomes, ultimately blocking autophagic flux and impairing degradation (126,127). Specifically, in the case of cationic polystyrene nanoparticles, a “proton sponge” effect was proposed as a potential mechanism of lysosomal membrane permeabilization. It was suggested that the high H⁺ buffering capacity of the amino groups on polystyrene nanoparticle surface could interfere with acidification of lysosomes, impair the proton pump activity, and ultimately induce lysosomal membrane permeabilization (127). This evidence suggests that nanomaterial properties play an important role in determining the nature of the autophagic response that is induced upon
internalization of nanoparticles. Specifically, I hypothesized that a variety of nanosized materials can induce activation of the autophagy system. This hypothesis is based on published evidence demonstrating that nanoparticles of different composition, shape, and charge were observed to induce autophagy (125,128-134). I also hypothesized that the specific physicochemical properties of nanoparticles, such as composition and surface charge, determine the specific nature of the autophagic response and whether nanoparticle-induced activation of autophagy is associated with enhanced autophagic clearance or impairment of downstream steps of the autophagy system, such as lysosomal function, lysosome-autophagosome fusion, and autophagic degradation.

The design rules for engineering nanoparticles with desired and precisely controlled impact on the autophagy system, however, are still unclear. Thus, I investigated a series of representative nanoparticles with the ultimate goal to map the physicochemical properties of nanoparticles with the specific autophagic response induced upon uptake of nanoparticles. Results from this study will generate the design rules for engineering nanoparticles with desired effect on the lysosome-autophagy system and will inform the design of nanotherapeutics for the treatment of diseases characterized by inefficient autophagic clearance.
Chapter 2

Objectives

The objective of this research is to develop chemical and biological strategies to modulate the cellular quality control machineries that control protein folding, processing, and degradation and restore cellular homeostasis under conditions of proteotoxic stress. Specifically, this work focuses on manipulating the lysosome-autophagy system to enhance folding and processing of lysosomal enzymes as well as to enhance the cellular clearance capacity. I hypothesized that modulating expression and activity of TFEB, a master regulator of autophagy and lysosomal biogenesis, affects the phenotypes associated with deficiencies in lysosomal hydrolytic activities and accumulation of undegraded lysosomal substrates, which characterize the development of lysosomal storage disorders (LSDs). This hypothesis was investigated by achieving the following specific objectives:
Objective 1. Understanding the role of TFEB in modulating lysosomal proteostasis. This objective was accomplished by exploring chemical and genetic approaches to activate TFEB in cells derived from patients affected by LSDs characterized by misfolding, non-inactivating mutations in genes encoding lysosomal hydrolases. Chemical activation of TFEB was achieved by culturing cells in media supplemented with sucrose, a known activator of TFEB. Genetic induction of TFEB was achieved by overexpressing wild type TFEB as well as TFEB variants that are known to accumulate preferentially in the nucleus and thus induce activation of TFEB-target genes. To investigate the role of TFEB in regulating lysosomal proteostasis, I monitored the folding, trafficking and activity of unstable lysosomal enzyme variants in in vitro models of LSDs. Results from this study provide evidence that TFEB activation mediates rescue of unstable, degradation-prone lysosomal enzyme variants and establish the role of TFEB as regulator of lysosomal proteostasis. This work was recently published in Human Molecular Genetics (135).

Objective 2. Exploring pharmacologic activation of TFEB to enhance autophagic clearance. Because of the similarities between the biological effects of 2-hydroxypropyl-β-cyclodextrin (HPβCD) administration and TFEB activation – including the ability to activate autophagy and promote cellular clearance – I hypothesized that TFEB mediates activation of autophagy observed upon HPβCD administration. In this study, I investigated this hypothesis by testing the molecular
mechanism of autophagy induction activated in response to cell treatment with HPβCD using an \textit{in vitro} model system of TFEB activation, namely HeLa cells that overexpress TFEB. To investigate whether HPβCD treatment induces enhancement of autophagic clearance mediated by TFEB and autophagy induction is accompanied by activation of apoptosis, I used fibroblasts derived from a patient with late infantile neuronal ceroid lipofuscinosis (LINCL). LINCL cells present accumulation of proteolipid aggregates and provide an \textit{in vitro} model system of lysosomal storage (described in Chapter 1), which allows evaluating whether autophagy activation parallels enhanced clearance of storage material. HPβCD treatment was found to activate TFEB-mediated autophagy and enhance clearance of storage material. This study provides a mechanistic understanding of the cellular response to HPβCD treatment, which will inform the development of safe HPβCD-based therapeutic modalities and may enable engineering HPβCD as a platform technology to reduce the accumulation of lysosomal storage material. This work was recently published in \textit{Journal of Biological Chemistry} (136).

**Objective 3: Understanding the design rules for engineering nanoparticles with desired autophagy-inducing properties: A focus on size and charge.** This study aims to understand the design rules for engineering nanoparticles with desired and precisely controlled impact on the autophagy system. I hypothesized that the physicochemical properties of nanoparticles and, specifically, the charge and size, affect the autophagic response induced upon nanoparticle uptake. To test
this hypothesis, I investigated the impact of a library of polystyrene nanoparticles of different size and surface charge on the lysosome-autophagy system. I evaluated each step of the autophagic flux to determine the effect of nanoparticle size and surface charge on i) TFEB-mediated autophagy activation, ii) lysosomal integrity, iii) autophagy-associated cell death and iv) autophagic flux. All the polystyrene nanoparticles investigated in this study were found to activate TFEB-mediated autophagy. However, activation of autophagic clearance was found to depend on nanoparticle charge. Neutral and anionic polystyrene nanoparticles were observed to activate autophagy without inducing cytotoxicity and to promote clearance of toxic aggregates, while cationic nanoparticles were found to result in impairment of lysosome integrity, reduced autophagolysosome formation and blockage of autophagic flux. Results of this study elucidate the mechanism of nanoparticle-induced autophagy and map the physicochemical properties of nanoparticle to the nature of autophagic response.

**Objective 4: Exploring activation of autophagy in response to ceria nanoparticles stabilized by organic surface coatings.** Ceria nanoparticles are used in an increasingly diverse number of applications including UV filters and catalysts (137-139). The expanding commercial scale production and use of ceria nanoparticles have inevitably increased the risk of release of nanoceria into the environment as well as the risk of human exposure. The use of nanoceria in biomedical applications is also being currently investigated due to its recently
characterized anti-oxidative properties. The goal of this study is to investigate the impact of ceria nanoparticles on the lysosome-autophagy system with the ultimate goal to understand whether cell exposure to ceria nanoparticles induce activation of autophagy and enhance autophagic clearance. Experiments were conducted using a battery of ceria nanoparticles functionalized with different types of biocompatible coatings (N-Acetylglucosamine, polyethylene glycol and polyvinylpyrrolidone) expected to have minimal effect on lysosomal integrity and function. Ceria nanoparticles were found to activate TFEB-mediated autophagy and enhance degradation of autophagic cargo. This study provides a mechanistic understanding of the interaction of ceria nanoparticles with the lysosome-autophagy system. These findings will inform the design of nanotherapeutics for the treatment of diseases with defective autophagic function and accumulation of lysosomal storage material as well as the use of nanoceria as drug delivery vehicles.
Chapter 3

TFEB Regulates Lysosomal Proteostasis


3.1. Introduction

Gaucher’s disease (GD) is the most common among lysosomal storage disorders (LSDs) caused by loss of lysosomal glucocerebrosidase (GC) activity and consequent accumulation of glucosylceramide (140) (refer to Chapter 1 for a detailed description). As described before, the most prevalent mutations in GD patients consist of single amino acid substitutions that do not impair the protein’s catalytic activity but rather destabilize the protein’s native structure, resulting in extensive degradation of the misfolded enzyme via ER-associated degradation.
(ERAD) (66). Although the causative relationship between genotype and phenotype is unclear, evidence points to a correlation between the extent of enzyme ERAD, lysosomal trafficking, and residual activity of mutated GC and the severity of the clinical manifestations in GD patients (141). Loss of lysosomal GC activity was in fact shown to correlate with a decrease in the concentration of the mutated enzyme due to ERAD and not with impaired (and thus lowered) enzyme activity (73). Accordingly, most unstable GC variants containing misfolding mutations retain biologic activity if forced to fold into their native 3D structure (63,142). On the other hand, overexpressing unstable GC variants did not result in increase in properly folded, functional enzymes (143), suggesting that the innate cell quality control system cannot rescue severely destabilized GC variants.

In an attempt to promote native folding, trafficking and activity of mutated GC variants, considerable effort was devoted to modulating the proteostasis network in GD cells (144). As described before, proteostasis modulators typically influence cellular components or pathways that control protein folding and degradation, including molecular chaperones, the UPS (70,101), Ca\(^{2+}\) homeostasis (71,102), and ERAD (74). Specifically, an increase in L444P GC activity to about 30% of wild type GC activity was previously observed in GD cells treated with modulators of Ca\(^{2+}\) homeostasis (102) or with ERAD inhibitors (74). This rescue in GC activity increased to about 40 to 50% of wild type GC activity when these compounds were used in combination with other proteostasis modulators (102). It is important to note that the abnormal storage of glucosylceramide can be efficiently reduced by
enhancing residual GC activity to as little as 20% of wild type activity, which, in turn, is expected to ameliorate GD symptoms (140).

Despite their diverse mechanisms of action, proteostasis modulators were found to typically induce i) dramatic upregulation of GC transcription as well as ii) activation of folding and trafficking pathways (70,71,74,102). I speculated that upregulating GC expression while simultaneously enhancing the cellular folding and trafficking capacity would result in an increase in the pool of natively folded enzyme variant that reaches the lysosome. This study aims to identify strategies to induce upregulation of GC expression and protein processing through the secretory pathway, thereby establishing the molecular basis for the development of proteostasis modulators that enhance GC activity for therapeutic intervention.

Interestingly, GBA, the gene encoding GC, was shown to be a direct target of TFEB, a master regulator of lysosomal biogenesis and function (42). As described in Chapter 1, TFEB modulates lysosomal clearance by regulating the expression of genes encoding proteins involved in the lysosome-autophagy system (42). Given its general roles in modulating lysosomal biogenesis and function, I speculated that TFEB might also regulate lysosomal proteostasis. Specifically, I hypothesized that TFEB activation enhances the folding and trafficking of unstable enzyme variants, thereby resulting in increase in enzymatic activity in the lysosome. This hypothesis was tested in fibroblasts derived from LSD patients with misfolding mutations in the genes encoding lysosomal enzymes that result in low residual enzymatic activity in the lysosome. I found that TFEB activation results in enhanced folding, trafficking
and lysosomal activity of a severely destabilized glucocerebrosidase (GC) variant associated with the development of GD, the most common LSD. TFEB specifically induces the expression of GC and of key genes involved in folding and lysosomal trafficking, thereby enhancing both the pool of mutated enzyme and its processing through the secretory pathway. TFEB activation also rescues the activity of a β-hexosaminidase mutant associated with the development of another LSD, Tay-Sachs disease, thus suggesting general applicability of TFEB-mediated proteostasis modulation to rescue destabilizing mutations in LSDs. In summary, these findings identify TFEB as a regulator of lysosomal proteostasis and suggest that TFEB may be used as a therapeutic target to rescue enzyme homeostasis in LSDs.

3.2. Results

3.2.1. Chemical activation of TFEB enhances native folding, trafficking and activity of L444P GC in GD patient-derived fibroblasts

To investigate the role of TFEB in regulating lysosomal proteostasis in LSDs, I first studied the effects of sucrose, a known TFEB activator (42), in patient-derived fibroblasts harboring L444P GC. This enzyme variant is severely destabilized, and is typically targeted to ERAD, leading to almost complete loss of lysosomal GC activity (69). L444P GC fibroblasts were cultured with a range of sucrose concentrations for 8 days, and GC activity was measured every 2 days using the intact cell activity
assay, as previously described (71). L444P GC activity was observed to increase 4.4-fold in cells treated with 150 mM sucrose (final medium concentration; p<0.01) for 4 days compared with untreated cells, which corresponds to about 55% of the wild type GC activity (Figure 3.1). Prolonging the time of incubation with sucrose resulted in even higher increase in GC activity, reaching 85% of wild type GC activity after 8 days of treatment with 100 mM sucrose (p<0.01).

![Graph showing the effect of sucrose concentration on GC activity over time](image)

**Figure 3.1. Sucrose treatment enhances L444P GC activity in GD patient-derived fibroblasts.** Time course of relative L444P GC activities in cells treated with a range of concentrations of sucrose for 8 days. GC activities were normalized to the activity of untreated cells (left y axis) (p<0.01). The corresponding fraction of wild type GC activity is reported (right y axis). Data are reported as mean ± SD (n = 3).

Polyol osmolytes, such as sucrose, can act as chemical chaperones and stabilize the protein native structure (145). Particularly, sucrose was shown to promote native folding and activity of denatured aminoacylase and prevent its
aggregation *in vitro* (146). Glycerol and glucose were also reported to stabilize proteins prone to misfolding. Glycerol treatment was shown to rescue the native conformation of a severely destabilized variant of the cystic fibrosis transmembrane conductance regulator (F508 CFTR) and to restore its function to a level comparable with wild type CFTR in NIH 3T3 cells (147). Glucose was shown to stabilize the native state of purified wild type transthyretin under denaturing conditions or conditions inducing aggregation (148). To investigate whether the increase in L444P GC activity observed upon sucrose treatment was due to sucrose function as a chemical chaperone, the activity of L444P GC was measured in cells treated with other known chemical chaperones, glycerol and glucose (the latter being the degradation product of sucrose) (145,148). L444P GC patient-derived fibroblasts were treated with glycerol or glucose under the same conditions applied to test sucrose, and GC activities were evaluated every 2 days for up to 8 days. No significant change was observed in GC activity even after 8 days of treatment (Figure 3.2A-B), indicating that, unlike sucrose, glycerol and glucose do not promote native folding of L444P GC. Together, these data suggest that the main mechanism involved in sucrose-mediated rescue of GC activity is likely not limited to its function as a chemical chaperone or to the chaperone-like activities of its breakdown product (i.e., glucose).
Figure 3.2. Cell treatment with glycerol or glucose does not promote rescue of L444P GC activity in GD patient-derived fibroblasts. Relative L444P GC activities were evaluated in cells treated with glycerol (A) or glucose (B) every 2 days for up to 8 days. GC activities were calculated as described in Figure 3.1. The data are reported as the mean ± SD (n=3).

To investigate whether sucrose treatment enhances L444P GC trafficking to the lysosome, the glycosylation state and subcellular localization of GC was tested. GD fibroblasts were treated with 150 mM sucrose for 4 days and L444P GC
glycosylation state was investigated by subjecting the total protein content to endoglycosidase H (EndoH) treatment followed by Western blot analyses using a GC-specific antibody, as previously described (74). EndoH hydrolyzes immature high mannose N-linked glycoproteins retained in the ER. EndoH treatment typically leads to the detection of a low molecular weight (MW) band corresponding to partially glycosylated, ER-retained GC (EndoH-sensitive) and a high MW GC band corresponding to fully glycosylated, lysosomal GC (EndoH-resistant) in Western blot experiments (149). A representative Western blot and the quantification of EndoH-resistant and EndoH-sensitive GC bands are reported (Figure 3.3A). In untreated cells, nearly all L444P GC was detected as EndoH-sensitive at 0 hr and 4 days, as expected (74). However, treatment with sucrose for 4 days resulted in an increase of the GC EndoH-resistant form up to 50% of the total GC protein, which also increased by 8.5-fold (Figure 3.3A). L444P GC subcellular localization was also tested by confocal microscopy by testing colocalization of GC with calnexin (CNX) and with lysosomal-associated membrane protein 1 (LAMP-1) to evaluate GC accumulation in the ER and in the lysosome, respectively. L444P GC was barely detectable in untreated cells due to extensive ERAD, as expected (150), but a significantly larger pool of GC accumulated both in the ER and in the lysosomes of cells treated with 150 mM sucrose for 4 days (Figure 3.3B-C). Together, these results suggest that sucrose treatment promotes rescue of L444P GC folding, resulting in GC escape of ERAD, and increased lysosomal transport and enzymatic activity.
Figure 3.3. Sucrose treatment enhances L444P GC trafficking in GD patient-derived fibroblasts. (A) Western blot analyses of total protein content from L444P GC fibroblasts treated with sucrose (150 mM for 4 days) and with EndoH, and detected using a GC antibody. The solid and dashed arrows indicate EndoH-resistant and EndoH-sensitive bands, respectively. Quantification of GC bands was obtained with ImageJ analysis software. Quantification of the lower molecular weight (MW), EndoH-sensitive band corresponding to ER-retained GC is reported in the white portion of the bars; quantification of higher MW, EndoH-resistant bands corresponding to lysosomal GC is reported in the black portions. (B-C) Confocal microscopy analyses of GC and CNX (an ER marker) (B) and GC and LAMP-1 (a lysosomal marker) (C) in L444P GC fibroblasts. Cells were treated with sucrose (150 mM) for 4 days. Colocalization of CNX (blue, column 1) and GC (red, column 2) is shown in purple (column 3). Colocalization of LAMP-1 (blue, column 1) and GC (red, column 2) is shown in purple (column 3). Heatmaps of colocalization images were obtained with ImageJ analysis software (column 4). Hot colors represent positive correlation (colocalization), whereas cold colors represent negative correlation (exclusion). UT, untreated; Sucr, sucrose. The scale bar is 10 μm.

A number of small molecule proteostasis modulators including MG-132 (70) were reported to enhance L444P GC residual activity in patient-derived fibroblasts at the expense of significant cytotoxicity and induction of apoptosis, most likely due to activation of the Unfolded Protein Response (UPR) (71,74). To evaluate whether treatment with sucrose affects cell viability under conditions promoting rescue of mutated GC activity, membrane rearrangement [characteristic of early apoptosis (annexin V binding)] and membrane fragmentation [characteristic of late apoptosis (propidium iodide (PI) binding)] were evaluated using the Cyto-GLO™ annexin V-FITC apoptosis detection kit as previously described (74). MG-132 (0.6 μM) was used for comparison. Sucrose treatment (150 mM, 4 days) did not cause any significant changes in activation of apoptosis compared with untreated cells (Figure 3.4A-B).
Figure 3.4. Sucrose treatment does not induce apoptosis. (A) Annexin V binding affinity change (%) in L444P GC patient-derived fibroblasts treated with sucrose (150 mM) or MG-132 (0.6 µM) for 16 hr normalized to untreated cells (p<0.01). (B) Propidium iodide (PI) binding population change (%) of cells treated with sucrose (150 mM) or MG-132 (0.6 µM) for 16 hr normalized to untreated cells (p<0.01). The number of cells analyzed in each experiment was 10,000. The data are reported as the mean ± SD (n = 3).

As described in Chapter 1, TFEB localizes predominantly in the cytoplasm in resting cells and translocates into the nucleus upon activation under conditions of lysosomal stress (42). Sucrose treatment, a model of lysosomal stress in cultured cells (151), was shown to cause progressive nuclear translocation of TFEB in HeLa cells stably transfected for TFEB expression (42). I asked whether sucrose treatment under conditions that promote rescue of L444P GC activity induces TFEB nuclear translocation. L444P GC fibroblasts and wild type fibroblasts were transfected with a plasmid encoding TFEB fused to mRuby (45) and treated with sucrose (150 mM). TFEB intracellular localization was evaluated using confocal microscopy by monitoring the DAPI nuclear stain and mRuby fluorescence (45) (Figure 3.5A). As expected, TFEB was barely detectable in the nucleus of untreated
wild type cells (42). Partial nuclear translocation of TFEB was observed in GD cells likely due to glucosylceramide-induced lysosomal stress (42). Administration of sucrose resulted in an increase of TFEB nuclear localization in both wild type and GD fibroblasts (Figure 3.5A). TFEB nuclear localization in GD cells was quantified by calculating the fraction of transfected cells that present nuclear localization of TFEB (Figure 3.5B). TFEB nuclear localization was observed in 31.3 ± 2.3% of untreated L444P GC fibroblasts transfected with TFEB-mRuby. The fraction of cells presenting TFEB nuclear localization increased to 71.8 ± 3.8% after 1 day and to 85.5 ± 4.2% after 2 days of treatment with sucrose (Figure 3.5B; p<0.01), demonstrating that sucrose activates TFEB in GD fibroblasts.
Figure 3.5. Sucrose treatment promotes TFEB nuclear localization in GD patient-derived fibroblasts.  (A) Confocal microscopy analyses of TFEB subcellular localization in wild type and L444P GC fibroblasts transfected with a plasmid encoding mRuby-TFEB and treated with 150 mM sucrose for 24 hr. Colocalization of DAPI (blue, column 1) and mRuby-TFEB (red, column 2) is shown in purple (column 3). Heatmaps of colocalization images were obtained as described in Figure 3.3. The scale bar is 5 μm. (B) Percentage of cells with nuclear localization of mRuby-TFEB upon sucrose treatment. Representative fields containing 50 to 100 cells were analyzed to calculate the percentage of cells with mRuby-TFEB nuclear localization in cells that express mRuby-TFEB (p<0.01). UT, untreated. Data are reported as mean ± SD (n = 3).
3.2.2. Sucrose administration specifically activates the CLEAR network in GD fibroblasts

Gene Ontology (GO) and KEGG pathway analyses were conducted in collaboration with Dr. Marco Sardiello (Baylor College of Medicine).

To determine whether sucrose administration resulted in the activation of the CLEAR network, and whether additional transcriptional programs were also activated, expression microarray analyses were performed in treated and untreated GD fibroblasts and global changes in gene expression. The microarray platform HT-12 V4.0 (Illumina) was used to profile the transcriptome changes in total RNA extracted from GD fibroblasts before and after treatment with sucrose for 4 days. After treatment, 925 genes were induced by a fold change of at least 2. Gene Ontology (GO) and KEGG pathway analyses of induced genes showed that the only significantly over-represented class was related to lysosomal metabolism (N = 53; fold enrichment = 5.4; adjusted p-value < 10^{-20}) (Table 3.1). Comparison with previous work (44) showed that most of these genes are part of the CLEAR network and are, therefore, bona fide TFEB direct targets (Figure 3.6A). Additional TFEB targets of unknown function or not annotated as genes participating in the lysosomal function were also induced by sucrose (Figure 3.6A). No known lysosomal gene was downregulated upon sucrose administration. A network analysis of upregulated genes performed using coregulation analysis (44,152) and Cytoscape (153) for graphical visualization showed that TFEB targets are located at the core of the regulatory network formed by the genes induced by sucrose (Figure
These data suggest that the primary response of the cell to sucrose administration is the activation of the CLEAR network through TFEB. Additional induced genes that are not direct TFEB targets might be part of secondary pathways that are activated following sucrose administration or TFEB activation. However, no specific cellular processes other than those related to lysosomal biogenesis and function emerged from the analysis of induced genes.

Table 3.1. Lysosomal genes upregulated upon sucrose administration to Gaucher fibroblasts.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Protein</th>
<th>Fold change</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Hydrolases</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FUCA1</td>
<td>fucosidase, alpha-L-1, tissue</td>
<td>7.88</td>
</tr>
<tr>
<td>CTSK</td>
<td>cathepsin K</td>
<td>5.61</td>
</tr>
<tr>
<td>CTNS</td>
<td>cystinosis, nephropathic</td>
<td>5.23</td>
</tr>
<tr>
<td>PLA2G15</td>
<td>phospholipase A2, group XV</td>
<td>4.96</td>
</tr>
<tr>
<td>NEU1</td>
<td>sialidase 1 (lysosomal sialidase)</td>
<td>4.05</td>
</tr>
<tr>
<td>GBA</td>
<td>glucosidase, beta; acid (includes glucosylceramidase)</td>
<td>3.62</td>
</tr>
<tr>
<td>TPP1</td>
<td>tripeptidyl peptidase I</td>
<td>3.47</td>
</tr>
<tr>
<td>CTS4</td>
<td>cathepsin A</td>
<td>2.97</td>
</tr>
<tr>
<td>HEXA</td>
<td>hexosaminidase A (alpha polypeptide)</td>
<td>2.75</td>
</tr>
<tr>
<td>HEXB</td>
<td>hexosaminidase B (beta polypeptide)</td>
<td>2.67</td>
</tr>
<tr>
<td>HYAL3</td>
<td>hyaluronoglu cosaminidase 3</td>
<td>2.62</td>
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<tr>
<td>ASAH1</td>
<td>N-acylsphingosine amidohydrolase (acid ceramidase)</td>
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<td>CTSD</td>
<td>cathepsin D</td>
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<td>NAGLU</td>
<td>N-acetylglucosaminidase, alpha-</td>
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<td>HYAL2</td>
<td>hyaluronoglu cosaminidase 2</td>
<td>2.41</td>
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<tr>
<td>GLB1</td>
<td>galactosidase, beta 1</td>
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<td>DNASE2</td>
<td>deoxyribonuclease II, lysosomal</td>
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<td>GLA</td>
<td>galactosidase, alpha</td>
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<tr>
<td>LIPA</td>
<td>lipase A, lysosomal acid, cholesterol esterase</td>
<td>2.19</td>
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<tr>
<td>GNS</td>
<td>glucosamine (N-acetyl)-6-sulfatase</td>
<td>2.16</td>
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<td>SMPD1</td>
<td>sphingomyelin phosphodiesterase 1, acid lysosomal</td>
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<td>IDS</td>
<td>iduronate 2-sulfatase</td>
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<td>CTSB</td>
<td>cathepsin B</td>
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<td>DPP7</td>
<td>dipeptidyl-peptidase 7</td>
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**Accessory proteins**

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<td>GM2A</td>
<td>GM2 ganglioside activator</td>
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<td>NPC2</td>
<td>Niemann-Pick disease, type C2</td>
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**Transport of lysosomal proteins**

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<th>Fold Change</th>
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<tr>
<td>IGF2R</td>
<td>insulin-like growth factor 2 receptor</td>
<td>3.27</td>
</tr>
<tr>
<td>SORT1</td>
<td>sortilin 1</td>
<td>2.38</td>
</tr>
<tr>
<td>LIMP2</td>
<td>scavenger receptor class B, member 2</td>
<td>2.04</td>
</tr>
<tr>
<td>M6PR</td>
<td>mannose-6-phosphate receptor (cation dependent)</td>
<td>1.96</td>
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**Membrane proteins**

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<td>C1orf85</td>
<td>chromosome 1 open reading frame 85</td>
<td>4.98</td>
</tr>
<tr>
<td>NPC1</td>
<td>Niemann-Pick disease, type C1</td>
<td>3.76</td>
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<tr>
<td>CLN3</td>
<td>ceroid-lipofuscinosis, neuronal 3</td>
<td>3.33</td>
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<tr>
<td>PNPLA7</td>
<td>patatin-like phospholipase domain containing 7</td>
<td>3.07</td>
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<tr>
<td>TMEM192</td>
<td>transmembrane protein 192</td>
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<tr>
<td>TMEM55B</td>
<td>transmembrane protein 55B</td>
<td>2.67</td>
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<td>CLCN7</td>
<td>chloride channel 7</td>
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<td>TMEM55A</td>
<td>transmembrane protein 55A</td>
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<tr>
<td>SLC15A4</td>
<td>solute carrier family 15, member 4</td>
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<tr>
<td>LAMP2</td>
<td>lysosomal-associated membrane protein 2</td>
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<tr>
<td>Gene</td>
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<tr>
<td>CD68</td>
<td>CD68 molecule</td>
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<tr>
<td>STX3</td>
<td>Syntaxin 3</td>
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<td>SYT7</td>
<td>Synaptotagmin VII</td>
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<tr>
<td>MCOLN1</td>
<td>Mucolipin 1</td>
<td>3.37</td>
</tr>
<tr>
<td>RAB7A</td>
<td>RAB7A, member RAS oncogene family</td>
<td>2.27</td>
</tr>
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**Vesicle fusion and lysosomal exocytosis**

**Autophagy**

<table>
<thead>
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<th>Description</th>
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<tr>
<td>DRAM1</td>
<td>DNA-damage regulated autophagy modulator 1</td>
<td>2.87</td>
</tr>
<tr>
<td>VPS18</td>
<td>Vacuolar protein sorting 18 homolog (S. cerevisiae)</td>
<td>2.03</td>
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**Other functions**

<table>
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<tbody>
<tr>
<td>HLA-DMA</td>
<td>Major histocompatibility complex, class II, DM alpha</td>
<td>3.59</td>
</tr>
<tr>
<td>IFI30</td>
<td>Interferon, gamma-inducible protein 30</td>
<td>2.59</td>
</tr>
</tbody>
</table>

Gene Set Enrichment Analysis (GSEA) is a statistically powerful framework to analyze how a subset of genes of interest is distributed in a set of differentially expressed genes (154). GSEA ranks the list of differentially expressed genes from the most upregulated to the most downregulated, without imposing arbitrary thresholds (such as considering only genes with a fold change greater than 2), and calculates an enrichment score (ES, ranging from -1 to +1) that expresses the tendency of the gene subset of interest to be upregulated (ES between 0 and +1) or downregulated (ES between -1 and 0). GSEA of genes previously reported to be involved in lysosomal metabolism (42,44,155) showed that the vast majority of these genes were induced by sucrose administration, resulting in an ES of 0.62 (p<0.0001) (Figure 3.6C). Additional analyses showed that 87% of TFEB targets that
were differentially expressed were upregulated by sucrose (ES = 0.53; p<0.0001) (Figure 3.6D). The analysis of TFEB targets with a known role in lysosomal functions showed an even more skewed distribution, with 100% of these targets falling within the group of upregulated genes and an ES of 0.66 (p<0.0001) (Figure 3.6E). Together, these data confirm that the cell reacts to sucrose administration by activating TFEB, which in turn induces its downstream targets and in particular those involved in lysosomal functions.

To confirm these results, the expression of representative genes of the CLEAR network was measured by Quantitative RT-PCR (qRT-PCR) at various time points of sucrose administration. GD fibroblasts harboring the L444P GC allele were treated with sucrose (150 mM; 2, 4, and 7 days). GBA transcription was dramatically upregulated by sucrose treatment (23- and 34-fold after 4 and 7 days of incubation, respectively; Figure 3.7A). The expression of the CLEAR genes, HEXA (Hexosaminidase A), TPP1 (Tripeptidyl-peptidase 1), LAMP1 (Lysosome-associated membrane glycoprotein 1), ATP6V1H (V-type proton ATPase subunit H), GRN (granulin), and PSAP (proactivator polypeptide), was also significantly upregulated by sucrose treatment (Figure 3.7A). Additional expression analyses showed that glycerol, a chemical chaperone that did not affect the activity of L444P GC (Figure 3.7B), caused only minimal changes in the expression of genes of the CLEAR network (Figure 3.7B). Together, these data suggest a positive association among sucrose administration, TFEB activation, induction of the CLEAR network and rescue of L444P GC activity.
Figure 3.6. Sucrose induces upregulation of the CLEAR network in GD patient-derived fibroblasts. (A) Venn diagram of genes with 2-fold difference expression levels in GD fibroblasts before and after sucrose treatment (upregulated and downregulated genes, red and blue sets, respectively), compared with the genes included in the CLEAR network (471 bona fide TFEB targets, green set) and genes participating in lysosomal functions (yellow set). (B) Cytoscape-generated network representing genes upregulated by sucrose administration. Genes (colored dots) are connected by blue lines whose color intensity is proportional to the extent of their co-regulation. The network is organized in a core of genes with more tight expression relationships and containing an enrichment of TFEB targets (center of the network), and in a set of genes with less tight expression relationships (periphery of the network). (C-E) Gene Set Enrichment Score Analysis (GSEA) of transcriptome changes following sucrose administration to GD fibroblasts. GSEA of lysosomal genes (C), TFEB targets (D) and TFEB targets with a known role in lysosomal metabolism (E) are reported. Upper panels show enrichment plots for each dataset, generated by GSEA of ranked gene expression data (left: upregulated,
red; right: downregulated, blue). The enrichment score is shown as a blue line. Yellow shades mark the genes that have at least a 2-fold variation in treated cells compared to untreated cells and that were used to build the Venn diagram in (A). In the middle panels, vertical blue bars indicate the position of genes in the selected gene sets within the ranked lists. Lower panels show the cumulative distribution of gene sets within the ranked lists. The ranking positions that include 50% of analyzed genes are indicated. The analysis shows that TFEB lysosomal targets have a higher ES score compared with general lysosomal genes or TFEB targets, indicating that TFEB targets participating in lysosomal function were preferentially upregulated by sucrose administration in GD fibroblasts.

Figure 3.7. Sucrose treatment upregulates the expression of representative genes of the CLEAR network in GD patient-derived fibroblasts. Relative mRNA expression levels of representative genes of the CLEAR network in L444P GC fibroblasts treated with (A) 150 mM sucrose and (B) 150 mM glycerol for 2, 4, and 7 days. GBA, HEXA, TPP1, LAMP1, ATP6V1H, GRN and PSAP mRNA expression levels were obtained by qRT-PCR, corrected by the expression of the house-keeping genes GAPDH and ACTB, and normalized to those of untreated cells (p<0.01). Data are reported as mean ± SD (n = 3).
3.2.3. TFEB modulates L444P GC activity in patient-derived GD fibroblasts

To directly assess the role of TFEB in the rescue of L444P GC activity, modulation of the levels of TFEB and its activation via sucrose in L444P fibroblasts was combined and tested. L444P GC fibroblasts were transfected with a vector encoding TFEB, and GC activity with or without sucrose treatment was measured. Overexpression of TFEB resulted in a 2.4-fold increase in L444P GC activity (30% of wild type GC activity; Figure 3.8A) compared with the transfection control (cells transfected with the empty vector, pcDNA4™/TO). Sucrose treatment (150 mM, 4 days) in GD cells transfected with the empty vector resulted in a 3.9-fold increase in L444P GC activity corresponding to about 49% of wild type GC activity (p<0.01; Figure 3.8A), which is consistent with the results reported above (Figure 3.1A). Interestingly, the combination of sucrose administration and TFEB transfection led to a higher increase in L444P GC activity than the two treatments alone (6.9-fold increase compared with control cells or 87% of wild type GC activity; p<0.01; Figure 3.8A), suggesting that the observed sucrose-mediated rescue of L444P GC activity depends on TFEB concentration. Wild type fibroblasts were also tested for comparison: while sucrose treatment increased GC activity by 1.7-fold in wild type fibroblasts and by 3.0-fold in wild type fibroblasts transfected with TFEB, TFEB overexpression alone did not increase GC activity (Figure 3.8B). The comparison with results obtained in GD cells (Figure 3.8A) suggests that TFEB is not significantly activated in non-diseased cells, while it is at least partially activated in
GD cells, as previously shown in cells derived from patients affected by other LSDs (42).

**Figure 3.8. TFEB activation mediates rescue of L444P GC activity in GD patient-derived fibroblasts.** (A) Relative L444P GC activities in L444P GC fibroblasts transfected with TFEB and treated with 150 mM sucrose for 4 days. Cell lysates were normalized to the same total protein concentration and GC activities were normalized to the activity of untreated control sample (cells transfected with empty vector pcDNA4™/TO) (p<0.01). (B) Relative GC activities of wild type fibroblasts transfected as in (A) and treated with 150 mM sucrose for 3 days. GC activities were calculated as described in (A) (p<0.01). (C) Relative L444P GC activities of L444P GC fibroblasts transfected with plasmids encoding wild type TFEB, TFEB S142A and TFEB-NLS. GC activities were calculated as described in (A) (p<0.01). (D) Relative GC activities of L444P GC fibroblasts incubated with TFEB siRNA and sucrose (black bars). GC activities were normalized to the activity measured in control cells.
To test the hypothesis that TFEB activation is needed for TFEB to promote rescue of L444P GC activity, GD fibroblasts were transfected with two TFEB variants that localize preferentially in the nucleus: TFEB containing a serine to alanine substitution at position 124 (TFEB S142A), which inactivates a TFEB phosphorylation sites and promotes TFEB nuclear translocation (46); and TFEB fused to a constitutive nuclear localization signal, which forces TFEB to translocate into the nucleus (TFEB-NLS-3xFLAG). GC activities were found to increase 2.3-, 3.4- and 3.2-fold in GD fibroblasts transfected with wild type TFEB, S142A TFEB and TFEB-NLS, respectively (p<0.05; Figure 3.8C), therefore showing that changes in TFEB localization that mimic its activation enhance the extent of L444P GC activity rescue.

Finally, small-interfering RNA (siRNA) was used to silence TFEB expression in cells treated with sucrose. TFEB silencing resulted in approximately 29-36% residual expression of TFEB compared with cells transfected with a control siRNA (Figure 3.8D), as evaluated by qRT-PCR. Accordingly, L444P GC activity decreased from 3.9-fold in cells treated with sucrose and control siRNA to 3.1-fold in cells treated with sucrose and TFEB siRNA (p<0.01; Figure 3.8D). The residual increased GC activity observed in TFEB-silenced cells was likely due to sucrose-mediated activation of residual TFEB.
Taken together, these results show that sucrose-mediated rescue of L444P GC activity depends on TFEB concentration, and that TFEB activation is needed to increase the activity of wild type and L444P GC.

**3.2.4. TFEB regulates the expression of genes involved in lysosomal targeting**

Previous studies showed that modulation of the proteostasis network to induce upregulation of *GBA* and enhancement of cellular folding and trafficking results in an increase in the amount of active GC in the lysosome, particularly in cells expressing mutated variants otherwise prone to misfolding and degradation (71,74,102). However, the overexpression of a highly unstable GC variant such as L444P GC was shown to be not sufficient to promote rescue of enzyme folding and activity (143), suggesting that the innate cellular folding capacity cannot cope with the destabilizing effect of the L444P substitution. Proteostasis regulators mediate an increase in L444P GC activity by inducing both *GBA* upregulation and enhancement of the cellular folding capacity (71,74,102). I thus asked whether cell treatment with sucrose, in addition to upregulating GC expression, also influences the expression of genes involved in folding and trafficking through the secretory pathway. To address this question, L444P GC fibroblasts were treated with sucrose or glycerol (150 mM, 2, 4, and 7 days), and changes were measured in the expression of representative genes encoding proteins of the ER chaperone system (BiP (binding immunoglobulin protein), CNX (calnexin), CRT (calreticulin)), proteins involved in the UPR pathway (CHOP (C/EBP homologous protein), ATF4 (activating transcription factor 4)) and
proteins that mediate trafficking through the secretory pathway (LIMP2 (lysosomal integral membrane protein II), M6PR (mannose-6-phosphate receptor) and SORT1 (sortilin)). Among genes involved in folding of secretory proteins, HSPA5 (BiP) and CNX were found to be upregulated by sucrose, and to a level significantly higher than glycerol (Figure 3.9A-B). CRT, a direct TFEB target (44), was upregulated 6.2-fold after treatment with sucrose for 4 days, compared with 1.1-fold increase in glycerol-treated cells. ATF4 and CHOP were mildly affected by treatment with either sucrose or glycerol. These data show that sucrose treatment upregulates the folding network in GD cells without causing activation of the UPR, likely contributing to the rescue of L444P GC activity.

Figure 3.9. Sucrose treatment upregulates the folding network in GD patient-derived fibroblasts. Relative mRNA expression levels of representative genes of the folding and trafficking network in L444P GC fibroblasts treated with (A) 150 mM sucrose and (B) 150 mM glycerol for 2, 4, and 7 days. HSPA5 (BiP), CNX, CRT, CHOP, ATF4, LIMP2, M6PR, and SORT1 mRNA expression levels were obtained by qRT-PCR, and calculated as described in Figure 3.7 (p<0.05). Data are reported as mean ± SD (n = 3).
M6PR facilitates the transport of most lysosomal hydrolases, whereas LIMP2 and SORT1 mediate M6PR-independent transport. LIMP2 was recently reported to specifically mediate M6PR-independent transport of GC to the lysosome (156). M6PR is a known TFEB target gene (44) and, not surprisingly, was upregulated 7.4-fold upon sucrose treatment, but not affected by glycerol treatment (Figure 3.9A-B). SORT1 was found to be upregulated 6-fold by sucrose treatment but not by glycerol (Figure 3.9A-B). Interestingly, LIMP2 expression was also dramatically increased to 7-fold after 4 days of sucrose treatment, compared with 1.8-fold increase observed upon glycerol treatment (Figure 3.9A-B). To further evaluate whether TFEB regulates the trafficking of lysosomal proteins through the secretory pathway, the expression of M6PR, LIMP2 and SORT1 was monitored in L444P GC fibroblasts transfected with TFEB. As shown in Figure 3.10A, the transcription of M6PR, LIMP2 and SORT1 was upregulated 3.1-, 3.6-, and 3.1-fold, respectively, upon TFEB overexpression compared with nontransfected cells (p<0.01). The expression of GC-encoding gene, GBA, a known TFEB target (44), was also measured for comparison and was found to be upregulated 3.9-fold (p<0.01). The expression of the gene encoding hepatocyte growth factor (HGF), which is not part of the CLEAR network (44), and is not involved in folding and trafficking of secretory proteins was measured here as negative control and was not altered by TFEB overexpression.
Figure 3.10. TFEB activation upregulates expression of lysosomal targeting genes. (A) Relative mRNA expression levels of trafficking genes in L444P GC fibroblasts transfected with TFEB. GBA, LIMP2, M6PR, SORT1 and HGF mRNA expression levels in cells transfected with TFEB were obtained by qRT-PCR, corrected by the expression of the house-keeping genes GAPDH and ACTB, and normalized to those of cells transfected with empty vector (p<0.01). (B-E) Relative mRNA expression levels of trafficking genes in L444P GC fibroblasts upon TFEB silencing. Relative mRNA expression levels of GBA (p<0.01) (B), LIMP2 (p<0.01) (C), M6PR (p<0.05) (D) and SORT1 (p<0.05) (E) in L444P GC fibroblasts treated with TFEB siRNA and sucrose were obtained by qRT-PCR, corrected to the expression of the house-keeping genes GAPDH and ACTB, and normalized to the activity of cells treated with control siRNA. Data are reported as mean ± SD (n = 3).
The effect of TFEB on the trafficking network was also investigated by silencing TFEB expression. L444P GC fibroblasts were treated with TFEB siRNA and sucrose followed by qRT-PCR analyses. As expected, GBA was upregulated by sucrose treatment. However, TFEB silencing in sucrose-treated cells lowered GBA expression (Figure 3.10B). Transcriptions of LIMP2, M6PR and SORT1 were upregulated 10.7-, 1.6-, and 2.7-fold, respectively, in cells treated with sucrose (Figure 3.10C-E). Silencing TFEB expression decreased the expression of LIMP2, M6PR and SORT1 by 0.9-, 0.4- and 0.6-fold, respectively (Figure 3.10C-E). TFEB silencing in cells treated with sucrose also caused decrease in expression of LIMP2, M6PR and SORT1 compared with cells only treated with sucrose (GBA: from 9.6- to 4.7-fold, p<0.01; LIMP2: from 10.7- to 2.1-fold, p<0.01; M6PR: from 1.6- to 0.5-fold, p<0.05; SORT1: from 2.7- to 1.5-fold, p<0.05; Figure 3.10C-E). In summary, these results confirm that TFEB regulates genes involved in lysosomal targeting and that sucrose treatment results in TFEB-dependent upregulation of lysosomal trafficking.

3.2.5. LIMP2 mediates TFEB-dependent rescue of L444P GC activity in GD fibroblasts

Previous studies demonstrated that LIMP2 interacts with GC and targets it to the lysosome (156). Based on our results showing that LIMP2 is induced by both sucrose treatment and TFEB activation – treatments that result in rescue of GC activity – I hypothesized that LIMP2 is a key player in TFEB-dependent rescue of
mutated GC activity in GD fibroblasts. To test this hypothesis, GC activity was evaluated in L444P GC fibroblasts transfected with LIMP2 and treated with sucrose. Overexpression of LIMP2 did not result in significant increase in GC activity (Figure 3.11A), probably due to the severe destabilizing effect of the L444P substitution, which results in nearly complete degradation (64). Addition of sucrose (150 mM, 4 days) to cells transfected with LIMP2 led to a 6.0-fold increase (75% of wild type GC activity; p<0.01; Figure 3.11A), which is higher than the activity observed in cells treated only with sucrose (4.5-fold; p<0.01) or only for LIMP2 overexpression.

L444P GC activity upon silencing of LIMP2 followed by sucrose treatment was measured. GC activity upon treatment with LIMP2 siRNA was observed to decrease to 0.8-fold compared with cells treated with a control siRNA (Figure 3.11B). LIMP2 silencing also caused a decrease in L444P GC activity in cells treated with sucrose (from 3.2- to 1.6-fold; p<0.01; Figure 3.11B), suggesting that LIMP2 plays a key role in sucrose-mediated rescue of L444P GC activity.

Collectively, our results suggest that TFEB activation results in the rescue of L444P GC activity by modulating the CLEAR network and, particularly, by 1) inducing GBA upregulation, and thus enhancing the pool of GC folding intermediates amenable to folding rescue, 2) inducing genes of the folding network, thereby assisting folding of mutated GC and favoring mutated GC ERAD escape, and 3) by inducing LIMP2 upregulation, and thus enhancing trafficking of unstable L444P GC from the ER to the lysosome, where it is highly stable due to the acidic environment for which this enzyme was evolutionarily selected (64). To further investigate this
hypothesis, L444P GC activity was tested in GD fibroblasts treated to upregulate GC expression and to enhance the cellular folding and trafficking capacity. Specifically, GD cells transfected with LIMP2 were treated with proteostasis modulators previously reported to upregulate GBA, enhance the ER folding capacity, and promote native folding of unstable GC variants, namely the ERAD inhibitor, Eeyarestatin I (74), and the proteasome inhibitor, MG-132 (70,71). Addition of Eeyarestatin I (EerI, 6 µM) and MG-132 in cells transfected with LIMP2 resulted in the rescue of L444P GC to 19% and 30% of wild type GC activity, respectively (p<0.01 for both compounds; Figure 3.11C). In both experiments, the combination of LIMP2 overexpression and treatment with a proteostasis modulator resulted in an increase in L444P GC activity higher than what observed using either strategy alone (e.g. LIMP2 transfection and MG-132 or EerI administration). Collectively, these results suggest that LIMP2 overexpression, by enhancing the lysosomal trafficking capacity of GD cells, increases lysosomal transport of native GC that is rescued by upregulation of ER chaperones. This proteostasis modulation approach involving upregulation of GC expression and enhancement of cellular trafficking recapitulates the features of TFEB activation and motivates the development of chemical strategies to activate TFEB for therapeutic intervention.
Figure 3.11. LIMP2 mediates TFEB-dependent rescue of L444P GC activity in GD patient-derived fibroblasts. (A) Relative L444P GC activities of cells overexpressing LIMP2 and treated with 150 mM sucrose for 4 days. GC activities were calculated as described in Figure 3.8A (p<0.01). (B) Relative L444P GC activities of L444P GC fibroblasts treated with LIMP2 siRNA and sucrose. GC activities were calculated as described in Figure 3.8D (p<0.01). (C) Relative L444P GC activities of cells overexpressing LIMP2 and treated with Eerl (6 µM) or MG-132 (0.6 µM). GC activities were normalized to the activity of untreated cells transfected with empty vector (p<0.05). Data are reported as mean ± SD (n = 3).
3.2.6. Sucrose treatment enhances activity of unstable, degradation-prone lysosomal enzyme variants in fibroblasts derived from patients with LSDs

To test whether TFEB activation via sucrose treatment is unique to the L444P GC variant, or more generally applicable to other GC variants containing different destabilizing mutations, GD fibroblasts carrying N370S GC, which is the most prevalent mutation found in GD patients were analyzed. Fibroblasts were treated with sucrose as described above and GC activities were evaluated every 2 days for up to 10 days. GC activity was observed to increase up to 39% of the wild type GC activity after 4 days of incubation (sucrose 100 mM; p<0.01) and to 61% after 10 days (sucrose 150 mM; p<0.01; Figure 3.12A). Interestingly, the increase in residual activity observed in N370S GC fibroblasts was less pronounced than that detected in L444P GC fibroblasts. This difference in rescue of GC activity is likely due to the different destabilizing effects of the N370S and L444P substitutions and the corresponding level of residual activity that responds differently to proteostasis modulation, as previously reported (74).
Figure 3.12. Sucrose treatment rescues lysosomal activities of mutated enzyme variants in cells derived from patients with LSDs. (A) GC activity of GD fibroblasts carrying the N370S GC allele treated with sucrose. Relative GC activities were calculated as described in Figure 3.1 (p<0.01). (B) HexA activity of Tay-Sachs fibroblasts carrying G269S/1278insTATC HexA and cultured with sucrose. HexA activities were normalized to the activity of untreated cells (left y axis) (p<0.01). The corresponding fraction of wild type HexA activity is reported (right y axis). Data are reported as mean ± SD (n = 3).
I also investigated whether sucrose treatment was able to rescue β-hexosaminidase A (HexA) activity in fibroblasts derived from patients with Tay-Sachs disease. Specifically, I focused on one of the most prevalent mutations, the G269S substitution in the HexA subunit, which destabilizes the protein native structure causing a loss of enzymatic activity to ~10% of wild type HexA activity (76). Patient-derived fibroblasts harboring G269S HexA were cultured with sucrose for up to 14 days and HexA activity was measured as previously described (70). Treatment with sucrose (100 mM) for 9 days led to a 2.4-fold increase in G269S HexA activity (24% of wild type HexA activity; p<0.01; Figure 3.12B). This increase in HexA activity was still observed after 14 days of incubation, suggesting that sucrose treatment enhances native folding, lysosomal concentration and activity of mutated HexA.

Taken together, our data show that the activation of the CLEAR network promotes rescue of trafficking and enzymatic activity of degradation-prone lysosomal enzyme variants. These results identify TFEB as a new strategic target for the development of treatments for LSDs caused by destabilizing missense mutations.
3.3. Discussion

Modulation of the proteostasis network has emerged as a promising strategy to upregulate the synthesis, folding and processing of proteins harboring destabilizing mutations and to enhance the innate cellular capacity to maintain protein homeostasis and sustain functions otherwise deficient in disease conditions (70,157,158). In an attempt to identify a modulator specific to lysosomal proteostasis, I focused on TFEB, a recently identified master regulator of lysosomal biogenesis and function (42) (refer to Chapter 1 for a detailed description). In this study, TFEB activation was shown to mediate rescue of folding and lysosomal targeting of L444P GC and enhance lysosomal GC activity in GD fibroblasts. TFEB activation was achieved either chemically using sucrose or genetically by transfecting GD fibroblasts with TFEB variants that localize preferentially in the nucleus (TFEB S142A (46) and TFEB-NLS-3xFLAG). Interestingly, overexpression of TFEB resulted in partial rescue of L444P GC activity in GD fibroblasts but did not increase GC activity in wild type fibroblast in the absence of external stimuli that promote its activation, supporting the notion that TFEB is activated under conditions of lysosomal impairment such as in LSDs (42). Moreover, overexpression of wild type TFEB resulted in sucrose-dependent rescue of L444P GC activity, while overexpression of TFEB variants that accumulate preferentially in the nucleus resulted in an increase in L444P GC activity in the absence of sucrose treatment, suggesting that TFEB activation mediates the observed increase in a natively folded GC variant that localizes in the lysosome.
It was also found that the main transcriptional response to sucrose administration in GD fibroblasts consisted of activation of the CLEAR network, which is composed of various lysosome-associated functions that are modulated by TFEB (42,44). In particular, LIMP2, a gene involved in lysosomal targeting of GC (156), was dramatically upregulated by sucrose treatment as well as upon genetic activation of TFEB and was found to play a critical role in TFEB-mediated rescue of GC folding and activity. These results indicate that the rescue of folding and lysosomal targeting of this highly unstable variant is dependent on simultaneous upregulation of GBA transcription (which enhances the pool of unstable GC amenable to folding rescue), and enhancement of the cellular folding and trafficking capacity. In summary, rescue of L444P GC activity was promoted by TFEB-mediated induction of genes encoding lysosomal proteins and accessory proteins needed for their processing.

Single-residue substitutions are the most common causative variations encountered in LSD patients with deficiencies in lysosomal enzyme activities (159,160). Interestingly, TFEB activation was also found to enhance activity of a distinct GC variant – N370S GC, the most prevalent mutant among GD patients – and of a mutated HexA variant associated with the development of Tay-Sachs disease. While these results confirm the role of TFEB as a modulator of lysosomal proteostasis, additional studies are needed to prove generality of this strategy for the treatment of other LSDs characterized by misfolding, non-inactivating mutations in lysosomal enzymes.
In summary, this study provided evidence that TFEB activation mediates rescue of unstable, degradation prone lysosomal enzyme variants by simultaneously upregulating their transcription and enhancing the cellular trafficking capacity, thus facilitating processing of proteins that traverse the secretory pathway. These findings demonstrate a new role of TFEB in regulating lysosomal proteostasis and suggest TFEB as a candidate therapeutic target to rescue enzyme homeostasis in LSDs.

3.4. Materials and Methods

3.4.1. Reagents, plasmids and cell cultures

Sucrose was purchased from Calbiochem. Glycerol was purchased from Acros Organics. Eeyarestatin I was from ChemBridge. MG-132 was obtained from Cayman Chemical. Lacidipine and Conduritol B Epoxide (CBE) were from Toronto Research Chemicals. 4-Methylumbelliferyl β-D-glucoside (MUG) was obtained from Sigma-Aldrich. Cell culture media were obtained from Lonza.

GD patient-derived fibroblasts homozygous for the L444P (1448T>C) mutation (GM10915) were obtained from Coriell Cell Repositories. Fibroblasts were grown at 37°C in 5% CO₂ in minimal essential medium with Earle’s salts,
supplemented with 10% heat-inactivated fetal bovine serum and 1% glutamine Pen-Strep. Medium was replaced every 3 or 4 days. Monolayers were passaged with TrypLE Express.

A TFEB-NLS-3xFLAG plasmid was obtained by mutagenesis of a TFEB-3xFLAG plasmid using QuikChange® II XL site-directed mutagenesis kit and a reverse primer that contained the NLS coding sequence, 5’-CCAAAGAAGAAGCGTAAG-3’ (161,162), just downstream of the last TFEB coding codon. The correct insertion of the NLS coding sequence was verified by sequencing, and the localization of the TFEB-NLS-3xFLAG protein product was determined by confocal microscopy analyses.

3.4.2. Enzyme activity assays

The intact cell glucocerebrosidase (GC) activity assay was performed as previously described (70). Briefly, 100 μl aliquots of 10⁴ cells were plated in each well of a 96-well plate and incubated overnight to allow cell attachment. The medium was replaced with fresh medium containing small molecules (small molecule concentrations and time of incubation are specified in each experiment), and plates were incubated at 37°C. The medium was then aspirated and cells were washed with PBS three times. The assay reaction was started by the addition of 50 μl of 2.5 mM 4-methylumbelliferyl β-D-glucoside (MUG) in 0.2 M acetate buffer (pH 4.0) and stopped after 7 hr of incubation at 37°C by the addition of 150 μl of 0.2 M
glycine buffer (pH 10.8) to each well. Liberated 4-methylumbelliferone was measured (excitation 365 nm, emission 445 nm) with a SpectraMax Gemini plate reader (Molecular Device). Non-lysosomal GC activity was evaluated by measuring GC activities in the presence of Conduritol B Epoxide (CBE) at 1 mM final concentration. Relative GC activities were calculated by subtracting the background of non-lysosomal activity and normalizing the obtained values by the activity of untreated cells.

The lysed cell GC activity assay was performed as previously described (163). Briefly, the cells were collected and lysed with the complete lysis-M buffer containing the protease inhibitor cocktail (Roche). Total protein concentrations were determined by the Bradford assay (Thermo Scientific) and each sample was diluted to the same protein concentration. The assay was performed by adding 50 μl of 2.5 mM MUG in 0.2 M acetate buffer (pH 4.0) containing 0.15% Triton X-100 (v/v, Fisher) and 0.15% taurodeoxycholate (w/v, Calbiochem) to 10 μl aliquots of cell lysates. The assay reaction was stopped by the addition of 150 μl of 0.2 M glycine buffer (pH 10.8) to each well after 7 hr of incubation at 37°C. Liberated 4-methylumbelliferone was measured (excitation 365 nm, emission 445 nm) with a SpectraMax Gemini plate reader (Molecular Device). CBE at 2 mM final concentration was used as a control to evaluate non-lysosomal GC activity. Relative GC activities were calculated by subtracting the background of non-lysosomal activity and normalizing the obtained values by the activity of untreated cells. Fold
changes in GC activity were then corrected by TFEB mRNA expression level (evaluated by qRT-PCR) in cells transfected with TFEB.

β-Hexosaminidase A (HexA) activity assay was performed as described previously (70,74).

### 3.4.3. Western blot analyses

Cells were incubated with sucrose for 4 days, collected and lysed with the complete lysis-M buffer containing the protease inhibitor cocktail (Roche). Total protein concentrations were determined by Bradford assay (Thermo Scientific), and each sample was diluted to the same protein concentration. Treatment with endoglycosidase H (EndoH) was performed by incubating samples at 95°C for 10 min, followed by incubation with EndoH (New England Biolabs) for 1 hr at 37°C. Aliquots of cell lysates were separated by 10% SDS-PAGE gel. Western blot analyses were performed using primary antibodies (rabbit anti-Glucocerebrosidase (Sigma-Aldrich), or rabbit anti-GAPDH (Santa Cruz Biotechnology)) and HRP-conjugated goat anti-rabbit (Santa Cruz Biotechnology) as the secondary antibody. Blots were visualized using Luminata Forte Western HRP substrate (Millipore) and quantified by NIH ImageJ analysis software.
3.4.4. Immunofluorescence assays

Fibroblasts were seeded on glass coverslips, cultured in the presence of sucrose for 4 days, and fixed with 4% paraformaldehyde for 30 min. Cells were permeabilized with 0.1% Triton-X for 5 min and incubated with 8% bovine serum albumin for 1 hr. Following incubation for 1 hr with primary antibodies (rabbit anti-β-glucocerebrosidase and mouse anti-CNX antibodies, Sigma-Aldrich), the cells were washed three times with 0.1% Tween-20/PBS, and then incubated with secondary antibodies for 1 hr (Dylight 488 goat anti-mouse IgG and Dylight 549 goat anti-rabbit IgG from KPL, and FITC anti-LAMP1 from Biolegend). Images were obtained using an Olympus IX81 confocal microscope and co-localized using the Fluoview software. Colocalization heatmap images were analyzed using NIH ImageJ analysis software.

3.4.5. Microarray experiments

Total RNA from GD fibroblasts before and after treatment with sucrose (100 mM, 4 days) was used to prepare cDNA for hybridization to the Illumina Human HT-12 V4.0 array platform. Hybridizations were performed in triplicates at the Microarray Core and Cell and Regulatory Biology, University of Texas, Houston, TX, USA. A p-value of < 0.01 was used to assess significant gene differential expressions. Gene Set Enrichment Analysis was performed as previously described (154). The cumulative distribution function was constructed by performing 1,000 random gene
set membership assignments. A nominal $p$-value < 0.01 and an FDR < 10% were used to assess the significance of the Enrichment Score (ES). GO analyses were performed with the web tool DAVID (164) using default parameters. Redundant terms were manually removed from the resulting lists. Pathway co-expression analyses were performed as previously described (44,152), and the expression correlation data were analyzed with Cytoscape (153) to draw a visual representation of relationships among co-expressed genes.

### 3.4.6. Quantitative RT-PCR

Quantitative RT-PCR (qRT-PCR) was performed as previously described (74). Cells were incubated with small molecules for indicated time lengths before total RNA was extracted using RNA GEM™ reagent (ZyGEM). cDNA was synthesized from total RNA using qScript™ cDNA SuperMix (Quanta Biosciences). Total cDNA amount was measured by NanoDrop 2000 (Thermo Scientific). qRT-PCR reactions were performed using cDNA, PerfeCTa™ SYBR Green FastMix™ (Quanta Biosciences) and the corresponding primers (Table A.1) in the CFX96™ Real-Time PCR detection system (Bio-Rad). Samples were heated for 2 min at 95°C and amplified in 45 cycles for 1 s at 95°C, 30 s at 60°C, and 30 s at 72°C. Analyses were conducted using CFX manager software (Bio-Rad) and the threshold cycle ($C_T$) was extracted from the PCR amplification plot. The $\Delta C_T$ value was calculated as previously described (165) to normalize the $C_T$ of each target gene to that of the house-keeping genes $GAPDH$. 
and ACTB. The relative mRNA expression level of each target gene in treated cells was normalized to that measured in untreated cells: relative mRNA expression level = $2^{\Delta C_T}$ (treated cells) - $\Delta C_T$ (untreated cells)). Each data point was evaluated in triplicate and measured three times.

3.4.7. Cell transfections

Transfection procedures were performed as follows: $10^6$ cells in 10 ml of growth medium were plated in a 10 cm culture dish and incubated overnight to allow cell attachment. Transfection reactions were conducted using jetPRIME™ reagent (Polyplus Transfection) when cells reached 80% confluency. After 16-hr incubation, the transfection medium was replaced by fresh medium or medium containing 150 mM sucrose. Lysosomal GC activities were measured using the lysed cell GC activity assays. GC activities were corrected for TFEB expression level (evaluated by qRT-PCR) to eliminate differences in enzymatic activities due to variability of transfection efficiency.

3.4.8. siRNA transfections

siRNA transfection was performed using HiPerFect® Transfection Reagent as described in manufacturer’s manual (Qiagen). Each well of a 96-well plate was spotted with 12.5 ng siRNA in 3µl RNase-free water. 0.75 µl of HiPerFect
Transfection Reagent was diluted with 24 µl of serum-free culture medium and added to each well. The mixture was incubated for 10 min at RT to allow formation of transfection complexes. A set of $10^4$ cells in 175 µl of culture medium were seeded into each well on top of the transfection complexes and incubated for 2 days. The medium was replaced by medium or medium containing 150 mM sucrose. Lysosomal GC activities were measured after 3 days by intact cell GC activity assays. TFEB siRNA (Cat. No. SI00094969), LIMP2 siRNA (Cat. No. SI02777215) and control siRNA (Cat. No.1027280) were purchased from Qiagen.

### 3.4.9. Apoptosis assays

Toxicity assay was conducted as previously described (74). Briefly, the cells were collected after incubating with small molecules for 24 hr. Cell toxicity was tested using the CytoGLO™ Annexin V-FITC Apoptosis Detection Kit (IMGENEX) according to the manufacturer’s instructions and analyzed by flow cytometry (FACSCanto™ II, Beckon Dickingson) with a 488-nm argon laser.

### 3.4.10. Statistical analyses

All data are presented as mean ± SD, and statistical significance was calculated using a two-tailed t-test.
HPβCD Promotes TFEB-mediated Activation of Autophagy


4.1. Introduction

HPβCD is an FDA-approved excipient used to improve the stability and bioavailability of drugs (111). It was previously demonstrated that HPβCD can mediate clearance of cholesterol in the brain of mice with Niemann-Pick type C (NPC) and in fibroblasts derived from NPC patients (112,116-118). Despite its wide use as a drug delivery vehicle and the recent approval of a clinical trial to evaluate
its potential for the treatment of NPC (114,115), the cellular pathways involved in the adaptive response that is activated upon exposure to HPβCD are still poorly defined. Recent findings demonstrated that depletion of cholesterol using HPβCD triggers activation of autophagy, an important lysosomal pathway involved in cellular clearance (110). However, the molecular mechanisms that regulate activation of autophagy observed upon HPβCD treatment remain unclear. Moreover, whether HPβCD treatment may lead to depletion of additional cellular substrates that are normally cleared by autophagy is not known.

Autophagy is an evolutionarily conserved and highly regulated catabolic pathway that mediates bulk degradation of long-lived proteins, macromolecules, and organelles (166) (refer to Chapter 1 for a detailed description), thus providing an important survival mechanism to supply energy resources under nutrient deprivation (18-20). However, it was found that activation of autophagy is associated with apoptosis induction (167-169), suggesting that in addition to pro-survival function, autophagy may also have a pro-death role. Although the molecular mechanism underlying activation of autophagy-associated apoptosis remains a subject of intense debate (170), a detailed characterization of the cellular response associated with HPβCD-mediated autophagy activation, including its relationship with cell death mechanisms, is critically needed to develop improved guidelines for using HPβCD in commercial products and therapeutic applications.

Inefficient autophagic activity has been linked to the development of a number of diseases characterized by aberrant accumulation of intracellular
substrates. For instance, neuronal ceroid lipofuscinoses (NCLs) described in Chapter 1 are a group of lysosomal storage disorders (LSDs) characterized by aberrant intracellular accumulation of autofluorescent ceroid lipopigment (80). NCL patient-derived cell lines exhibit slowed growth and increased propensity to undergo apoptosis (171). Interestingly, TFEB activation was shown to lower the accumulation of ceroid lipopigment in fibroblasts derived from a patient with juvenile NCL (172), suggesting a potential role of TFEB as a therapeutic target for the treatment of NCLs.

Because of the similarities between the biological effects of HPβCD administration and TFEB activation — including the ability to activate autophagy and promote cellular clearance — I hypothesized that TFEB mediates activation of autophagy observed upon HPβCD administration. This hypothesis was investigated by testing the molecular mechanism of autophagy induction activated in response to cell treatment with HPβCD using an in vitro model system of TFEB activation, consisting of HeLa cells that overexpress TFEB (42). To investigate whether HPβCD treatment induces enhancement of autophagy-mediated clearance and whether enhancement of autophagic activity is accompanied by activation of apoptosis, I used fibroblasts derived from a patient with late infantile neuronal ceroid lipofuscinosis (LINCL). LINCL cells were used in this study because i) they provide an in vitro model system of lysosomal storage, which allows evaluating whether autophagy activation parallels enhanced clearance of storage material, and ii) they are prone to activation of cell death pathways, and thus enable detecting even basal
activation of autophagy associated cell death. In this study, cell treatment with HPβCD was found to activate TFEB and enhance the cellular autophagic clearance capacity. HPβCD administration promotes TFEB-mediated clearance of proteolipid aggregates in LINCL fibroblasts. Interestingly, HPβCD-mediated activation of autophagy was found not to be associated with activation of apoptotic pathways. In summary, this study demonstrates that HPβCD treatment results in enhancement of the cellular autophagic capacity and that this response is mediated by TFEB. These findings provide a mechanistic understanding of the cellular response to HPβCD treatment, which will inform the development of safe HPβCD-based therapeutic modalities and may enable engineering HPβCD as a platform technology to reduce the accumulation of lysosomal storage material.

4.2. Results

This study was conducted in collaboration with Dr. Fan Wang (Rice University).

4.2.1. HPβCD treatment promotes TFEB activation

To investigate the role of TFEB in regulating autophagy activation upon HPβCD treatment, TFEB subcellular localization was tested. Specifically, TFEB subcellular localization was monitored in a cellular model system of TFEB activation, consisting of HeLa cells stably transfected with a TFEB-3xFLAG construct (HeLa/TFEB cells) (42). Cells were cultured in the presence of HPβCD (0.1-10 mM;
24 hr) and TFEB subcellular distribution was evaluated by confocal microscopy using DAPI nuclear staining and an anti-FLAG antibody (Figure 4.1A). The extent of TFEB nuclear localization was quantified by calculating the fraction of HeLa/TFEB cells exposed to HPβCD that presented nuclear localization of TFEB (Figure 4.1B). The results showed that, as expected, TFEB localizes predominantly in the cytoplasm of untreated HeLa/TFEB cells. HPβCD treatment, however, resulted in increased translocation of TFEB into the nucleus (Figure 4.1A-B). The extent of TFEB nuclear accumulation correlated with HPβCD concentration, as it increased progressively in cells treated with HPβCD ranging from 0.1 to 10 mM HPβCD. Colocalization of TFEB and DAPI nuclear staining in cells treated with 1 mM HPβCD was comparable to that observed in cells treated with sucrose under conditions (100 mM; 24 hr) previously reported to result in maximum activation of TFEB (42).

To investigate whether nuclear translocation of TFEB observed in cells treated with HPβCD results in activation of the CLEAR network, the expression of representative genes of the CLEAR network that are involved in lysosomal function was tested upon HPβCD administration. HeLa/TFEB cells were treated with HPβCD (1 mM), and the mRNA expression levels of TFEB targets were monitored by quantitative RT-PCR (Figure 4.1C). Significant upregulation of TFEB targets, namely, GBA (Glucocerebrosidase; 5.1-fold, p<0.05), HEXA (Hexosaminidase A; 2.7-fold, p<0.05), and LAMP1 (Lysosome-associated membrane glycoprotein 1; 3.5-fold, p<0.05) was observed upon HPβCD treatment.
**Figure 4.1. HPβCD treatment results in activation of TFEB and the CLEAR network.** (A) Confocal microscopy analyses of TFEB subcellular localization in HeLa/TFEB cells treated with 0.1, 1, and 10 mM HPβCD or sucrose 100 mM for 1 day. Colocalization of DAPI (blue, column 1) and an anti-FLAG antibody (red, column 1) is shown in purple color (column 3). The scale bar is 10 μm. UT, untreated; Sucr, sucrose (B) Percentage of cells presenting nuclear localization of TFEB-FLAG upon treatment with HPβCD (0.1, 1, and 10 mM) or sucrose (100 mM). Representative (~30) fields containing approximately 50 cells were analyzed (p<0.05; *p<0.01). UT, untreated. Data are reported as mean ± SD. (C) Relative mRNA expression levels of representative genes of the lysosome system in HeLa/TFEB cells treated with 1 mM HPβCD for 1 day. mRNA expression levels of GBA, HEXA, LAMP1 were obtained by qRT-PCR, corrected by the expression of the house-keeping gene GAPDH, and normalized to those of untreated cells (dashed line). Data are reported as mean ± SD (n ≥ 3; p<0.05).

LC3 is a protein found on the membrane of autophagosomes (173) and widely used as a marker of autophagy activation (20). Upon activation of autophagy, the cytosolic form of LC3 (LC3-I) is conjugated to phosphatidylethanolamine (LC3-II), which is recruited to autophagosomal membranes (174). To investigate whether HPβCD treatment, under conditions that result in TFEB activation, induces activation of autophagy, HeLa/TFEB cells were transfected with a vector encoding LC3 gene fused to GFP to facilitate visualization of LC3 structures. As expected, a diffuse GFP signal was observed in untreated cells, indicative of basal autophagic activity, and punctate GFP structures in cells treated with HPβCD, indicative of the formation of autophagic vesicles (Figure 4.2A). Activation of autophagy upon HPβCD treatment was also confirmed by immunoblotting of LC3 isoforms. Although the actual molecular weight of LC3-II is larger than that of LC3-I, LC3-II migrates faster than LC3-I on SDS-PAGE due to its hydrophobicity and thus displays a lower apparent molecular weight (14 kD) (174). LC3-II can be therefore detected and
distinguished from LC3-I by immunoblotting analyses using an LC3-specific antibody. The increase in LC3-II in cells treated with HPβCD compared to untreated cells suggests increased formation of autophagic vesicles (Figure 4.2B). The further increase in LC3-II levels observed in cells treated with HPβCD in the presence of the autophagy inhibitor bafilomycin (100 nM), compared to cells treated only with HPβCD (p<0.01) and to cells treated only with bafilomycin (p<0.05), is indicative of an increase of autophagic flux upon HPβCD treatment. A time dependent analysis of LC3 conversion and consumption upon HPβCD was also conducted to confirm upregulation of autophagy. The ratio of LC3-II over LC3-I levels was observed to increase with time of cell exposure to HPβCD (Figure 4.2C, *p<0.05). These results confirm that HPβCD treatment induces activation of autophagy (174).
Figure 4.2. HPβCD treatment results in activation of autophagy. (A) Confocal microscopy analyses of LC3 expression in HeLa/TFEB cells transfected for the expression of LC3-GFP for 20 hr and treated with 1 mM HPβCD for additional 24 hr. The scale bar is 10 μm. UT, untreated. (B) Western blot analyses of LC3 isoforms and GAPDH (used as loading control) in HeLa/TFEB cells treated with 1 mM HPβCD and 100 nM bafilomycin for 24 hr and quantification of LC3-II bands. Band intensities were quantified with ImageJ analysis software, corrected by GAPDH band intensities, and divided by the values obtained in untreated samples (p<0.01, *p<0.05). Baf, bafilomycin. (C) Western blot analyses of LC3 isoforms and GAPDH (used as loading control) in HeLa/TFEB cells treated with 1 mM HPβCD for 0, 12, 24, 48, and 72 hr and quantification of LC3-II/LC3-I ratios at each time point. Band intensities were quantified as described in (A) (*p<0.05). Data are reported as mean ± SD (n ≥ 3; p<0.01). (D) Relative mRNA expression levels of representative genes of the autophagy system in HeLa/TFEB cells treated with 1mM HPβCD for 1 day. mRNA expression levels of MAPLC3B, SQSTM1, and BECN1 were obtained as described in Figure 4.1. Data are reported as mean ± SD (n ≥ 3; p<0.01).

To further confirm that HPβCD treatment mediates activation of autophagy, the expression of genes involved in different steps of the autophagic pathway was tested. Cells were treated with HPβCD (1 mM), and mRNA levels were tested by quantitative RT-PCR (Figure 4.2D). Upregulation was observed upon HPβCD treatment for MAPLC3 (microtubule-associated light chain protein 3 (LC3); 4.8-fold, p<0.01), which is essential for the formation of autophagic vesicles; for SQSTM1 (p62; 2.1-fold, p<0.01), which is essential for cargo recognition; and for BECN1 (Beclin-1; 3.0-fold, p<0.01), which is required for the formation of autophagosomes. Interestingly, MAPLC3B and SQSTM1 are known to be direct targets of TFEB (51,175).
Taken together, these results demonstrate that HPβCD treatment results in activation of TFEB, transcriptional upregulation of genes involved in the lysosome-autophagy system, and upregulation of the autophagic flux.

4.2.2. HPβCD treatment results in clearance of ceroid lipopigment in LINCL fibroblasts

As described before, previous studies associated autophagy activation with HPβCD-induced cholesterol depletion in cultured human fibroblasts (110). To investigate whether the link between HPβCD-induced clearance and autophagy is specific for cholesterol storage or is a more general cellular response activated upon uptake of HPβCD, I focused on a different model of lysosomal storage, namely neuronal ceroid lipofuscinoses (NCLs). Cells derived from NCL patients are characterized by accumulation of ceroid lipopigment, a lipofuscin-like autofluorescent material that is readily visible in microscopic analyses (80). Specifically, I used fibroblasts derived from a patient with late infantile neuronal ceroid lipofuscinosis (LINCL), a disease caused by deficiency of Tripeptidyl peptidase (TPP1) activity. Previous studies showed that cells derived from patients with LINCL or other NCLs have increased tendency to undergo apoptosis (171). Thus, apoptosis-sensitive LINCL fibroblasts were selected for this study to investigate whether HPβCD treatment affects autophagic clearance of ceroid lipofuscin and whether HPβCD-induced modulation of autophagy also activates cell
death mechanisms (see below). I used cells carrying two heterozygous compound mutations in the *TTP1* gene: a missense mutation (Arg208→stop codon) and a splicing mutation resulting in the retention of intron 5 in the spliced transcript (176). Enzymatic activity assays confirmed that this cell line has null TPP1 enzyme activity compared to fibroblasts derived from a non-affected individual (Figure 4.3).

![Graph showing TPP1 enzymatic activity](image)

**Figure 4.3.** LINCL fibroblasts present null TPP1 enzyme activity and accumulation of ceroid lipopigment. **(A)** TPP1 enzymatic activity in fibroblasts derived from a healthy individual (wild type) and from a patient affected by late-infantile neuronal ceroid lipofuscinosis (LINCL) conducted as described in the *Materials and Methods* (*n* ≥ 3; *p*<0.01). **(B)** Confocal microscopy analyses of ceroid lipopigment in fibroblasts derived from a healthy individual (wild type) and from a patient affected by late-infantile neuronal ceroid lipofuscinosis (LINCL). The scale bar is 50 μm.
To test the effects of HPβCD treatment on the storage of the autofluorescent material in LINCL fibroblasts, the culture medium was supplemented with a range of HPβCD concentrations and incubated with cells for 3 days. The autofluorescence of ceroid lipopigment in LINCL fibroblasts was monitored using confocal microscopy as described in the Materials and Methods. Confocal microscopy analyses showed that HPβCD treatment resulted in clearance of ceroid lipopigment as observed by monitoring the loss of autofluorescence (Figure 4.4A-B). Notably, ceroid lipopigment-associated autofluorescence decreased with increasing concentrations of HPβCD, and maximum clearance occurred upon treatment with 1-10 mM HPβCD (Figure 4.4A-B), which was previously reported to activate autophagy (110).

To test whether clearance of ceroid lipopigment parallels activation of TFEB in cells treated with HPβCD, TFEB subcellular localization was also evaluated in LINCL cells treated with a range of HPβCD concentrations. Confocal microscopy analyses revealed that TFEB preferentially accumulates in the nucleus in LINCL fibroblasts treated with HPβCD, and that the extent of TFEB nuclear translocation increases with increasing concentrations of HPβCD in the culture medium (Figure 4.4A). Partial nuclear translocation of TFEB was observed in untreated LINCL fibroblasts, as expected, due to storage–induced lysosomal stress (42).
Figure 4.4. HPβCD treatment results in reduced storage of ceroid lipopigment. (A) Confocal microscopy analyses of ceroid lipopigment (top, green) and TFEB (bottom, red) in LINCL patient-derived fibroblasts treated with 0.1, 1, and 10 mM HPβCD evaluated by detecting green autofluorescence and binding of an anti-TFEB antibody, respectively. The scale bar is 20 μm. UT, untreated. (B) Quantification of ceroid lipopigment autofluorescence in LINCL patient-derived fibroblasts treated as described in (A). Representative fields containing approximately 50 cells were analyzed. UT, untreated. Data are reported as mean ± SD (p<0.05, *p<0.01). (C) Confocal microscopy analyses of ceroid lipopigment (top, green) and TFEB (bottom, red) in LINCL fibroblasts treated with 1 mM HPβCD for 1, 3 and 7 days and
evaluated as described in Figure 4.4A. The scale bar is 20 μm. (D-E) Relative mRNA expression levels of representative genes of the lysosome-autophagy system in LINCL fibroblasts treated with 1 mM HPβCD for 3 days. GBA, HEXA, LAMP1, MAPLC3B, SQSTM1, and BECN1 mRNA expression levels were obtained as described in Figure 4.1 (p<0.01).

To confirm that the clearance of ceroid lipopigment in LINCL fibroblasts depends on HPβCD treatment, autofluorescence of ceroid lipopigment was monitored in LINCL fibroblasts treated with a fixed concentration of HPβCD (1 mM) for up to 7 days. Confocal microscopy analyses showed that both ceroid lipopigment clearance and TFEB nuclear translocation increased with increasing time of incubation with HPβCD (Figure 4.4C).

To investigate whether the nuclear translocation of TFEB observed in LINCL fibroblasts treated with HPβCD results in the activation of the CLEAR network, the expression of representative genes of the CLEAR network was measured upon HPβCD administration. LINCL cells were treated with HPβCD (1 mM) and the mRNA expression levels of TFEB targets were monitored by quantitative RT-PCR (Figure 4.4D). HPβCD treatment was found to result in transcriptional upregulation of all genes of the lysosomal system tested: GBA (2.7-fold; p<0.01), HEXA (2.8-fold, p<0.01), and LAMP1 (1.6-fold, p<0.01). To test whether the autophagy system was also transcriptionally activated, the mRNA expression levels of representative genes involved in different steps of the autophagy pathway was measured as described above. Significant upregulation of MAPLC3 (3.2-fold, p<0.01), SQSTM1 (2.5-fold, p<0.01), and BECN1 (2.5-fold, p<0.01) was detected in LINCL fibroblasts upon
HPβCD treatment (Figure 4.4E). Taken together, these results demonstrate that the reduced deposition of ceroid lipopigment observed upon HPβCD administration parallels activation of TFEB and transcriptional upregulation of genes involved in the lysosome-autophagy system. These results therefore suggest that the clearance of ceroid lipopigment correlates with the activation of TFEB.

4.2.3. TFEB mediates the autophagic clearance observed upon HPβCD treatment

To determine whether clearance of ceroid lipopigment observed in HPβCD-treated LINCL fibroblasts depends on TFEB activation, TFEB expression was silenced using specific small interfering RNAs (siRNA). An increase in ceroid lipopigment accumulation was observed in cells treated with TFEB siRNA compared to cells treated with a control siRNA (Figure 4.5A, top panels and Figure 4.5B), suggesting that TFEB is involved in the clearance of lipopigment deposits. TFEB silencing resulted in a 60% reduction in TFEB expression levels compared to cells transfected with a control siRNA, as evaluated by quantitative RT-PCR (Figure 4.5C, solid yellow and solid blue bars). Interestingly, the clearance of autofluorescent storage material upon HPβCD treatment was decreased by TFEB silencing (Figure 4.5A, bottom panels and Figure 4.5B). The decrease in ceroid lipopigment storage observed upon administration of HPβCD to TFEB silenced cells was likely due to HPβCD-mediated activation of residual TFEB that is present upon partial silencing.
of TFEB expression (Figure 4.5C). Accordingly, the residual amount of TFEB detected in silenced cells was found to localize preferentially in the cytoplasm in untreated cells and in the nucleus in cells treated with HPβCD (Figure 4.5A).

Figure 4.5. HPβCD-induced activation of clearance is regulated by TFEB. (A) Confocal microscopy analyses of ceroid lipopigment (green) and TFEB (red) in LINCL fibroblasts treated with control siRNA or TFEB siRNA and with 1 mM HPβCD, evaluated by detecting green autofluorescence and binding of an anti-TFEB
antibody, respectively. The scale bar is 20 μm. UT, untreated. (B) Quantification of ceroid lipopigment autofluorescence in LINCL patient-derived fibroblasts treated as described in (A) Representative fields containing approximately 50 cells were analyzed. UT, untreated; siCtrl, control siRNA; siTFEB, TFEB siRNA. Data are reported as mean ± SD (p<0.05, *p<0.01). (C) Relative mRNA expression levels of representative genes of the lysosome-autophagy system in LINCL fibroblasts treated with control siRNA or TFEB siRNA for 2 days and with 1 mM HPβCD for 3 days. TFEB, HEXA, MAPLC3B, and BECN1 mRNA expression levels were obtained as described in Figure 4.1 (n≥3; p<0.01).

To further investigate the effect of TFEB silencing in LINCL fibroblasts treated with HPβCD, the expression of genes that encode proteins involved in the lysosome-autophagy system was examined. LINCL fibroblasts were incubated with TFEB siRNA for 2 days and with HPβCD for 3 additional days, and mRNA expression levels were measured by quantitative RT-PCR (Figure 4.5C). As mentioned above, TFEB silencing reduced TFEB transcription to 40% of that measured in control LINCL cells. Interestingly, HPβCD treatment resulted in a 6.9-fold increase (p<0.01) in TFEB transcription in non-silenced LINCL cells. Similar results were obtained upon TFEB silencing: HPβCD treatment resulted in a 5.6-fold increase (p<0.01) in TFEB transcription compared to untreated (silenced) cells, suggesting that administration of HPβCD causes upregulation of TFEB. Representative genes of the lysosomal system, GBA and HEXA, were found to be upregulated in cells treated with HPβCD and control siRNA (3.4- and 2.7-fold, respectively, confirming the results reported in Figure 4.4D; p<0.01), downregulated in cells treated with TFEB siRNA (0.65- and 0.6-fold, respectively; p<0.01), and upregulated in cells treated with TFEB siRNA and HPβCD (1.7- and 1.4-fold, respectively; p<0.01), compared to cells
treated with control siRNA. The increase in expression levels of TFEB target genes observed in cells treated with HPβCD in both control and silenced cells suggests that HPβCD treatment has a dual effect and results in transcriptional upregulation of TFEB as well as TFEB protein activation. In summary, HPβCD treatment was found to cause an increase in expression of TFEB target genes that is subsequent to TFEB nuclear translocation, confirming that LINCL cells respond to HPβCD treatment by activating TFEB and the CLEAR network.

To investigate the role of TFEB in the activation of autophagy observed upon HPβCD treatment, the expression of representative genes involved in the autophagy pathway, namely MAPLC3B and BECN1 was examined. As observed for genes involved in lysosomal function, MAPLC3B and BECN1 were found to be upregulated in LINCL fibroblasts treated with HPβCD and control siRNA (2.4- and 3.2-fold; p<0.01, respectively), downregulated in cells treated with TFEB siRNA (0.5-fold; p<0.01), and upregulated in cells treated with TFEB siRNA and HPβCD (1.6- and 1.3-fold; p<0.01, respectively), compared to cells treated with control siRNA (Figure 4.5C).

Taken together, these results demonstrate that HPβCD treatment results in coordinated upregulation of lysosome biogenesis and autophagy and enhanced clearance of autophagic material. Importantly, these data also demonstrate that TFEB plays a key role in mediating autophagy activation observed upon HPβCD administration and that the upregulation of genes involved in the lysosome-
autophagy system and the reduction of ceroid lipopigment accumulation correlate with TFEB expression levels and parallel TFEB activation.

4.2.4. HPβCD treatment results in activation of autophagy without inducing apoptosis

To investigate whether clearance of ceroid lipopigment in LINCL cells treated with HPβCD depends on activation of autophagy, the expression of LC3 was analyzed by confocal microscopy. Endogenous LC3 was visualized as diffuse cytoplasmic pool in untreated cells but it appeared as punctate structures, which primarily represent autophagosomes and autophagolysosomes (174), in cells treated with HPβCD (1 mM, 3 days). Interestingly, the increase in expression of LC3 and punctate appearance – indicative of an increase in autophagosome formation – paralleled a reduction in autofluorescence of storage material (Figure 4.6A, left panel).

To confirm that that clearance of ceroid lipopigment observed upon HPβCD treatment depends on activation of autophagy, LINCL fibroblasts were treated with bafilomycin (100 nM). Bafilomycin, a specific inhibitor of vacuolar H+ ATPase (V-ATPase) that prevents fusion of autophagosomes with lysosomes, thereby impairing autophagic flux (177), did not result in a significant increase in lipopigment autofluorescence, confirming that LINCL cells have a defective autophagy (178). However, lipopigment autofluorescence was partially reduced by addition of HPβCD
(1 mM). HPβCD treatment in cells cultured in the presence of bafilomycin was found not only to reduce accumulation of ceroid lipopigment, but also to enhance the appearance of punctate LC3 structures, compared to cells only treated with bafilomycin (Figure 4.6A, right panel).

**Figure 4.6. HPβCD treatment activates autophagic clearance.** (A) Confocal microscopy analyses of ceroid lipopigment (green) and LC3 (red) in LINCL fibroblasts treated with 1 mM HPβCD and 100 nM bafilomycin for 3 days, evaluated by detecting green autofluorescence and binding of an anti-LC3 antibody, respectively. The scale bar is 20 μm. UT, untreated; Baf, bafilomycin. (B) Western blot analyses of LC3 isoforms and GAPDH (used as loading control) in LINCL fibroblasts treated with 1 mM HPβCD and 100 nM bafilomycin for 24 hr and quantification of LC3-II bands. Band intensities were quantified with ImageJ analysis software, corrected by GAPDH band intensities, and divided by the values obtained in untreated samples (p<0.05). Baf, bafilomycin.
Activation of autophagy upon treatment of LINCL cells with HPβCD was also confirmed by immunoblotting of LC3 isoforms. HPβCD treatment was observed to increase the amount of LC3-II, which is indicative of autophagy induction (Figure 4.6B). The increase in LC3-II levels observed in cells treated with HPβCD in the presence of bafilomycin, compared to cells treated only with HPβCD, confirmed that HPβCD treatment results in an increase in autophagic flux.

To verify activation of the autophagic flux, the extent to which HPβCD treatment resulted in fusion of autophagosomes with lysosomes and subsequent formation of autophagolysosomes was tested. To this end, the colocalization of LC3 with LAMP-2, a protein that resides on the lysosomal membrane was evaluated. LINCL fibroblasts were treated with HPβCD (1 mM), bafilomycin (100 nM), or a combination thereof for 3 days. Accumulation of ceroid lipopigment was evaluated under all conditions tested in this experiment by monitoring autofluorescence using confocal microscopy (Figure 4.7, column 1). The overlap of LC3 (Figure 4.7, column 2) and LAMP-2 (Figure 4.7, column 3) signals was evaluated using the ImageJ script Colocalization Colormap (Figure 4.7, column 4 and 5). HPβCD treatment resulted in enhanced formation of autophagolysosomes as indicated by the punctate LC3 appearance and by the hot colors in the co-localization heatmap. As expected, the formation of autophagolysosomes decreased in cells treated with bafilomycin and was partially restored upon addition of HPβCD.
Figure 4.7. HPβCD treatment enhances formation of autophagolysosomes. Confocal microscopy analyses of ceroid lipopigment (green, column 1), LC3 (red, column 2), LAMP-2 (blue, column 3) in LINCL fibroblasts treated with 1 mM HPβCD and 100 nM bafilomycin for 3 days, evaluated by detecting green autofluorescence, binding of anti-LC3 antibody, and binding of anti-LAMP-2 antibody, respectively. Colocalization of LC3 and LAMP-2 is shown in purple color (column 4). Heatmaps of co-localization images were obtained with ImageJ analysis software (column 5). Hot colors represent positive correlation (colocalization), whereas cold colors represent negative correlation (exclusion). The scale bar is 20 μm. UT, untreated; Baf, bafilomycin.

To confirm that the HPβCD treatment of LINCL fibroblasts results in clearance of ceroid lipopigment specifically through the autophagy pathway, ATG7 expression was silenced using specific small interfering RNAs (siRNA). ATG7 is an essential autophagy gene required for basal as well starvation-induced autophagy (179). An increase in ceroid lipopigment accumulation was observed in cells treated
with ATG7 siRNA compared to cells treated with a control siRNA (Figure 4.8A, top panels and Figure 4.8B), confirming that ATG7 is required for clearance of lipopigment deposits. Interestingly, ATG7 silencing decreased clearance of autofluorescent storage material even upon HPβCD treatment (Figure 4.8A, bottom panels and Figure 4.8B). As expected, ATG7 silencing did not alter HPβCD-induced activation of TFEB. These results suggest a model in which HPβCD treatment results in TFEB-induced activation of the autophagy system, but blockage of downstream steps of the autophagic flux (e.g., blockage of ATG7 expression) prevents clearance of ceroid lipopigment.

![Figure 4.8](image)

**Figure 4.8. HPβCD-induced activation of autophagy is reduced upon genetic inhibition of the autophagic flux.** (A) Confocal microscopy analyses of ceroid lipopigment (green) and TFEB (red) in LINCL fibroblasts treated with control siRNA or ATG7 siRNA and with 1 mM HPβCD, evaluated by detecting green autofluorescence and binding of an anti-TFEB antibody, respectively. The scale bar is 20 μm. UT, untreated. (B) Quantification of ceroid lipopigment autofluorescence in LINCL fibroblasts treated as described in (A). Representative fields containing approximately 50 cells were analyzed. UT, untreated; siCtrl, control siRNA; siTFEB, TFEB siRNA. Data are reported as mean ± SD (*p<0.01).
Activation of the apoptotic pathway is often observed in association with excessive autophagic activity (168,180). To investigate whether administration of HPβCD increases apoptosis under the conditions used in this study, I used LINCL fibroblasts, which present higher propensity to undergo apoptosis compared to wild type fibroblast (171). Induction of early and late apoptosis was monitored by measuring membrane rearrangement, characteristic of early apoptosis (annexin V binding), and membrane fragmentation, characteristic of late apoptosis (propidium iodide (PI) binding), using the Cyto-GLO™ annexin V-FITC apoptosis detection kit (181). HPβCD (0.1-10 mM, 3 days) did not cause significant changes in activation of early or late apoptosis compared with untreated cells (FITC binding affinity, p<0.01; PI binding population, p<0.01; respectively). Taxol (50 nM) was used as positive control in this study because it is known to stabilize microtubules leading to cell cycle arrest and apoptosis induction (182) (Figure 4.9). These data suggest that LINCL fibroblasts respond to treatment with HPβCD by activating the pro-survival autophagy pathway, which, under the conditions used in this study, results in extensive clearance of ceroid lipopigment deposits.

In summary, these results demonstrate that (i) HPβCD treatment results in clearance of ceroid lipopigment deposits in a model of neuronal ceroid lipofuscinosis, (ii) clearance of ceroid lipopigment observed in cells treated with HPβCD is dependent on the activation of the autophagy pathway, and (iii) induction of autophagy observed in cells treated with HPβCD does not parallel activation of apoptosis.
Figure 4.9. HPβCD treatment does not induce activation of apoptosis. (A) Annexin V binding affinity change (%) in LINCL patient-derived fibroblasts treated with HPβCD (0.1, 1, and 10 mM) or taxol (50 nM) for 1 day normalized to untreated cells (ANOVA, p<0.01). (B) PI binding population change (%) of LINCL patient-derived fibroblasts treated with HPβCD (0.1, 1, and 10 mM) or taxol (50 nM) for 1 day normalized to untreated cells (ANOVA, p<0.01). The number of cells analyzed in each experiment was 10,000. The data are reported as the mean ± SD (n ≥ 3).

4.3. Discussion

Exposure to natural and anthropogenic nanosized particles induces activation of a series of compensatory mechanisms to maintain cellular homeostasis. As described in Chapter 1, engineered nanomaterials are typically perceived by the cell as foreign or toxic, such as virus and pathogens (23-25), and may stimulate the reaction of cellular clearance mechanisms. In this study, the autophagic response that is activated upon exposure to HPβCD was investigated. Results from this study provide evidence for the first time that cell treatment with HPβCD induces a series of adaptive changes mediated by TFEB and that culminate in enhancement of the innate cellular clearance capacity.
Although cyclodextrins were first described over a century ago and have been widely used for a variety of industrial and pharmaceutical applications, their potential use for applications beyond the solubilization and stabilization of small molecules was only recently recognized. It has long been known that cyclodextrins can trap cholesterol into their hydrophobic core (107,108) (refer to Chapter 1). However, the molecular mechanism involved in HPβCD-mediated reduction of cholesterol accumulation in cells is unclear. This study reveals that HPβCD administration induces a series of adaptive changes in the lysosome-autophagy systems that are mediated by activation of TFEB, a master regulator of lysosomal biogenesis and autophagy. Importantly, HPβCD treatment was found to enhance TFEB-mediated clearance of ceroid lipopigment deposits in disease cells that have a defective lysosomal system. I propose a model in which cellular uptake of HPβCD and accumulation into late endosomes and lysosomes (117,183) results in activation of the cellular clearance response mediated by the autophagy pathway, which, in turn, results in degradation of the autophagic substrate ceroid lipopigment (Figure 4.10). Importantly, activation of autophagy observed upon HPβCD administration, under conditions that result in activation of TFEB and clearance of autophagic material, is not associated with activation of apoptosis, suggesting that cells respond to HPβCD treatment by activating the pro-survival autophagic pathway.
Figure 4.10. Schematic representation of the proposed adaptive cellular response to HPβCD treatment. Administration of HPβCD results in activation of TFEB. Upon translocation from the cytoplasm to the nucleus, TFEB regulates the expression of genes involved in biogenesis and fusion of lysosomes and autophagosomes. As a result, HPβCD administration results in enhanced clearance of the autophagic substrate ceroid lipopigment.

This study provides, for the first time, a detailed mechanistic understanding of the changes induced by HPβCD administration on this important catabolic pathway, which plays a key role in the regulation of cellular metabolism with
implications ranging from cell survival under nutrient deprivation to defense against virus and parasites infections. On the one hand, these results provide a solid mechanistic groundwork to reevaluate the use of HPβCD as a drug delivery vector in conditions that may be negatively impacted by deregulation of lysosomal pathways and autophagy. On the other hand, our findings will extend the capability of designing therapeutic solutions based on the use of HPβCD for the treatment of diseases characterized by inefficient autphagic clearance and accumulation of storage material.

As described in Chapter 1, impairment or deregulation of autophagy is linked to the development and progression of a number of human diseases, ranging from neurodegenerative diseases to cancer. For instance, the accumulation of lysosomal substrates typically observed in affected cells from patients with LSDs was shown to impair fusion of lysosomes with autophagosomes, and, ultimately, lower the autophagic clearance capacity (184). Among LSDs, NCLs are among the most devastating inherited disorders of childhood and the most common cause of neurodegeneration in children in the US. In light of these findings, it is important to reevaluate the design of therapeutic strategies based on the use of HPβCD as the drug delivery vehicle or as the active agent.
4.4. Materials and Methods

4.4.1. Reagents, plasmids and cell cultures

2-Hydroxypropyl-β-cyclodextrin was purchased from Sigma-Aldrich, sucrose was from Calbiochem, bafilomycin was from Cayman Chemical, and DAPI nuclear stain was from Enzo Life Sciences. Cell culture media were from Lonza. TFEB siRNA (Cat# SI00094969) and control siRNA (Cat#1027280) were from Qiagen. pBABEpuro GFP-LC3 plasmid was from Addgene.

HeLa cells stably transfected for the expression of TFEB-3xFLAG were a generous gift of Dr. Sardiello (42). Fibroblasts derived from patients with LINCL were obtained from Coriell Cell Repositories. Direct sequencing of TPP1 coding sequences showed compound heterozygous variations: an Arg208-to-stop codon mutation and a G-to-C transversion of the consensus AG 3-prime splice acceptor site at exon 6, resulting in the retention of intron 5 in the spliced transcript. Cells were grown at 37°C in 5% CO₂ in minimal essential medium with Earle’s salts, supplemented with 10% heat-inactivated fetal bovine serum and 1% glutamine Pen-Strep. Medium was replaced every 2 or 3 days. Monolayers were passaged with TrypLE Express.
4.4.2. Enzyme activity assays

TPP1 activity assays were conducted as described previously (185). Briefly, fibroblasts derived from a patient with LINCL and from a healthy individual were plated and cultured overnight at 37°C in 5% CO₂. Cells were collected using TrypLE, washed with PBS and incubated with lysis buffer (150 mM NaCl, 50 mM Tris pH 8, 0.5% sodium deoxycholate, 1% Triton, 0.1% SDS) supplemented with 1% protease inhibitor for 1 hr at 4°C. Lysed cells were centrifuged and the supernatant collected for activity assays. The protein concentration was measured using BCA assay and TPP1 activity was assayed as previously described (185) using 3.5 µg of total protein in the assay reaction.

4.4.3. Immunofluorescence assays

Cells were seeded on glass coverslips and cultured in the presence of HPβCD. Immunofluorescence studies were conducted as described in Section 3.4.4. Cells were incubated with primary antibodies for 1 hr (rabbit anti-3xFLAG (Sigma-Aldrich), rabbit anti-LC3 (MBL International), mouse anti-LAMP2 (BioLegend) or mouse anti-TFEB (Abcam)), washed with 0.1% Tween-20/PBS, and incubated with secondary antibodies for 1 hr (Dylight 549 goat anti-mouse IgG or Dylight 633 goat anti-rabbit IgG (KPL)). Images were obtained using an Olympus IX81 confocal microscope and co-localized using the Fluoview software as described in Section 3.4.4.
4.4.4. Quantitative RT-PCR

Cell were cultured with HPβCD for 24 hr and 72 hr. Quantitative RT-PCR (qRT-PCR) was conducted as described in Section 3.4.3 using corresponding primers reported in Table A.1.

4.4.5. LC3 Western blot analyses

Cell lysates were prepared as described in Section 3.4.3. Samples were separated by 15% SDS-PAGE gel and Western blot analyses were performed using primary antibody (rabbit anti-LC3 (MBL) and rabbit anti-GAPDH (Santa Cruz Biotechnology)) and secondary antibody (HRP-conjugated goat anti-rabbit (Santa Cruz Biotechnology)). Blots were visualized and quantified by as previous described in Section 3.4.3.

4.4.6. Ceroid lipopigment analyses

The accumulation of ceroid lipopigment was quantified by measuring the brightness density of each image and calculating the ratio of brightness density of HPβCD treated cells over that of untreated cells. Average values were taken over multiple images from replicate samples.
4.4.7. Apoptosis assays

LINCL patient-derived cells were treated with HPβCD or Taxol for 24 hr at 37 °C. Induction of apoptosis was measured as described in Section 3.4.9.

4.4.8. Cell transfections

Cells (10^5) in 2 ml of growth medium were seeded in each well of a 6-well plate. 6 µl of jetPRIME™ reagent (Polyplus Transfection) was mixed with 0.6 µg of the plasmid pBABEpuro GFP-LC3 to form transfection complexes. Transfection complexes were added to each well of the plate when cells reached 70-80% confluency. After incubation for 20 hr, the transfection medium was replaced with fresh medium containing HPβCD.

4.4.9. siRNA transfections

siRNA transfection was performed using HiPerFect® Transfection Reagent as described in manufacturer's manual (Qiagen). Briefly, each well of a 24-well plate was spotted with 75 ng siRNA diluted in 18 µl RNase-free water. HiPerFect reagent (4.5 µl) was resuspended in 145.5 µl of culture medium without serum and was added to the pre-spotted siRNA. The mixture was incubated for 10 min at room
temperature to allow formation of transfection complexes. Cells ($3 \times 10^4$) in 1 ml of culture medium were seeded into each well on top of the transfection complexes and incubated for 2 days. Medium was replaced with fresh medium or medium containing HPβCD. Ceroid lipopigment autofluorescence intensity level and relative mRNA expression levels of representative TFEB or autophagy genes were evaluated after 3 days of HPβCD treatment.

### 4.4.10. Statistical analyses

All data are presented as mean ± SD, and statistical significance was calculated using a two-tailed t-test.
Chapter 5

Surface Charge-dependent Autophagic Clearance by Polystyrene Nanoparticles

5.1. Introduction

As described in Chapter 1, a number of synthetic nanoparticles are sequestered in autophagosomes when internalized into cells (119-123), suggesting that they may activate the autophagy pathway. However, activation of autophagy by nanoparticles is not necessarily always followed by clearance of autophagosomes but may result instead in autophagy dysfunction and accumulation of autophagosomes (125-127). For instance, cationic nanoparticles were reported to induce autophagy, but also to disrupt lysosomes, ultimately blocking autophagic flux and impairing degradation (126,127). Specifically, a “proton sponge” effect was proposed as a potential mechanism of lysosomal membrane permeabilization. It
was suggested that the high H+ buffering capacity of the amino groups on nanoparticle surface could interfere with acidification of lysosomes, impair the proton pump activity, and ultimately induce lysosomal membrane permeabilization (refer to Chapter 1 for a detailed description) (127). Thus, I hypothesized that the surface charge of nanoparticles plays an important role in determining the nature of the autophagic response that is induced upon nanoparticle internalization. This hypothesis was investigated by testing the impact of polystyrene nanoparticles of different sizes that were functionalized to present different surface charges on the lysosome-autophagy system.

Polystyrene nanoparticles are often used to study nano-bio interactions because they are biologically inert and can be easily functionalized. In addition, polystyrene nanobeads can be obtained in a wide range of sizes and in fluorescently labeled forms allowing localization and tracking in live cells. In this study, polystyrene nanoparticles of 50 nm, 100 nm and 200 nm diameter were selected. Nanoparticles of each size were functionalized with different groups according to the manufacturer’s specifications (refer to the Materials and Methods): non functionalized nanoparticles (PS) present neutral surfaces, carboxyl-functionalized nanoparticles (PS-COOH) present anionic surfaces, and amino-functionalized nanoparticles (PS-NH₂) present cationic surfaces. Neutral and anionic surfaces are expected not to change acidification of lysosomes or lysosomal integrity (133), whereas nanoparticles presenting cationic surface modifications were reported to
cause lysosome rupture through a “proton sponge” effect (127) and consequently block autophagic flux and induce cytotoxicity (126,127,186).

To investigate the molecular mechanism of autophagy induction by polystyrene nanoparticles, I used HeLa cells overexpressing TFEB as well as PC12 cells, which are known to be more resistant to cytotoxicity induced by cationic nanoparticles (127). I found that cell exposure to polystyrene nanoparticles of different size (50 - 200 nm) and surface charge (neutral, anionic, cationic) results in activation of TFEB. Interestingly, cationic polystyrene nanoparticles were found to induce TFEB activation to a higher level than neutral or anionic nanoparticles. Moreover, cationic nanoparticles induce TFEB activation when used at lower concentrations than neutral and anionic nanoparticles. However, cationic nanoparticles were also found to induce considerable cytotoxicity, which was not observed in cells treated with either neutral or anionic nanoparticles.

To investigate whether activation of TFEB observed in cells treated with different polystyrene nanoparticles leads to upregulation of autophagic flux, I used fibroblasts derived from a patient with late infantile neuronal ceroid lipofuscinosis (LINCL) that accumulate the autophagic substrate ceroid lipopigment, as described in Chapter 4 (80). I found that cell exposure to neutral and anionic polystyrene nanoparticles results in enhanced cellular clearance of ceroid lipopigment whereas cell exposure to cationic nanoparticles impairs lysosomal integrity and results in blockage of autophagic flux. These results suggest that clearance of autophagic cargo upon polystyrene nanoparticle treatment is surface charge dependent.
In summary, this study provides a detailed mechanistic understanding of nanoparticles as autophagy activators with respect to nanoparticle size and surface charge. Our findings provide evidence that nanoparticle surface charge plays a key role in determining whether nanoparticle-mediated autophagy activation results in effective autophagic clearance or blockage of autophagic flux.

5.2. Results

5.2.1. Characterization of polystyrene nanoparticles

The polystyrene nanoparticles used in this study are functionalized with neutral, anionic and cationic surfaces according to the manufacturer’s specifications. Zeta potential measurements were conducted to verify the surface charge on each nanoparticle (Table 5.1). Results indicate that PS present neutral or slightly anionic surface charge, PS-COOH present anionic surface charge, and PS-NH\textsubscript{2} present cationic surface charge (Table 5.1).

Table 5.1. Zeta potential measurement of polystyrene nanoparticles

<table>
<thead>
<tr>
<th>Nanoparticle</th>
<th>Zeta Potential (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PS 50nm</td>
<td>- 8.0 ± 2.0</td>
</tr>
<tr>
<td>PS 100nm</td>
<td>- 10.0 ± 1.2</td>
</tr>
<tr>
<td>PS 200nm</td>
<td>- 16.1 ± 1.3</td>
</tr>
<tr>
<td>PS-COOH 50nm</td>
<td>- 21.3 ± 2.2</td>
</tr>
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5.2.2. Polystyrene nanoparticles activate TFEB

To investigate whether polystyrene nanoparticles activate induction of the autophagic response, I first monitored TFEB subcellular localization in HeLa/TFEB cells. Cells were treated with polystyrene nanoparticles of different size and surface charge at concentrations ranging from 10 to 100 ppm and compared to untreated cells. TFEB intracellular localization was evaluated by confocal microscopy using DAPI nuclear stain and an anti-FLAG antibody. The percentage of TFEB nuclear localization was calculated by normalizing the fluorescence intensity of TFEB that localizes in the nucleus by the total fluorescence intensity of TFEB in each cell. The average fraction of TFEB that localizes in the nucleus was calculated by quantifying the percentage of TFEB nuclear localization in each cell of representative (over 30) fields each containing approximately 50 cells as described in the Materials and Methods.

TFEB was found to localize predominantly in the cytoplasm of untreated HeLa/TFEB cells, as expected (42). Specifically, the average fraction of TFEB that localizes in the nucleus was observed to be 29.1 ± 1.7% of the total pool of TFEB in

<table>
<thead>
<tr>
<th>Nanoparticle</th>
<th>Percentage (± SD)</th>
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<tbody>
<tr>
<td>PS-COOH 100nm</td>
<td>43.3 ± 3.6</td>
</tr>
<tr>
<td>PS-COOH 200nm</td>
<td>20.6 ± 3.2</td>
</tr>
<tr>
<td>PS-NH₂ 50nm</td>
<td>20.4 ± 1.3</td>
</tr>
<tr>
<td>PS-NH₂ 100nm</td>
<td>19.1 ± 1.6</td>
</tr>
<tr>
<td>PS-NH₂ 200nm</td>
<td>21.6 ± 2.6</td>
</tr>
</tbody>
</table>
untreated cells. Significant increase in TFEB translocation from the cytoplasm to the nucleus was observed upon cell treatment with PS (Figure 5.1A) or PS-COOH (Figure 5.1B). The average fraction of TFEB that localizes in the nucleus was found to increase to over 40% after 24 hr of cell treatment with PS of different sizes at concentrations of 50 ppm and 100 ppm. Interestingly, while no significant changes in TFEB nuclear localization were detected in cells treated with lower concentrations of PS (data not shown), the extent of TFEB nuclear localization was also not found to change upon increase in nanoparticle concentration in the culturing medium (PS 50nm: 45.4 ± 3.3% at 50 ppm, 47.5 ± 1.0% at 100 ppm; PS 100nm: 44.4 ± 3.8% at 50 ppm, 42.6 ± 2.4% at 100 ppm; PS 200nm: 44.9 ± 2.2% at 50 ppm, 49 ± 2.8% at 100 ppm. Figure 5.1A; p<0.01). Similar results were obtained from cells treated with PS-COOH (PS-COOH 50nm: 40.9 ± 1.4% at 50 ppm, 49.5 ± 2.8% at 100 ppm; PS-COOH 100nm: 42.0 ± 2.0% at 50 ppm, 48.6 ± 2.7% at 100 ppm; PS-COOH 200nm: 48.9 ± 2.7% at 50 ppm, 52.3 ± 1.3% at 100 ppm. Figure 5.1B; p<0.01). Treatment with PS-NH₂, on the other hand, was observed to induce a dramatic increase in TFEB nuclear localization when cells were treated with lower concentrations of nanoparticles (10 ppm and 25 ppm; Figure 5.1C) compared to PS and PS-COOH. The average fraction of TFEB that localizes in the nucleus was found to increase to over 60% after 24 hr of cell treatment with 10 ppm and 25 ppm PS-NH₂ (PS-NH₂ 50nm: 62.4 ± 1.0% at 10 ppm, 66.0 ± 3.7% at 25 ppm; PS-NH₂ 100nm: 70.5 ± 4.7% at 10 ppm, 75.4 ± 3.4% at 25 ppm; PS-NH₂ 200nm: 62.3 ± 2.7% at 10 ppm, 64.0 ± 2.2% at 25 ppm) (Figure 5.1C; p<0.05). Interestingly, cell treatment
with higher concentrations of PS-NH$_2$ (50–100 ppm) resulted in considerable cytotoxicity and cell death, precluding accurate evaluation of TFEB nuclear localization. Representative images of cells treated with PS, PS-COOH and PS-NH$_2$ under different conditions are reported in Figure 5.2.

Collectively, these results indicate that cationic polystyrene nanoparticles induce TFEB activation in HeLa/TFEB cells to a higher extent than neutral and anionic nanoparticles. These results also suggest that the minimal concentration of cationic polystyrene nanoparticles that is needed to observe TFEB nuclear localization is lower than that of neutral and anionic nanoparticles. However, once the minimal medium concentration to achieve increase in TFEB activation is reached, the extent of TFEB nuclear localization does not increase with nanoparticle concentration. Moreover, no significant difference in the extent of nuclear TFEB was observed between nanoparticles of different size among the nanoparticle tested (50–200 nm), suggesting that the extent of TFEB nuclear localization is independent of nanoparticle size in this size range.
Figure 5.1. Polystyrene nanoparticles promote TFEB activation in HeLa/TFEB cells. The average fraction of TFEB that localizes in the nucleus of HeLa/TFEB cells treated with (A) 50 ppm and 100 ppm PS, (B) 50 ppm and 100 ppm PS-COOH, (C) 10 ppm and 25 ppm PS-NH₂ for 24 hr. Representative fields (~30) each containing approximately 50 cells were analyzed to calculate the average fraction of TFEB presenting nuclear localization in HeLa/TFEB cells (p<0.01). Data are reported as mean ± SD (n=3).
Figure 5.2. TFEB subcellular localization in HeLa/TFEB cells. Representative confocal microscopy images of TFEB subcellular localization in HeLa/TFEB cells treated with (A) 50 ppm and 100 ppm PS, (B) 50 ppm and 100 ppm PS-COOH, (C) 10 ppm and 25 ppm PS-NH₂ for 24 hr. UT, untreated. The scale bar is 10 μm.
To quantify cellular uptake of polystyrene nanoparticles in HeLa/TFEB cells and, particularly, to investigate the correlation between cellular uptake and TFEB activation, I measured the extent of nanoparticles internalization. Cells were incubated with fluorescently labeled polystyrene nanoparticles for 24 hr and washed with PBS to remove nanoparticles from the outer cell membrane. Cell fluorescence was measured as described in the Materials and Methods. The extent of polystyrene nanoparticle uptake was calculated by normalizing the fluorescence intensity of each sample to that of each corresponding nanoparticle. Cellular uptake was observed to be concentration- and size- dependent for all polystyrene nanoparticles (non functionalized, carboxylated and aminated) (Figure 5.3). Specifically, the uptake of polystyrene nanoparticles was found to generally increase with nanoparticle concentration, suggesting a positive correlation between cellular uptake and nanoparticle concentration (Figure 5.3). A number of studies demonstrated that nanoparticle uptake is also size-dependent (125,132,187-195). In this study, I observed different patterns of cellular uptake in cells treated with nanoparticles presenting different surface charges. Specifically, the uptake of PS was found to decrease with size (Figure 5.3A), which is in agreement with previous studies (133,195). Analyses conducted using PS-COOH revealed that the cellular uptake of PS-COOH of intermediate size (100 nm) is significantly lower than that of PS-COOH of small (50 nm) and large (200 nm) size (Figure 5.3B). Previous studies suggested that the electrostatic repulsion between nanoparticles presenting negative surface charge and the cell membrane, which is also negatively charged,
prevents efficient cellular uptake (196,197). Not surprisingly, cell treatment with 100 nm PS-COOH, which present the most negatively charged surface among the three anionic polystyrene nanoparticles (50, 100 and 200 nm; Table 5.1), results in the lowest cellular uptake (Figure 5.3B). Unlike PS or PS-COOH, the cellular uptake of PS-NH₂ increases with size (Figure 5.3C), suggesting that the positively charged functional groups on the surface of polystyrene nanoparticles may also affect cellular uptake, a notion in agreement with previously reported studies (190,192).

In summary, these results show that cellular uptake of polystyrene nanoparticles of different sizes and surface charges varies with nanoparticle concentration in the culturing medium, nanoparticle size, and nanoparticle charge (Figure 5.3). On the other hand, the results of TFEB nuclear localization studies (Figure 5.1) suggest the extent of TFEB activation does not depend on nanoparticle size or concentration. Taken together, these results suggest that TFEB activation does not depend on the extent of nanoparticle uptake (compare Figure 5.1 and Figure 5.3). More specifically, the extent of TFEB nuclear localization does not change with cellular uptake when the intracellular concentration of polystyrene nanoparticles reaches the minimal concentration that is needed to activate TFEB, which is specific for each nanoparticle surface charge but does not vary with nanoparticle size. Collectively, these results suggest that TFEB activation is a switch-like response that is activated upon uptake of a critical nanoparticle concentration.
Figure 5.3. Cellular uptake of polystyrene nanoparticles in HeLa/TFEB cells. Cellular uptake of polystyrene nanoparticles in HeLa/TFEB cells treated with (A) PS (25 ppm, 50 ppm and 100 ppm; 24hr), (B) PS-COOH (25 ppm, 50 ppm and 100 ppm; 24hr), (C) PS-NH$_2$ (10 ppm and 25 ppm; 24hr) (p<0.05). Data are reported as mean ± SD (n=3).
Nanoparticles presenting cationic surface modifications may cause lysosomal rupture through a “proton sponge” effect (186) and were showed to induce cytotoxicity (127,198). To evaluate polystyrene nanoparticle-induced toxicity, HeLa/TFEB cells were treated with polystyrene nanoparticles under conditions that result in activation of TFEB and cytotoxicity was measured using the LIVE/DEAD® Viability/Cytotoxicity assay. Specifically, binding of calcein AM, which is converted to green-fluorescent calcein by cellular esterase in live cells, was monitored to detect live cells. Binding of EthD-1, which exhibits red fluorescence upon entering the cell through damaged cell membrane and binding to nucleic acid, was monitored to detect dead cells. Cells were treated with polystyrene nanoparticles for 24 hr and cytotoxicity was evaluated by confocal microscopy. I found that neither PS nor PS-COOH induce cytotoxicity in HeLa/TFEB cells under conditions (50 ppm; 24 hr) that result in TFEB activation (Figure 5.4). Cell treatment with PS-NH₂ under the conditions that induce TFEB activation (10 ppm; 24 hr), on the other hand, resulted in higher toxicity (Figure 5.4), possibly due to elevation of lysosomal pH and impairment of lysosomal integrity (127,198,199).
Figure 5.4. Cytotoxicity induced by polystyrene nanoparticles in HeLa/TFEB cells. Confocal microscopy analyses of calcein (green) and EthD-1 binding (red) in HeLa/TFEB cells treated with 50 ppm PS, 50 ppm PS-COOH or 10 ppm PS-NH$_2$ for 24 hr. UT, untreated. The scale bar is 40 μm.
In summary, these studies indicate that cell treatment with neutral and anionic polystyrene nanoparticles (50 ppm) does not induce cytotoxicity and results in activation of TFEB in HeLa/TFEB cells. However, the same cell line was found to respond to treatment with lower concentrations of cationic polystyrene nanoparticles (10 ppm) with a higher extent of activation of TFEB compared to neutral and anionic nanoparticles. Interestingly, higher concentrations of cationic nanoparticles (> 25 ppm) resulted in high cytotoxicity and cell death. To further investigate these findings, I tested the impact of this battery of polystyrene nanoparticles on TFEB subcellular localization in PC12 cells, which are more resistant to cytotoxicity caused by cationic nanoparticles (127). PC12 cells were treated with polystyrene nanoparticles at concentrations ranging from 10 to 100 ppm for 24 hr and TFEB intracellular localization was evaluated and quantified as described above (Figure 5.5). Similar to what was observed in HeLa/TFEB cells, TFEB localizes predominantly in the cytoplasm of untreated PC12 cells. Specifically, the average fraction of TFEB that localizes in the nucleus was found to be 30.7 ± 0.7% of the total pool of TFEB in untreated cells. TFEB nuclear translocation was found not to increase in cells treated with PS or PS-COOH at the concentration tested (10–100 ppm) compared to untreated cells (Figure 5.5A-B). Interestingly, the extent of TFEB nuclear localization in PC12 cells treated with PS and PS-COOH was found to increase only upon exposure to much higher concentrations of nanoparticles (500–600 ppm; data not shown). However, a significant increase in TFEB activation compared to untreated cells was observed in cells treated with PS-
NH$_2$ (Figure 5.5C). Specifically, the average fraction of TFEB that localizes in nucleus increased to 50% after 24 hr of treatment (PS-NH$_2$ 50nm: 51.8 ± 5.3% at 50 ppm, 51.7 ± 7.1% at 100 ppm; PS-NH$_2$ 100nm: 57.5 ± 5.6% at 50 ppm, 53.1 ± 5.5% at 100 ppm; PS-NH$_2$ 200nm: 59.9 ± 8.2% at 50 ppm, 57.5 ± 2.1% at 100 ppm) (Figure 5.5C; p<0.01). Representative images of cells treated with PS, PS-COOH and PS-NH$_2$ under different conditions are reported in Figure 5.6.

The cytotoxic effect of polystyrene nanoparticles of different size and charge on PC12 cells was also investigated. Cells were treated with polystyrene nanoparticles (50 ppm; 24 hr) and cytotoxicity was evaluated as described above. Neither PS nor PS-COOH were found to induce toxicity in PC12 cells (Figure 5.7). PS-NH$_2$, on the other hand, were found to induce high cytotoxicity in PC12 cells (Figure 5.7), in analogy to what was observed in HeLa/TFEB cells (Figure 5.4).
Figure 5.5. **PS-NH₂ promote TFEB activation in PC12 cells.** The average fraction of TFEB that localizes in the nucleus of PC12 cells treated with 50 ppm and 100 ppm (A) PS (B) PS-COOH (C) PS-NH₂ for 24 hr. Representative fields (~30) containing approximately 50 cells were analyzed to calculate the average fraction of TFEB presenting nuclear localization in PC12 cells (p<0.01). Data are reported as mean ± SD (n=3).
Figure 5.6. TFEB subcellular localization in PC12 cells. Representative confocal microscopy images of TFEB subcellular localization in PC12 cells treated with 50 ppm and 100 ppm (A) PS (B) PS-COOH (C) PS-NH₂ for 24 hr. UT, untreated. The scale bar is 10 μm.
Figure 5.7. Cytotoxicity induced by polystyrene nanoparticles in PC12 cells. Confocal microscopy analyses of calcein (green) and EthD-1 binding (red) in PC12 cells treated with 50 ppm PS, PS-COOH or PS-NH₂ for 24 hr. UT, untreated. The scale bar is 40 μm.
Collectively, these results indicate that cell exposure to polystyrene nanoparticles of different size (50–200 nm) and surface charge (neutral, anionic, cationic) result in activation of TFEB. These results also show that cationic polystyrene nanoparticles induce TFEB activation to a higher level than neutral or anionic polystyrene nanoparticles, both in terms of the extent of TFEB translocation to the nucleus (compare Figure 5.1 AB and C) and in terms of the minimal concentration of nanoparticle needed to observe increase in TFEB activation. Cationic nanoparticles were found to induce TFEB activation when used at lower concentrations than neutral and anionic nanoparticles in both cell lines tested. Moreover, the minimal concentration of cationic nanoparticles that results in activation of TFEB in PC12 cells, which are particularly resistant to cytotoxicity induced by cationic nanoparticles (127), was found to be higher than that inducing TFEB activation in HeLa/TFEB cells.

5.2.3. Autophagic clearance induced upon internalization of polystyrene nanoparticles depends on nanoparticle surface charge

I asked whether TFEB activation induced by polystyrene nanoparticles of different size and surface charge results in enhancement of autophagic clearance. To address this question, I first tested the activity of the autophagy system using fibroblasts derived from a patient with LINCL. Preliminary studies were conducted to evaluate the minimal concentration of polystyrene nanoparticles that activate
TFEB in LINCL fibroblasts. Cells were exposed to nanoparticles for a longer time of incubation (3 days), which is needed to monitor autophagic clearance. Cell treatment with 25 ppm of polystyrene nanoparticles with different surface charge (neutral, anionic and cationic) for 3 days was found to induce increase of TFEB nuclear localization (Figure 5.8A, red), without inducing considerable cytotoxicity (data not shown). TFEB activation was observed upon treatment with all three types of polystyrene nanoparticles under the same concentration (25 ppm) possibly due to the prolonged time of incubation (3 days) with low nanoparticle concentration used in LINCL fibroblasts compared to the conditions investigated using HeLa/TFEB cells and PC12 cells (24 hr). I then monitored the accumulation of ceroid lipopigment in LINCL fibroblasts treated with polystyrene nanoparticles under conditions found to activate TFEB. Specifically, I monitored the autofluorescence of ceroid lipopigment using confocal microscopy. Confocal microscopy analyses showed that accumulation of ceroid lipopigment was reduced in cells treated with PS or PS-COOH (Figure 5.8A, green). The accumulation of ceroid lipopigment in cells exposed to nanoparticles was quantified and reported in Figure 5.8B. Specifically, the fluorescence intensity of ceroid lipopigment in LINCL fibroblasts decreased to approximately 60% of that of untreated cells after treatment with PS or PS-COOH (PS 50nm: 60.7 ± 15.0%; PS 100nm: 65.6 ± 10.0%; PS 200nm: 63.0 ± 7.0%; PS-COOH 50nm: 60.5 ± 6.5%; PS-COOH 100nm: 62.1 ± 8.9%; PS-COOH 200nm: 59.1 ± 6.9%) (Figure 5.8B; p<0.01). Treatment with PS-NH₂ under the same condition, however, was found not to reduce accumulation of ceroid
lipo pigment (Figure 5.8A, green). PS-NH$_2$ treatment at higher concentration was not evaluated due to considerable induction of cytotoxicity (data not shown).
Figure 5.8. Accumulation of ceroid lipopigment in LINCL fibroblasts treated with polystyrene nanoparticles. (A) Confocal microscopy analyses of ceroid lipopigment (green) and TFEB (red) in LINCL fibroblasts treated with PS, PS-COOH or PS-NH₂ (25 ppm for 3 days) and evaluated by detecting green autofluorescence and binding of anti-TFEB antibody, respectively. UT, untreated. The scale bar is 20 μm. (B) Quantification of ceroid lipopigment fluorescence intensity calculated as described in the Materials and Methods. Data are reported as mean ± SD (n=15; p<0.01).

5.2.4. Cationic polystyrene nanoparticles impair lysosomal integrity and block autophagic flux

As described above, autophagy-mediated cellular clearance relies on the coordinated activation of lysosome and autophagosome biogenesis and function, which are essential for degradation of autophagic substrates (39). Hence, not only
both branches of the lysosome-autophagy system need to be transcriptionally upregulated, but their functions also need to be functionally active to avoid blockage of the autophagic flux. Previous studies suggest that, depending on the material’s charge and composition, nanoparticles may cause impairment of lysosomal function (125,127,199). Under these conditions, transcriptional upregulation of autophagy genes and increased formation of autophagosomes are ultimately associated with decreased autophagic clearance (125). To investigate the impact of the surface charge of polystyrene nanoparticles on lysosomal function, I assessed lysosomal integrity in fibroblasts treated with PS, PS-COOH and PS-NH₂. This study was conducted using neutral, anionic and cationic nanoparticles of one representative size (50 nm) because neither the extent of TFEB activation nor the accumulation of ceroid lipopigment was found to vary with nanoparticle size. Fibroblasts were treated with polystyrene nanoparticles (50 nm; 25 ppm) and assayed every 24 hr for up to 72 hr. Lysosomal integrity was then evaluated using acridine orange (AO), a lysosomotropic probe that accumulates in acidic organelles and exhibits red fluorescence. The red fluorescence of AO dissipates upon lysosomal membrane permeabilization, when the dye is released in a more neutral environment. Therefore, a decrease in fluorescence intensity indicates abnormalities in lysosomal pH or disruption of the lysosomal membrane. In this study, I measured the fluorescence intensity of AO in cells treated with polystyrene nanoparticles using flow cytometry, and I evaluated impairment of lysosomal integrity by calculating the percentage of cells that present low AO fluorescence. The percentage of cells
presenting low AO fluorescence was calculated by gating the cell population as described in the Materials and Methods (Figure 5.9A). The average AO fluorescence was also quantified to evaluate the average lysosomal integrity in the entire cell population in each experiment (10,000 cells) (Figure 5.9B). The results showed that treatment with PS-NH₂ results in significant increase in the percentage of cells with low AO fluorescence, which was not observed in cells treated with PS or PS-COOH (Figure 5.9A), suggesting a significant increase in the number of cells with impaired lysosomal membrane upon PS-NH₂ treatment. In addition, I observed a significant decrease in the average AO fluorescence intensity in cells treated with PS-NH₂, suggesting an average decrease in lysosomal integrity (Figure 5.9B). Representative histograms are reported to compare AO fluorescence in cells treated with different types of polystyrene nanoparticles after 24 hr and 72 hr (Figure 5.9C). Collectively, these results suggest that cationic polystyrene nanoparticles impair lysosomal integrity.
Figure 5.9. Lysosomal integrity in fibroblasts treated with polystyrene nanoparticles. (A) Percentage of cells with low AO fluorescence \( (\text{AO}^{\text{low}}) \). AO fluorescence intensity was measured by flow cytometry every 24 hr for up to 72 hr in fibroblasts treated with PS, PS-COOH or PS-NH\(_2\) (25 ppm). The number of cells analyzed in each experiment was 10,000. The percentage of cells with low AO fluorescence \( (\text{AO}^{\text{low}}) \) was calculated by normalizing the cell number with low AO fluorescence by the total cell number. Data are reported as mean ± SD \((n=3; p<0.05)\). (B) Average AO fluorescence intensity measured by flow cytometry of cells treated with polystyrene nanoparticles as described in (A). Data are reported as mean ± SD \((n=3; p<0.05)\). (C) Representative histograms of cells treated with different types of polystyrene nanoparticle after 24 hr and 72 hr. UT, untreated.

Because cationic nanoparticles may cause impairment in lysosomal integrity by elevating lysosomal pH \((127,199)\), I asked whether the polystyrene nanoparticles investigated in this study accumulate in the lysosome, and whether lysosomal integrity is associated with lysosomal accumulation of nanoparticles. To this end, I tested whether neutral, anionic and cationic nanoparticles accumulate in the lysosomes of fibroblasts treated under the same conditions used to evaluate lysosomal integrity. The lysosomal localization of polystyrene nanoparticles was evaluated by testing the colocalization of polystyrene nanoparticles and LAMP-2, a protein that resides on the membrane of lysosomes, using confocal microscopy \((200)\). Confocal microscopy analyses revealed that all three types of polystyrene nanoparticles investigated in this study accumulate in the lysosomes at each time point investigated \((25 \text{ ppm}; 24 \text{ hr}, 48 \text{ hr} \text{ and } 72 \text{ hr})\) \((Figure 5.10)\). Because lysosomal function and integrity depends on the acidic pH of the lysosomal environment \((12,201,202)\), these results suggest that impairment of lysosomal integrity induced upon uptake of cationic nanoparticle is possibly due to the effect
of the surface charge of these nanoparticles on the lysosomes. Neutral and anionic nanoparticles, on the other hand, were also found to accumulate in the lysosomes at each time point investigated, but not to affect lysosomal integrity.

Lysosomal membrane permeabilization may compromise lysosome integrity and lysosome fusion with autophagosomes. To test whether cell treatment with polystyrene nanoparticles that induces lysosomal membrane permeabilization results in impairment of lysosome-autophagosome fusion, which would result in blockage of autophagic flux, I tested the extent to which nanoparticle treatment induces lysosome-autophagosome fusion and subsequent formation of autophagolysosomes. To this end, I evaluated the colocalization of LC3, a protein that is found on the membrane of autophagosomes (174), with LAMP-2, a protein that resides on the membrane of lysosomes (200). Fibroblasts were treated with polystyrene nanoparticles (50nm; 25 ppm) for up to 72 hr. Colocalization of LC3 and LAMP-2 was evaluated using confocal microscopy and was quantified as described in the Materials and Methods. Average values were calculated over multiple images and replicate samples (Figure 5.11A). Significant increase in the extent of LC3 and LAMP-2 colocalization was observed in cells treated with PS or PS-COOH compared to untreated cells (untreated: 10.1 ± 1.8% after 24 hr, 10.3 ± 2.1% after 48 hr, 9.7 ± 2.2% after 72 hr; PS 50nm: 19.1 ± 3.4% after 24 hr, 16.1 ± 1.7% after 48 hr, 17.7 ± 3.3% after 72 hr; PS-COOH 50nm: 18.4 ± 2.9% after 24 hr, 18.8 ± 3.8% after 48 hr, 16.9 ± 1.8% after 72 hr) (Figure 5.11A; p<0.01), indicating that neutral and anionic polystyrene nanoparticles promote fusion of autophagosomes and lysosomes and
formation of autophagolysosomes. The extent of colocalization of LC3 and LAMP-2 in cells treated with PS-NH₂ reached 24.3 ± 3.5% after 24 hr of incubation and 24.6 ± 4.4% after 48 hr, but decreased to 13.7 ± 1.7% after 72 hr (Figure 5.11A; p<0.01), indicating that formation of autophagolysosomes is impaired by cationic nanoparticle treatment. Representative images of untreated cells and cells treated with PS, PS-COOH and PS-NH₂ are reported in Figure 5.11B-E. Colocalization of LC3 (red) and LAMP-2 (blue) is shown in merged images (purple).

In summary, these results demonstrate that cell exposure to neutral and anionic nanoparticles activates the autophagy system, enhances autophagic activity, and promotes autophagic clearance of ceroid lipopigment in LINCL fibroblasts. However, treatment with cationic nanoparticle results in impairment of lysosomal integrity and activity, reduced formation of autophagolysosomes and ultimately blockage of autophagic flux.
Figure 5.10. Lysosomal accumulation of polystyrene nanoparticles. Confocal microscopy analyses of polystyrene nanoparticles (green) and LAMP-2 (blue) in fibroblasts treated with polystyrene nanoparticles (50nm; 25 ppm), evaluated every 24 hr for up to 72 hr by detecting the fluorescence intensity of fluorescently labeled polystyrene nanoparticles and binding of anti-LAMP-2 antibody, respectively. Colocalization of polystyrene nanoparticles (green) and LAMP-2 (blue) is shown in merged images. UT, untreated. The scale bar is 20 μm.
Figure 5.11. Colocalization of LC3 and LAMP-2 in fibroblasts treated with polystyrene nanoparticles. (A) Quantification of LC3-LAMP-2 colocalization in fibroblasts treated with polystyrene nanoparticles (50nm; 25 ppm) calculated as described in the Materials and Methods. UT, untreated. Data are reported as mean ± SD (n=15; p<0.01). (B-E) Representative confocal microscopy images of LC3 (red) and LAMP-2 (blue) in (B) untreated fibroblasts (UT) and in fibroblasts treated with (C) PS (50nm; 25 ppm), (E) PS-COOH (50nm; 25 ppm), (E) PS-NH₂ (50nm; 25 ppm) after 24, 48, and 72 hr evaluated by detecting binding of anti-LC3 antibody and binding of anti-LAMP-2 antibody, respectively. Colocalization of LC3 (red) and LAMP-2 (blue) is shown in merged images (purple). The scale bar is 20 μm.

5.3. Discussion

In this study, I investigated the response of the lysosome-autophagy system to exposure to polystyrene nanoparticles engineered with various sizes and surface charges. I first tested the impacts of polystyrene nanoparticles of different size and surface charge on TFEB activation. Cationic polystyrene nanoparticles were found to induce TFEB activation to a higher level than neutral or anionic polystyrene nanoparticles, both in terms of extent of TFEB nuclear localization and in terms of minimal concentration of nanoparticle needed to observe increase in TFEB activation. I found that the extent of TFEB nuclear localization does not correlate with nanoparticle uptake indicating that TFEB activation by nanoparticle treatment is a switch-like response. Moreover, the extent of TFEB activation seems also independent of nanoparticle size in the range of nanoparticle size investigated in this study. Nanoparticle aggregation is commonly observed upon cellular internalization typically due to the high ionic strength conditions of the cytosolic environment (129,203); whether nanoparticle aggregation plays a role in TFEB
activation observed in response to cell treatment with polystyrene nanoparticles remains to be determined.

The impact of nanoparticle size and surface charge on the nature of autophagic response was also investigated using cells that accumulate the autophagic substrate ceroid lipopigment. The results reported herein indicate that autophagic clearance of ceroid lipopigment induced upon uptake of polystyrene nanoparticles depends on nanoparticle surface charge. Specifically, cell treatment with neutral and anionic polystyrene nanoparticles was found to result in activation of autophagy and enhanced clearance of ceroid lipopigment. Cell treatment with cationic polystyrene nanoparticles, on the other hand, was found to result in impairment of lysosomal integrity, reduced formation of autophagolysosomes and ultimately blockage of autophagic flux. As a result, treatment with cationic nanoparticles prevents degradation of autophagic substrates.

This study provides a detailed mechanistic understanding of the interaction of polystyrene nanoparticles of different size and surface charge with the autophagic pathway and contributes to defining design rules for engineering nanotherapeutics for the treatment of diseases characterized by inefficient autophagic activity and accumulation of storage material. In addition to its therapeutic implications, this study also provides an experimental setup and the analytical tools for evaluating the impact of nanoparticles on biological systems and will ultimately inform the design of safe nanoparticle-containing products.
5.4. Materials and Methods

5.4.1. Reagents and cell cultures

Polystyrene nanoparticles used in this study are described in Table 5.2.

Table 5.2. Polystyrene Nanoparticles

<table>
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<tr>
<th>Name</th>
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<th>Manufacturer</th>
<th>Catalogue No.</th>
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<td>-COOH</td>
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<td>Magsphere</td>
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<td>Magsphere</td>
<td>CAF-100NM</td>
</tr>
</tbody>
</table>
Bafilomycin was purchased from Cayman Chemical. DAPI nuclear stain was from Enzo Life Sciences. Acridine Orange was from Invitrogen. Cell culture medium was purchased from Lonza.

Fibroblasts derived from LINCL patients (GM16486) were obtained from Coriell Cell Repositories. HeLa cells stably transfected for the expression of TFEB-3xFLAG were a generous gift of Dr. Sardiello (42). Cells were grown at 37°C in 5% CO₂ in Dulbecco’s Modified Eagle Medium supplemented with heat-inactivated fetal bovine serum (20% FBS for LINCL fibroblasts and 10% FBS for HeLa cells) and 1% glutamine Pen-Strep. The medium was replaced every 3 or 4 days and monolayers were passaged with TrypLE Express.

PC12 cells were from ATCC. Cells were grown at 37°C in 5% CO₂ in Dulbecco’s Modified Eagle Medium supplemented with 5% heat-inactivated fetal bovine serum, 10% horse serum and 1% glutamine Pen-Strep.
5.4.2. Characterization of polystyrene nanoparticles

Surface charges of polystyrene nanoparticles were analyzed by zeta potential using a ZetaPALS system by Brookhaven Instruments (Holtsville, NY).

5.4.3. Cellular uptake of polystyrene nanoparticles

The absolute fluorescence intensity of fluorescently labeled polystyrene nanoparticles was evaluated by measuring the fluorescence of solutions containing nanoparticles ranging from 1 to 100 ppm using a SpectraMax Gemini plate reader (Molecular Device) (excitation 488 nm, emission 530 nm). The fluorescence intensity per unit of nanoparticle concentration was obtained by calculating the slope fitting the fluorescence intensity by nanoparticle concentration. Nanoparticle uptake was evaluated as previously reported (204). Briefly, cells \((10^4)\) were plated in each well of 96-well plates and incubated overnight to allow cell attachment. Cells were then exposed to fluorescent polystyrene nanoparticles for 24 hr, washed three times with PBS to remove membrane-binding nanoparticles, and lysed with the complete lysis-M buffer containing the protease inhibitor cocktail (Roche). The fluorescence intensity of the cell lysate was quantified using a SpectraMax Gemini plate reader (Molecular Device) (excitation 488 nm, emission 530 nm). To calculate the intracellular concentration of polystyrene nanoparticles, the fluorescence intensity of cell lysates was normalized to the absolute fluorescence of each corresponding nanoparticle.
5.4.4. Immunofluorescence assays

Cells were seeded on glass coverslips and cultured in the presence of polystyrene nanoparticles as indicated in each experiment. Immunofluorescence studies were conducted as described in Section 3.4.4. Cells were incubated with primary antibodies and secondary antibodies as described in Section 4.4.3. Images were obtained using an Olympus IX81 confocal microscope and co-localized using the Fluoview software as described in Section 3.4.4.

The percentage of TFEB nuclear localization was calculated by dividing the fluorescence intensity of TFEB that localizes in the nucleus by the total fluorescence intensity of TFEB in each cell, measured using MATLAB. The average fraction of TFEB that localizes in the nucleus was calculated over 30 images each containing approximately 50 cells.

The accumulation of ceroid lipopigment was quantified as described in Section 4.4.6.

Colocalization of LC3 and LAMP-2 was quantified by calculating the number of pixels presenting brightness (0 - 255 grey scale) of red and blue above a predefined threshold (grey scale > 30) and with ratio of red to blue within a predefined range (0.5 - 2). The percentage of colocalization was calculated by normalizing the number of pixels presenting LC3 and LAMP-2 colocalization by the total number of pixels in each cell over the entire image. Average values were calculated over multiple images and replicate samples.
5.4.5. Cell viability assays

Cell viability assay was performed using LIVE/DEAD® Viability/Cytotoxicity Kit (Molecular Probes). Cells were seeded on glass coverslips and cultured in the presence of polystyrene nanoparticles for 24 hr. Cells were then washed three times with PBS and incubated with 2 μM calcein AM and 4 μM ethidium homodimer (EthD-1) according to the manufacturer’s instructions (Invitrogen). Images were obtained using an Olympus IX81 confocal microscope and co-localized using the Fluoview software.

5.4.6. Lysosomal integrity assays

Lysosomal integrity assay was conducted using acridine orange (AO) as previously described (127). Cells were incubated with polystyrene nanoparticles for 24 hr and then incubated with 1 μg/mL acridine orange (Molecular Probes) for 15 min at 37°C. Cells were then collected, washed with PBS and analyzed by flow cytometry (FACSCanto™ II, BeckonDickinson) with a 488-nm Argon laser and a 670 nm emission filter. The cell population presenting low AO fluorescence in each experiment was defined by setting the gate boundary to include 2% of the untreated cells presenting the lowest AO fluorescence intensity in the AO histogram. To determine the percentage of cells presenting low AO fluorescence, each sample was analyzed using the gate defined from the analysis of the corresponding untreated cell control sample.
5.4.7. Statistical analyses

All data is presented as mean ± s.d., and statistical significance was calculated using a two-tailed t-test.
Ceria Nanoparticles Stabilized by Organic Surface Coatings Activate the Lysosome-Autophagy System and Enhance Autophagic Clearance

6.1. Introduction

Cerium oxide nanoparticles (nanoceria) are used in an increasingly diverse number of applications. The optical properties and ability to filter ultraviolet rays make nanoceria a highly promising material for applications such as UV blockers and filters (137). Nanosized ceria are also used as catalyst or co-catalyst in a number of reactions, such as water-gas shift, due to its high oxygen storage capacity and high activity in redox reactions (138,139). Oxygen vacancies are rapidly formed and eliminated by redox cycling between cerium (III) and cerium (IV) (205-207), generating large clusters of Ce$^{3+}$ ion groups exposed to gas reactants that can
participate in a range of chemical reactions on the surface of CeO$_2$ (208-212). It was recently shown that CeO$_2$ nanoparticles can enhance the combustion of the soot collected on the diesel particulate filters, thus enhancing fuel efficiency and reducing the exhaust emissions when added to diesel fuels (213-215).

The expanding commercial scale production and use of CeO$_2$ nanoparticles have inevitably increased the risk of release of nanoceria to the environment as well as the risk of human exposure. Much of the concern associated with the use of nanoceria as industrial catalyst in fact is related to the potential effects not only on the environment (216) but also on human health (217-219). On the other hand, recent studies elucidated the antioxidant properties of CeO$_2$ nanocrystals, which were reported to scavenge free radicals or reactive oxygen species (ROS) and protect cells from oxidative stress (220-223). The antioxidant properties of nanoceria were specifically investigated in light of potential therapeutic implications (224) and, particularly, neuroprotective function (225). Ceria nanoparticles were shown to function as radical scavengers and protect from radiation-induced cellular damage (226,227). Furthermore, nanoceria can prevent radical oxygen-induced retinal degeneration (228,229). Collectively, these findings indicate that nanoceria may provide a promising material for biomedical applications. Evidence of the potential cytotoxic impact of ceria nanoparticles, however, was also reported as CeO$_2$ nanoparticles were found to lower viability and reduce membrane integrity in human lung cancer cells (230). CeO$_2$ nanoparticles were also observed to affect cell viability by inducing activation of apoptosis and
autophagic cell death (219,231-233). However, the molecular mechanism involved in CeO$_2$-induced activation of these cellular pathways remains unclear.

Previous evidence suggests that ceria nanoparticles, similar to a number of rare earth oxide nanocrystals (122), may impact the autophagy system (231,232). However, the molecular mechanisms underlying upregulation of the autophagic response upon exposure to ceria nanoparticles are still unclear. In this study, I investigated the impact of ceria nanoparticles on the lysosome-autophagy system with the ultimate goal to understand whether ceria-induced upregulation of autophagy results in coordinated activation of lysosome and autophagosome formation and fusion and consequent increase in autophagic clearance or in blockage of autophagic flux.

To investigate the impact of ceria nanoparticles on the autophagy system, I tested a library of ceria nanoparticles of 4.3 ± 0.5 nm inner core coated with different types of biocompatible coatings: N-Acetylglucosamine (GlcNAc), polyethylene glycol (PEG) and polyvinylpyrrolidone (PVP). These surface coatings are commonly used in biological and pharmaceutical applications of nanomaterials. For instance, GlcNAc-decorated nanoparticles enhance uptake and delivery of therapeutic molecules both in vitro and in vivo (234,235). PEG is widely used to enhance biocompatibility of nanoparticles in biomedical applications (236) and improves brain tissue penetration of large nanoparticles through the brain blood barrier (237). PVP is a non-toxic, biocompatible material that prevents agglomeration and enhances the blood circulation time of nanoparticles (238),
commonly in MRI contrast agents and drug delivery systems (239,240). Importantly, the three surface coatings selected in this study represent both negative and neutral interfaces, which do not change acidification of the lysosomes or lysosomal integrity (133), which are known mechanism of nanoparticle-induced cytotoxicity (127,186). Negative and neutral interfaces were thus selected to avoid potential confounding factors and investigate the impact of nanoceria on the lysosome-autophagy system.

To investigate the molecular mechanism of autophagy induction by ceria nanoparticles, I used HeLa cells overexpressing TFEB (42). To investigate the outcome of ceria-mediated induction of autophagy and determine whether it results in enhancement of autophagic clearance, I used fibroblasts derived from a patient with late infantile neuronal ceroid lipofuscinosis (LINCL) that accumulate the autophagic substrate ceroid lipopigment, as described in Chapter 4 (80). I found that all differently functionalized ceria nanoparticles used in this study activate TFEB and enhance autophagic clearance of ceroid lipopigment in LINCL fibroblasts. This study provides a detailed mechanistic understanding of the impact of ceria nanoparticles on the autophagy system, providing evidence for the first time that cell exposure to nanoceria results in autophagy induction and enhanced clearance of toxic cellular waste.
6.2. Results

6.2.1. Synthesis and characterization of ceria nanoparticles

The synthesis and characterization of ceria nanoparticles were conducted by Dr. Seung Soo Lee (Rice University).

Nanocrystalline CeO$_2$ were synthesized using the high temperature cerium precursor decomposition method, resulting in nanocrystals with near spherical shape and narrow diameter distribution ($D = 4.3 \pm 0.5$ nm in organic solvents; Figure 6.1A) (241). CeO$_2$ nanocrystals capped with oleylamine were first dispersed in hexane due to their hydrophobicity and subsequently phase transferred into water by the ligand exchange method, using different phase transfer agents (GlcNAc, PEG and PVP). Phase-transferred, water soluble CeO$_2$ nanocrystals presented unaltered morphologies and were colloidally stable as shape and hydrodynamic diameters remained constant for more than six months (data not shown). Representative results obtained using GlcNAc coated CeO$_2$ (CeO$_2$-GlcNAc) are reported in Figure 6.1B and C: phase transferred CeO$_2$-GlcNAc displayed a 4.3 ± 0.5 nm diameter, as determined using transmission electron microscopy (TEM); and x-ray diffraction (XRD) analyses revealed a cubic fluorite nanocrystal structure. Functionalization with GlcNAc, PEG, and PVP resulted in compact surface coating as shown in Figure 6.1D. The hydrodynamic diameters of aqueous dispersion of CeO$_2$ nanocrystals were measured by dynamic light scattering (DLS) (Figure 6.1D). CeO$_2$-GlcNAc and CeO$_2$-PEG200 were found to have small hydrodynamic diameters (20.2
± 0.9 and 20.5 ± 0.8 nm, respectively) due to the low molecular weight of the surface coatings. High molecular weight PEG (PEG 1K and 10K) and PVP (PVP 10K and 40K) coatings resulted in a larger hydrodynamic diameter \( (\text{CeO}_2\text{-PEG1K}: 25.9 ± 0.5 \text{ nm}; \text{CeO}_2\text{-PEG10K}: 29.6 ± 0.7 \text{ nm}; \text{CeO}_2\text{-PVP10K}: 25.9 ± 0.4 \text{ nm}; \text{and CeO}_2\text{-PVP40K}: 32.8 ± 0.5 \text{ nm}) \), with the PVP surface coatings leading to a slightly smaller hydrodynamic diameter compared to the same molecular weight PEG surface coatings because of the wrapping surface structure formed by the PVP coating (compare 25.9 ± 0.4 nm CeO\(_2\)-PVP10K to 29.6 ± 0.7 nm CeO\(_2\)-PEG10K)\(^{(242)}\).

Measurements of the nanoparticles’ zeta potentials were conducted to characterize the surface charge of water-soluble ceria nanoparticles. All the surface coatings used resulted in negative to neutral charges \( (\text{CeO}_2\text{-GlcNAc}: -13.6 ± 1.5 \text{ mV}; \text{CeO}_2\text{-PEG200}: -2.6 ± 1.8 \text{ mV}; \text{CeO}_2\text{-PEG1K}: -2.0 ± 1.5 \text{ mV}; \text{CeO}_2\text{-PEG10K}: -4.4 ± 2.4 \text{ mV}; \text{CeO}_2\text{-PVP10K}: -5.3 ± 1.1 \text{ mV}; \text{CeO}_2\text{-PVP40K}: -6.8 ± 3.0 \text{ mV}) \), as expected \((243,244)\), which I hypothesized induce minimal changes on lysosome integrity and function \((133)\). GlcNAc-coated nanoparticles presented the most negatively charged surface due to the presence of acetyl groups and hydroxyl groups on the nanoparticle surface. PEG- and PVP-coated nanoparticles presented more neutral surface charges because neither the ether groups of the PEG coating nor the pyrrolidone groups of the PVP coating present a local negative charge.
Figure 6.1. Characterization of ceria nanoparticles. (A) TEM analyses of monodisperse nanocrystals phase transferred to ultrapure water using GlcNAc (scale bar, 20nm). (B) Size distribution of ceria nanoparticles determined by TEM (4.3 ± 0.5 nm). (C) Structure (cubic fluorite) of nanocrystalline cerium oxide verified by XRD; the diffraction patterns (black) match to the reference (JCPDS Card #34-0394 (vertical red lines)). (D) Hydrodynamic diameter of CeO$_2$ nanoparticles coated with GlcNac, PEG200, PEG1K, PVP10K, PEG10K, PVP40K determined by DLS analyses. Data are reported as mean ± SD ($n = 3$)
6.2.2. Ceria nanoparticles activate TFEB and upregulate cellular clearance networks

To investigate whether CeO$_2$ nanoparticles activate autophagy, I first monitored TFEB subcellular localization in HeLa/TFEB cells. Cells were treated with CeO$_2$ nanocrystals (100 ppm; 24 hr) and TFEB intracellular localization was evaluated by confocal microscopy using DAPI nuclear stain and an anti-FLAG antibody (Figure 6.2A). As expected, TFEB was barely detectable in the nucleus of untreated cells. Administration of CeO$_2$ nanoparticles, however, promoted TFEB nuclear localization in cells exposed to CeO$_2$ nanoparticles coated with GlcNAc, PEG and PVP (Figure 6.2A). TFEB nuclear localization was quantified by calculating the fraction of HeLa/TFEB cells exposed to CeO$_2$ nanoparticles that presented nuclear localization of TFEB (Figure 6.2B). TFEB nuclear localization was observed in 3.8 ± 2.8% of untreated HeLa/TFEB cells. The fraction of cells presenting TFEB nuclear localization was found to increase to over 50% of the total population after 24 hr of treatment with the library of CeO$_2$ nanoparticles (CeO$_2$-GlcNAc: 61.8 ± 5.0%; CeO$_2$-PEG200: 62.1 ± 4.5%; CeO$_2$-PEG1K: 62.7 ± 4.8%; CeO$_2$-PVP10K: 57.1 ± 2.4%; CeO$_2$-PEG10K: 56.1 ± 2.1%; CeO$_2$-PVP40K: 53.8 ± 3.5%) (Figure 6.2B; p<0.01), demonstrating that CeO$_2$ nanoparticles activate TFEB in HeLa/TFEB cells.
Figure 6.2. Ceria nanoparticles promote TFEB activation in HeLa/TFEB cells. (A) Confocal microscopy analyses of TFEB subcellular localization in HeLa/TFEB cells treated with 100 ppm CeO₂ nanoparticles for 24 hr. Colocalization of DAPI (blue) and TFEB-3xFLAG (red) is shown in merged images (purple). Representative images are reported. UT, untreated. The scale bar is 10 μm. (B) Percentage of cells with nuclear localization of TFEB-3xFLAG upon nanoceria treatment. Representative fields containing 50 to 100 cells were analyzed to calculate the percentage of cells with TFEB-3xFLAG nuclear localization in HeLa/TFEB cells (p<0.01). UT, untreated. Data are reported as mean ± SD (n=3).

To test whether nanoceria-induced nuclear translocation of TFEB results in upregulation of the lysosome system, which is observed upon TFEB activation (42),
I monitored the expression of representative genes of the CLEAR network upon exposure to CeO$_2$ nanoparticles. HeLa/TFEB cells were treated with CeO$_2$ nanoparticles (100 ppm; 72 hr) and the mRNA expression levels of TFEB targets were monitored by quantitative RT-PCR (qRT-PCR) (Figure 6.3A). I found that CeO$_2$ nanoparticles coated with GlcNAc, PEG, and PVP caused significant upregulation of TFEB targets, namely, $GBA$ (Glucocerebrosidase; between 2.1- and 3.6-fold), $HEXA$ (Hexosaminidase A; between 2.3- and 4.6-fold), and $LAMP1$ (Lysosome-associated membrane glycoprotein 1; between 2.2- and 4.1-fold) (Figure 6.3A; p<0.05).

To investigate whether CeO$_2$ nanoparticle-induced activation of TFEB also results in upregulation of the autophagy system, I tested the expression of representative genes involved in different steps of the autophagic pathway. Cells were treated with CeO$_2$ nanoparticles (100 ppm; 72 hr), and mRNA levels were tested by quantitative RT-PCR. I detected upregulation of $MAPLC3$, (microtubule-associated light chain protein 3; between 1.4- and 4.0-fold), which is essential for the formation of autophagic vesicles; $SQSTM1$ (p62; between 2.1- and 3.6-fold), which is essential for cargo recognition; and $BECN1$ (Beclin-1; between 2.0- and 3.0-fold), which is required for the formation of autophagosomes (Figure 6.3B; p<0.05). Interestingly, $MAPLC3B$ and $SQSTM1$ are known to be direct targets of TFEB (44,46).
Figure 6.3. Ceria nanoparticles upregulate genes of the lysosome-autophagy system in HeLa/TFEB cells. (A) Relative mRNA expression levels of representative genes of the lysosome system in HeLa/TFEB cells treated with 100 ppm CeO$_2$ nanoparticles for 24 hr. mRNA expression levels of $GBA$, $HEXA$, $LAMP1$ were obtained by qRT-PCR, corrected by the expression of the house-keeping gene $GAPDH$ and $ACTB$, and normalized to those of untreated cells (dashed line). Data are reported as mean ± SD ($n$=3; $p<0.05$). (B) Relative mRNA expression levels of representative genes of the autophagy system in HeLa/TFEB cells treated with 100 ppm CeO$_2$ nanoparticles for 24 hr. mRNA expression levels of $MAPLC3B$, $SQSTM1$, and $BECN1$ were obtained as described in (A). Data are reported as mean ± SD ($n$=3; $p<0.05$).
Collectively, these results indicate that cell exposure to CeO\(_2\) nanoparticles results in activation of TFEB and transcriptional upregulation of genes involved in autophagy-mediated cellular clearance.

To quantify cellular uptake of CeO\(_2\) nanoparticles used in this study and, particularly, to determine whether the efficiency of cellular uptake affects activation of TFEB and autophagy, I measured the extent of CeO\(_2\) nanoparticle internalization in HeLa/TFEB cells using quantitative inductively coupled plasma mass spectrometry (ICP-MS). Cell growth was not affected by treatment with CeO\(_2\) nanoparticles (100 ppm; 24 hr), as determined by cell count (data not shown). Cells were washed with PBS and incubated with acidic solution to remove membrane-binding nanocrystals and allow accurate measurement of intracellular nanocrystal concentration. The concentration of cerium atoms was measured by ICP-MS and the number of CeO\(_2\) nanoparticles internalized by the cells was calculated as described in the Materials and Methods. The six differently functionalized CeO\(_2\) nanocrystals were observed to have comparable internalization efficiency, suggesting that the different surface coatings used in this study (GlcNAc, PEG 1K and 10K, and PVP 10K and 40K) have similar effect on the cellular uptake of CeO\(_2\) nanocrystals (Figure 6.4).
**Figure 6.4. Ceria nanoparticle uptake in HeLa/TFEB cells.** The intracellular concentration of CeO$_2$ nanoparticles in HeLa/TFEB cells was quantified by ICP-MS after 24 hr incubation with CeO$_2$ nanoparticles (100 ppm). Data are reported as mean ± SD ($n=3$; p<0.05).

### 6.2.3. Ceria nanoparticles activate the lysosome-autophagy system

Previous studies suggest that activation of autophagy is associated with apoptosis induction (167-169). To investigate whether cell treatment with CeO$_2$ nanoparticles, under conditions that result in activation of TFEB and transcriptional upregulation of the lysosome-autophagy system, affects the apoptotic pathway, I tested induction of early and late apoptosis in HeLa/TFEB cells exposed to CeO$_2$ nanoparticles (100 ppm; 24 hr) using the Cyto-GLO™ annexin V-FITC apoptosis detection kit (74). None of the CeO$_2$ nanoparticles tested caused a considerable apoptotic response compared to untreated cells (FITC binding affinity, p<0.01; PI binding population, p<0.01; Figure 6.5A-B). Taxol (50 nM) was used here as positive control (182). Minimal changes in induction of early apoptosis and no
significant changes in induction of late apoptosis were observed after prolonged exposure to the differently functionalized ceria nanoparticles under the same conditions (Figure 6.5C-D). To confirm that CeO$_2$ nanoparticles used in this study do not induce cytotoxicity under the conditions resulting in autophagy activation, cell viability and cytotoxicity was measured using the LIVE/DEAD® Viability/Cytotoxicity assay. Cells were treated with CeO$_2$ nanocrystals (100 ppm; 24 hr) and cell viability was evaluated by confocal microscopy. None of the six differently surface functionalized CeO$_2$ nanoparticles caused cell death under the conditions used in this study (Figure 6.5E).

These results suggest that the CeO$_2$ nanoparticles used herein presenting various surface coatings and hydrodynamic diameters (Figure 6.1D) do not induce activation of apoptosis and cytotoxicity in HeLa/TFEB cells under conditions that result in transcriptional upregulation of autophagy, in agreement with previously reported evidence of low acute cytotoxicity observed under similar conditions (223,245).
Figure 6.5. Ceria nanoparticles do not induce activation of apoptosis and cytotoxicity in HeLa/TFEB cells. (A) Annexin V binding affinity change (%) in HeLa/TFEB cells treated with CeO$_2$ nanoparticles (100 ppm) or taxol (50 nM) for 24 hr normalized to untreated cells (p<0.01). (B) Propidium iodide (PI) binding population change (%) of cells treated with CeO$_2$ nanoparticles (100 ppm) or taxol.
(50 nM) for 24 hr normalized to untreated cells (p<0.01). (C) Annexin V binding affinity change (%) and (D) PI binding population change (%) in HeLa/TFEB cells treated with CeO$_2$ nanoparticles (100 ppm) or taxol (50 nM) for 48 hr normalized to untreated cells (p<0.01). The number of cells analyzed in each experiment was 10,000. The data are reported as the mean ± SD (n=3). (E) Confocal microscopy analyses of calcein (green) and EthD-1 binding (red) in HeLa/TFEB cells treated with CeO$_2$ nanocrystals (100 ppm) for 24 hr.

Previous studies suggest that depending on the material’s charge and composition, nanoparticles may cause impairment of lysosomal function (refer to Chapter 1 for a detailed description) (125-127). Under these conditions, transcriptional upregulation of autophagy genes and increased formation of autophagosomes is ultimately associated with decreased autophagic clearance (125). To investigate the impact of CeO$_2$ nanoparticles on lysosomal function, I quantified the lysosomal degradation capacity of HeLa/TFEB cells treated with CeO$_2$ nanoparticles. Specifically, HeLa/TFEB cells treated with CeO$_2$ nanoparticles (100 ppm; 24 hr) were labeled with the fluorogenic substrate DQ Green BSA, a bovine serum albumin derivative conjugated to the self-quenched dye BODIPY® (246). Proteolysis of DQ-BSA by lysosomal hydrolases results in release of the fluorescent product BODIPY. Therefore, cell fluorescence intensity in this assay is an indication of lysosomal activity. Cells treated with CeO$_2$ nanocrystals displayed fluorescence intensity comparable to or higher than untreated cells, indicating that none of CeO$_2$ nanoparticles impairs lysosomal function (Figure 6.6A). The lysosomal inhibitor chloroquine (CQ; 10 and 20 µM) (247) was used as a control in this study and resulted in decreased cell fluorescence in a concentration-dependent fashion. These
results confirm that cell treatment with CeO$_2$ nanocrystals, under conditions that result in upregulation of the gene network underlying the lysosome-autophagy system, do not impair lysosomal function, which is necessary for productive clearance of autophagic material.

**Figure 6.6. Ceria nanoparticles activate the lysosome-autophagy system.** (A) Lysosomal activity of HeLa/TFEB cells treated with CeO$_2$ nanoparticles (100 ppm) or chloroquine (10 µM and 20 µM) for 24 hr evaluated using the fluorogenic substrate DQ Green BSA. Data are reported as mean ± SD (n=3). UT, untreated; CQ, chloroquine. (B) Western blot analyses of LC3 isoforms (cytoplasmic LC3-I and autophagosome-associated LC3-II) in HeLa/TFEB cells treated with CeO$_2$ nanoparticles (100 ppm) or chloroquine (10 µM) for 24 hr, and detected using an LC3 antibody. GAPDH served as loading control. UT, untreated; CQ, chloroquine.
To investigate whether TFEB activation and upregulation of autophagy genes upon exposure to CeO$_2$ nanoparticles results in altered formation of autophagosomes, I monitored the processing of LC3 (248). LC3 isoforms, namely cytoplasmic LC3-I and autophagosome-associated LC3-II, were analyzed by immunoblotting using an LC3-specific antibody. The increase in LC3-II in cells treated with HPβCD compared to untreated cells suggests increased formation of autophagic vesicles. The amount of LC3-II in HeLa/TFEB cells was found to increase upon exposure to CeO$_2$ nanoparticles (100 ppm; 24 hr), indicating that treatment with CeO$_2$ nanocrystals enhances the formation of autophagic vesicles (Figure 6.6B).

Taken together, these results confirm that cellular uptake of CeO$_2$ nanoparticles results in upregulation of the lysosome-autophagy system and does not cause impairment of lysosomal function and consequent activation of autophagy-associated cell death.

6.2.4. Ceria nanoparticles promote autophagic clearance of ceroid lipopigment in LINCL fibroblasts

To verify that cell exposure to CeO$_2$ nanoparticles under conditions that result in activation of TFEB and upregulation of autophagy leads to enhanced cellular clearance, I used fibroblasts derived from a patient with LINCL and monitored the accumulation of ceroid lipopigment (80) upon treatment with ceria nanoparticles. LINCL fibroblasts were incubated with the different types of CeO$_2$
nanoparticles (100 ppm for 3 days). The autofluorescence of ceroid lipopigment was monitored using confocal microscopy. The accumulation of autofluorescent material in cells exposed to nanoparticles was quantified (Figure 6.7A). The fluorescence intensity of ceroid lipopigment in LINCL fibroblasts decreased to approximately 50 to 60% of that of untreated cells after treatment with CeO$_2$ nanoparticles (CeO$_2$-GlcNAc: $51.2 \pm 11.5\%$; CeO$_2$-PEG200: $63.2 \pm 10.2\%$; CeO$_2$-PEG1K: $59.6 \pm 12.9\%$; CeO$_2$-PVP10K: $66.0 \pm 10.9\%$; CeO$_2$-PEG10K: $68.0 \pm 11.9\%$; CeO$_2$-PVP40K: $64.5 \pm 13.1\%$; Figure 6.7A; p<0.01), indicating that cell treatment with CeO$_2$ nanoparticles promotes clearance of ceroid lipopigment. Representative confocal microscopy images of cells treated with ceria nanoparticles are reported in Figure 6.7B.

To confirm that treatment of LINCL cells with CeO$_2$ nanoparticles, under conditions that result in clearance of ceroid lipopigment, induce TFEB activation, I also evaluated TFEB subcellular localization. Partial nuclear localization of TFEB was observed in untreated LINCL fibroblasts, likely due to lipopigment-induced lysosomal stress (42). Administration of CeO$_2$ nanoparticles resulted in an increase of TFEB nuclear localization in LINCL fibroblasts (Figure 6.7B, red), confirming that CeO$_2$-induced clearance of ceroid lipopigment parallels activation of TFEB.
Figure 6.7. Ceria nanoparticles promote TFEB activation and clearance of ceroid lipopigment in LINCL fibroblasts. (A) Confocal microscopy analyses of ceroid lipopigment and TFEB in LINCL fibroblasts treated with CeO$_2$ nanoparticles (100 ppm for 3 days). The fluorescence intensity of ceroid lipopigment was quantified as described in the Materials and Methods. Data are reported as mean ± SD (n=15; $p<0.01$). (B) Representative confocal microscopy images of ceroid lipopigment (green) and TFEB (red) in LINCL fibroblasts upon ceria nanoparticle treatment (100 ppm for 3 days). The scale bar is 20 μm.
Figure 6.8. Ceria nanoparticles upregulate genes of the lysosome-autophagy system in LINCL fibroblasts. [A] Relative mRNA expression levels of representative genes of the lysosome system in LINCL fibroblasts treated with 100 ppm CeO$_2$ nanoparticles for 3 days. mRNA expression levels of GBA, HEXA, LAMP1 were obtained as described in Figure 6.3A. Data are reported as mean ± SD (n=3; p<0.05). [B] Relative mRNA expression levels of representative genes of the autophagy system in LINCL fibroblasts treated with 100 ppm CeO$_2$ nanoparticles for 3 days. mRNA expression levels of MAPLC3B, SQSTM1, and BECN1 were obtained as described in Figure 6.3A. Data are reported as mean ± SD (n=3; p<0.05).
To confirm that nanoceria-induced nuclear translocation of TFEB in LINCL fibroblasts results in upregulation of genes involved in lysosomal biogenesis and function, I tested the expression of representative genes of the CLEAR network in LINCL cells treated with CeO$_2$ nanoparticles (100 ppm; 72 hr), as described above. I found CeO$_2$ nanoparticle treatment to cause significant upregulation of $GBA$ (from 1.5- to 2.8-fold), $HEXA$ (from 1.5- to 2.7-fold), and $LAMP1$ (from 1.5- to 2.7-fold) (Figure 6.8A; $p<0.05$). To test whether the autophagy system was also transcriptionally activated, I measured the expression of genes of the autophagic pathway. I detected significant upregulation of $MAPLC3$ (from 2.7- to 4.4-fold), $SQSTM1$ (from 1.8- to 2.9-fold), and $BECN1$ (from 2.2- to 3.7-fold) (Figure 6.8B; $p<0.05$). Taken together, these results demonstrate that TFEB activation and upregulation of genes of the lysosome-autophagy system parallels the reduced accumulation of ceroid lipopigment in cells exposed to CeO$_2$ nanoparticles.

To confirm enhancement of autophagic flux in cells presenting reduced accumulation of ceroid lipopigment, I tested the extent to which cell treatment with CeO$_2$ nanoparticles results in autophagosome-lysosome fusion and formation of autophagolysosomes, which is necessary for clearance of cargo (124). To this end, I evaluated the colocalization of LC3 and LAMP-2 as an indication of autophagosome-lysosome fusion. LINCL fibroblasts were treated with the library of CeO$_2$ nanoparticles (100 ppm; 3 days). Accumulation of ceroid lipopigment was also monitored by detecting cell autofluorescence using confocal microscopy (Figure 6.9A, green). Colocalization of LC3 (red) and LAMP-2 (blue) is shown in merged
images (purple). Treatment with CeO$_2$ nanoparticles resulted in enhanced formation of autophagosomes as indicated by the appearance of punctate LC3 structures. Colocalization of LC3 and LAMP-2 was quantified as described in Chapter 5 and average values were calculated over multiple images and replicate samples (Figure 6.9B). I observed up to 3-fold increase in the extent of colocalization of LC3 and LAMP-2 in LINCL fibroblasts treated with CeO$_2$ nanoparticles compared to untreated cells (untreated: 8.6 ± 3.7%; CeO$_2$-GlcNAc: 18.8 ± 2.0%, CeO$_2$-PEG200: 18.7 ± 2.6%; CeO$_2$-PEG1K: 18.8 ± 2.1%; CeO$_2$-PVP10K: 23.8 ± 2.5%; CeO$_2$-PEG10K: 20.9 ± 2.6%; CeO$_2$-PVP40K: 25.7 ± 1.2%) (Figure 6.9B; p<0.01), confirming that CeO$_2$ nanoparticles promote fusion of autophagosomes and lysosomes and formation of autophagolysosomes.

Intracellular localization of CeO$_2$ nanoparticles in LINCL fibroblasts was verified by TEM analyses of representative CeO$_2$ nanoparticles used in this study (CeO$_2$-PEG200 and CeO$_2$-PEG1K). TEM images revealed that the CeO$_2$ nanoparticles localize either in the cytoplasm or in autophagic vesicles in LINCL fibroblasts (Figure 6.10). Interestingly, ceria nanoparticles appeared to form aggregates both in the cytoplasm and in autophagic vesicles, most likely due to the high ionic strength conditions of the intracellular environment, which is conducive to nanoparticle aggregation (129,203). While the intracellular fate of CeO$_2$ nanoparticles remains to be determined and, particularly, whether cytoplasmic localization occurs upon endosomal escape and whether it precedes autophagosome localization, evidence of
CeO$_2$-PEG200 and CeO$_2$-PEG1K sequestration in double membrane autophagic vesicles confirms the interaction of nanoceria with the autophagy system.

Figure 6.9. Ceria nanoparticles induced clearance of ceroid lipopigment parallels activation of autophagy in LINCL fibroblasts. (A) Confocal microscopy analyses of ceroid lipopigment (green), LC3 (red), and LAMP-2 (blue) in LINCL fibroblasts treated with 100 ppm CeO$_2$ nanoparticles for 3 days, evaluated by
detecting green autofluorescence, binding of anti-LC3 antibody, and binding of anti-LAMP-2 antibody, respectively. Colocalization of LC3 and LAMP-2 is shown in merged images (purple). The scale bar is 20 μm. **(B)** Quantification of LC3-LAMP-2 colocalization. Data are reported as mean ± SD (n=15; p<0.01).

**Figure 6.10. Ceria nanoparticles localize in the cytoplasm and in autophagic vesicles in LINCL cells.** TEM analyses of LINCL fibroblasts treated with **(A)** CeO$_2$-PEG200 and **(B)** CeO$_2$-PEG1K. Representative images of nanoparticles accumulation in the cytoplasm (left) and in autophagic vesicles (right). Scale bar is 500 nm.

In summary, these results demonstrate that cell exposure to CeO$_2$ nanoparticles induces upregulation of the autophagy system, enhances autophagic
activity, and promotes clearance of the autophagic substrate ceroid lipopigment in a model of neuronal ceroid lipofuscinosis.

6.3. Discussion

In this study, I investigated the response of the lysosome-autophagy system to exposure to ceria nanoparticles. I report that ceria nanoparticles activate TFEB, thereby inducing upregulation of the pathway at the transcriptional level. Moreover, CeO$_2$ nanoparticles were found to activate autophagy without inducing cytotoxicity or apoptosis and promote clearance of toxic aggregates in disease cells that have an inefficient lysosomal system.

Recent studies suggest that lysosomal impairment may be associated with the mechanism of nanoparticle-induced cytotoxicity. Nanoparticles presenting cationic surfaces were specifically reported to disrupt lysosomes by inducing a "proton sponge" effect (127) (refer to Chapter 1 and Chapter 5 for a detailed description). It was suggested that some nanomaterials once internalized by cells induce enhanced formation of autophagosomes, but also impair lysosome function, thereby disrupting the autphagic flux (125,249,250). CeO$_2$ nanoparticles do not impair lysosomes, but promote clearance, thus ultimately resulting in enhanced degradation of autophagic cargo. This study provides a mechanistic understanding of the interaction of ceria nanoparticles with this important cellular catabolic
pathway, thus furthering our understanding of the environmental impact of ceria nanoparticles and laying the foundation for the design of nanotherapeutics for the treatment of diseases characterized by inefficient autophagic activity and accumulation of storage material.

The fate of the ceria nanoparticles upon activation of the autophagy system and upregulation of autophagic clearance remains to be determined. In vitro studies revealed that metal nanoparticles including gold, superparamagnetic iron oxide, and quantum dots, are removed from cells through exocytosis (251,252). Exocytosis of autolysosomes was previously reported as a mechanism of TFEB-mediated clearance (45), and it is thus likely to be induced by nanoparticle autophagy activators, such as the six differently surface functionalized CeO$_2$ nanoparticles analyzed in this study.

In this study, I demonstrated that nanoceria promotes clearance of ceroid lipopigment deposits in cells derived from a patient affected by LINCL, which is characterized by a defective lysosome-autophagy system. Although it remains to be demonstrated whether CeO$_2$ nanoparticles enhance clearance of other autophagic cargos, results from this study provide proof of principle evidence of the use of nanomaterials as a platform for the development of nanotherapeutics that enhance TFEB-mediated activation of autophagy, with tremendous implications for the treatment of diseases characterized by storage of autophagic substrates.
6.4. Materials and Methods

6.4.1. Reagents and cell cultures

Cerium nitrate hexahydrate (Ce(NO$_3$)$_3$·6H$_2$O), oleylamine, 1-octadecene, N-acetyl-D-glucosamine (GlcNAc), Polyethylene glycol (PEG 200, PEG 1K, PEG 10K), Polyvinylpyrrolidone (PVP 10K and PVP 40K) were purchased from Sigma-Aldrich. Hexane, acetone, and ethanol were purchased from Fisher Scientific.

Cell culture medium was purchased from Lonza. Chloroquine (CQ) was from Sigma-Aldrich. DQ Green BSA was from Invitrogen.

Fibroblasts derived from patients with late infantile neuronal ceroid lipofuscinosis (LINCL) were obtained from Coriell Cell Repositories (GM16486). HeLa cells stably transfected for the expression of TFEB-3xFLAG were a generous gift of Dr. Sardiello (42). Cells were grown at 37°C in 5% CO$_2$ in Dulbecco’s Modified Eagle Medium, supplemented with heat-inactivated fetal bovine serum (20% FBS for LINCL fibroblasts and 10% FBS for Hela cells) and 1% glutamine Pen-Strep. The medium was replaced every 3 or 4 days and monolayers were passaged with TrypLE Express.

6.4.2. Synthesis of monodisperse ceria nanocrystals

Nanocrystalline cerium oxides were synthesized using the cerium precursor decomposition method at high temperature (241). Cerium nitrate hexahydrate (10
mmol, 4.34 g) was mixed with oleylamine (30 mmol, 8.02 g) in 1-octadecene (120 mmol, 30 g) at room temperature. The reaction mixture was stirred vigorously at 80°C, forming yellow cerium-oleylamine mixture. This cerium precursor was decomposed at 260°C, and near-spherical ceria nanocrystals with narrow diameter distribution (σ < 10%) were prepared after 2 hr reaction at 260 °C under ultrahigh purity nitrogen. The resulting dark brown ceria nanocrystals were purified by addition of 25 ml of ethanol and precipitated by adding 25 ml of acetone. The precipitate was separated from unreacted cerium precursor, oleylamine, and 1-octadecene by centrifugation at 6000 rpm for 30 min. The purification process was repeated 3 times, and purified ceria nanocrystals were redispersed in hexane.

### 6.4.3. Phase transfer of ceria nanocrystals

Water soluble nanocrystals were produced using various phase transfer agents (GlcNAc, PEG 200, PEG 1K, PEG 10K, PVP 10K, and PVP 40K). Specifically, 1 ml of ceria nanocrystal/hexane solution ([Ce] = 6820 mg/L) was mixed with the phase transfer agent (GlcNAc (1.8 mg), PEG 200 (1.2 mg), PEG 1K (1.0 mg), PEG 10K (1.0 mg), PVP 10K (0.05 mg), or PVP 40K (0.08 mg)) in 15 ml of ultra-pure water (Millipore, 18.2 MΩ·cm). This suspension was then sonicated using a probe-sonicator (Hielscher UP50H) for 20 min at 80 % amplitude and full cycle. The phase-transferred nanocrystals were purified by ultracentrifugation at 45,000 rpm for 4 hr and by membrane filtration (Ultrafiltration cellulose membranes, 30 kDa MWCO)
using a stirred Cell (Amicon) to remove the excess of free phase transfer agents left in the supernatant. The purification was repeated 3 times, and phase-transferred ceria nanocrystals were stored in ultrapure water.

6.4.4. Characterization of ceria nanocrystals

TEM specimens were prepared by dropping the nanocrystal solution on ultra-thin carbon type A 400 mesh copper grids (Ted Pella Inc.). The TEM micrographs were taken on a JEOL 2100 field emission gun TEM operated at 200 kV with a single tilt holder. Inductively coupled plasma atomic emission spectroscopy (ICP-AES) was used to measure the concentration of ceria nanocrystals. ICP-AES analyses were carried out using a Perkin Elmer instrument equipped with autosampler. Hydrodynamic radius and surface charge of water soluble ceria nanocrystals were analyzed by DLS and zeta potential using a Malvern Nano ZS system by Malvern Instruments (Malvern Zetasizer Nanoseries, Malvern, UK).

6.4.5. Cellular uptake of ceria nanocrystals

Cells ($10^5$) were plated in each well of 6-well plates and incubated overnight to allow cell attachment. Cells were then exposed to ceria nanoparticles for 24 hr, washed with PBS, and incubated with acidic solution (50 mM glycine, 100 mM sodium chloride, 2 mg/ml polyvinylpyrrolidone (MW: 40K, pH 3) to remove
membrane-binding nanocrystals as previously described (253). After acid strip, cells were washed with DI water, collected and digested with 1% HNO₃. The amount of ceria nanoparticles internalized by the cells was quantified using inductively coupled plasma mass spectrometry (ICP-MS; Perkin Elmer ELAN9000): the concentration of cerium atoms in each well was measured and the number of ceria nanoparticles was calculated based on the number of atoms in each particle, as previously described (241).

To prepare TEM samples, LINCL fibroblasts were seeded in 6 well plates to 70-80% confluence, cultured in the presence of ceria nanoparticles for 24 hr, washed with PBS and fixed with 2% paraformaldehyde and 3% glutaraldehyde in 0.1 M PBS. After fixation, the samples were washed in 0.1 M cacodylate buffer and treated with 0.1% Millipore-filtered buffered tannic acid, postfixed with 1% buffered osmium tetroxide for 30 min, and stained en bloc with 1% Millipore-filtered uranyl acetate. The samples were washed several times in water, then dehydrated in increasing concentrations of ethanol, infiltrated, and embedded in LX-112 medium. The samples were polymerized in a 60°C oven for 2 days. Ultrathin sections were cut in a Leica Ultracut microtome (Leica, Deerfield, IL), stained with uranyl acetate and lead citrate in a Leica EM Stainer, and examined in a JEM 1010 transmission electron microscope (JEOL, USA, Inc., Peabody, MA) at an accelerating voltage of 80 kV. Digital images were obtained using AMT Imaging System (Advanced Microscopy Techniques Corp, Danvers, MA).
6.4.6. Immunofluorescence assays

Cells were seeded on glass coverslips and cultured in the presence of ceria nanoparticles as indicated in each experiment. Immunofluorescence studies were conducted as described in Section 3.4.4. Cells were incubated with primary antibodies and secondary antibodies as described in Section 4.4.3. Images were obtained using an Olympus IX81 confocal microscope and co-localized using the Fluoview software as described in Section 3.4.4.

The accumulation of ceroid lipopigment was quantified as described in Section 4.4.6.

Colocalization of LC3 and LAMP-2 was quantified as described in Section 5.4.4.

6.4.7. Quantitative RT-PCR

Cells were cultured with ceria nanoparticles for indicated time lengths. Quantitative RT-PCR (qRT-PCR) was conducted as described in Section 3.4.6 using corresponding primers reported in Table A.1.
6.4.8. Apoptosis assays

HeLa/TFEB cells were treated with ceria nanoparticles or Taxol for 24 hr and 48 hr at 37 °C. Induction of apoptosis was measured as described in Section 3.4.9.

6.4.9. DQ-BSA assays

DQ-BSA assay was conducted as previously described (125). Cells were incubated with ceria nanoparticles for 24 hr, washed with PBS, and then incubated in DMEM medium containing 10 μg/mL DQ Green BSA (Molecular Probes) for 3 hr at 37°C. Cells were then washed again with PBS and analyzed by flow cytometry (FACSCanto™ II, Beckon Dickingson) with a 488-nm Argon laser.

6.4.10. LC3 Western blot analyses

HeLa/TFEB cells were cultured in the presence of ceria nanoparticles or chloroquine for 24 hr. Western blot analyses were performed as described in Section 4.4.5.
6.4.11. Statistical analyses

All data is presented as mean ± s.d., and statistical significance was calculated using a two-tailed $t$-test.
Chapter 7

Summary and Future Directions

7.1. Summary

In this work, a number of chemical and biological strategies to modulate the lysosome-autophagy system were investigated. The ultimate goal was to restore cellular homeostasis in lysosomal storage disorders (LSDs), which are characterized by deficiencies in lysosomal hydrolytic enzymes and accumulation of undegraded lysosomal substrates (52,53). This goal was achieved by exploring strategies to i) rescue the folding and processing of unstable, degradation-prone lysosomal enzymes and restore lysosomal function, and ii) promote clearance of lysosomal substrates that accumulate due to inefficient lysosomal function, thereby restoring a physiologic ratio of lysosomal substrate to enzymatic activity. Specifically, I investigated the role of TFEB in regulating lysosomal proteostasis and autophagic clearance. To understand the role of TFEB in modulating lysosomal proteostasis, I
investigated chemical and genetic approaches to activate TFEB in cells derived from patients with LSDs harboring misfolding mutations in genes encoding lysosomal enzymes. TFEB activation was found to enhance folding, trafficking and activity of unstable, degradation-prone lysosomal enzymes associated with the development of Gaucher’s and Tay-Sachs (135). TFEB-mediated rescue of lysosomal enzyme activity depends on the simultaneous upregulation of the enzyme transcription and enhancement of the cellular folding and trafficking capacity (135). Results from this study demonstrate the role of TFEB as a target for modulating lysosomal proteostasis and potentially as a therapeutic target for the treatment of LSDs.

To investigate whether TFEB modulation activates autophagy and enhances autophagic clearance, I used cells derived from patients with LSDs characterized by accumulation of undegraded lysosomal substrates. Specifically, I used fibroblasts derived from a patient with late infantile neuronal ceroid lipofuscinosis (LINCL), which present accumulation of proteolipid aggregates (80). I demonstrated that HPβCD, a cyclic oligosaccharide known to activate autophagy (110), upregulates autophagic clearance by activating TFEB (136). Particularly, HPβCD treatment was found to promote clearance of ceroid lipopigment in LINCL fibroblasts (136).

To further investigate the molecular mechanism of autophagy induction and activation of autophagic clearance, I tested the impact of polystyrene nanoparticles of different size and surface charge on the lysosome-autophagy system. The goal of this study was to link the physicochemical properties of nanomaterial with the nature of autophagy response. I demonstrated that all polystyrene nanoparticles
investigated in this study activate autophagy and that autophagy induction is mediated by TFEB. However, activation of autophagic clearance depends on nanoparticle charge. Specifically, polystyrene nanoparticles with neutral and anionic surfaces activate autophagy without inducing cytotoxicity and promote clearance of ceroid lipopigment in LINCL fibroblasts. By contrast, cationic nanoparticle treatment results in impairment of lysosome integrity, reduced autophagolysosome formation and blockage of autophagic flux. This study furthers our understanding of the design rules for engineering nanoparticles with desired effect on the lysosome-autophagy system.

Nanoceria have found extensive industrial as well as biomedical applications due to its high oxygen storage capacity and high activity in redox reactions (138,139). The increasing production and use of nanoceria are potentially involving human exposure. Therefore, it is of particular interest to study biological impacts of ceria nanoparticles. Specifically, I investigated the impact of ceria nanoparticles on the lysosome-autophagy system using a battery of ceria nanoparticles functionalized with different biocompatible coatings. Each step of the autophagy pathway was evaluated upon nanoceria treatment. In this study, I demonstrated that ceria nanoparticles activate TFEB-mediated autophagy and enhance degradation of autophagic cargo. This study provides a mechanistic understanding of the interaction of ceria nanoparticles with this important cellular catabolic pathway, thus furthering our understanding of the environmental impact of ceria nanoparticles and laying the foundation for the design of nanotherapeutics for the
treatment of diseases characterized by inefficient autophagic activity and accumulation of storage material.

In summary, I developed a number of cell engineering strategies to modulate the lysosome-autophagy system. Specifically, I demonstrate that chemical and genetic modulation of TFEB enhances the capacity of the lysosome-autophagy system, thereby rescuing the folding, processing and activity of mutated lysosomal enzymes and promoting clearance of lysosomal substrates that accumulate due to inefficient lysosomal function. This study also provides a mechanistic understanding of the interaction of nanomaterials with the lysosome-autophagy system and will inform development of nanotherapeutics for the treatment of diseases associated with inefficient autophagic clearance.

7.2. Future Directions

HPβCD was recently approved for a clinical trial to evaluate its potential for the treatment of Niemann-Pick type C, a LSD characterized by aberrant storage of cholesterol (114,115). Results from this study may extend the capability of designing therapeutic solutions based on the use of HPβCD for the treatment of diseases characterized by inefficient autophagic clearance and accumulation of storage material. Testing the function of HPβCD in in vivo models of different LSDs would allow exploring whether HPβCD treatment ameliorates the phenotypes
associated with accumulation of different lysosomal substrate and evaluate HPβCD therapeutic potential for the treatment of multiple LSDs.

The rapid growth in production and use of nanoparticles in industrial and consumer products has led to an increased likelihood of human exposure and elevated concentration of nanoparticles in the environment. Thus, it is of critical importance to evaluate potential effects of nanomaterial on human health as well as on the environment. However, very little is currently known about the impact of nanomaterial on biological systems. Increasing evidence suggests a number of synthetic nanoparticles are sequestered into autophagic vesicles when internalized into cells (119-123). However, compartmentalization of nanoparticles into autophagosomes may cause autophagy dysfunction and accumulation of autophagosomes (125-127). In this study, I demonstrated that cell treatment with cationic polystyrene nanoparticles results in blockage of autophagic flux by impairing lysosomal integrity and fusion of autophagosomes with lysosomes. These results suggest nanoparticles surface charge play a critical role in activating autophagic degradation. Other physicochemical properties, such as nanoparticle composition, may also affect autophagy activation and efficient degradation of autophagic cargo. Gold nanoparticles, for instance, may impair lysosomal function, leading to accumulation of autophagosomes (125). Gold nanoparticles are the most extensively used nanomaterial with a variety of industrial and biomedical applications (254,255). However, the molecular mechanisms underlying activation of autophagosome formation and blockage of autophagic flux upon cell exposure to
gold nanoparticles are still unclear and should be investigated. I also recommend testing other nanomaterials that are extensively used in the industry or the biomedical field, such as carbon nanotubes (256-258), metal oxides nanoparticles (259,260), and quantum dots (261-263). Elucidating the interactions of these nanomaterials with the lysosome-autophagy system will be important to evaluate the potential impact of these nanomaterials on human health.
References


and cerium oxide nanoparticles based on dissolution and oxidative stress properties. *ACS Nano* **2**, 2121-2134


## Appendix

### Table A.1. Primer Sequences for Quantitative RT-PCR

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