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Neural Stem Cells as Model to Study Neurodegeneration and Novel Therapeutics in Lysosomal Storage Disorders

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Doctor of Philosophy, PhD

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Neural stem cells as model to study neurodegeneration

and novel therapeutics in lysosomal storage disorders
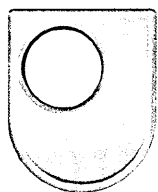
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TABLE OF CONTENTS

ABBREVIATIONS

AAV2/9	adeno-associated virus type 2/9
AD	Alzheimer's disease
ALS	Amyotrophic Lateral Sclerosis
ATG	autophagy-related proteins
BMP	bone morphogenetic proteins
CATD	cathepsin D
CDM6PR	cation-dependent M6PR
CGN	*cis*-Golgi network
CIM6PR	cation-independent M6PR
CLEAR	Coordinated Lysosomal Expression and Regulation
CLN3	Neuronal Ceroid Lipofuscinosls type 3
CMA	chaperone-mediated autophagy
CMV	cytomegalovirus
CNS	Central Nervous System
ECM	extracellular matrix
EGF	epidermal growth factor
ER	endoplasmic reticulum
ERT	enzyme replacement therapy
FGE	formylglycine-generating enzyme
FGF	fibroblast growth factor
Fz	Frizzled
GAG	glycosaminoglycans
GFAP	glial fibrillary acidic protein
GTPase	guanosine triphosphatases
HD	Huntington's disease
HDAC6	histone deacetylase 6

HEXB	hexosaminidase B
HGSNAT	heparan-α-glucosaminide N-acetyltransferase
HSCs	Hematopoietic Stem Cells
HSPGs	heparan sulfate proteoglycans
LAMP1	lysosome associated membrane protein 1
LAMP2	lysosome associated membrane protein 2
LAMP2A	lysosome-associated membrane protein 2
LC3 or MAP1LC3	microtubule-associated protein 1 (MAP1) light chain 3
LDH	lactate dehydrogenase
LIMP2	lysosome integral membrane protein 2
LMPs	lysosomal membrane proteins
LROs	lysosomal related organelles
LSD	Lysosomal Storage Disorder
M6P	mannose-6-phosphate
M6PR	mannose-6-phosphate receptor
MCOLN1	mucolipin 1
MHC	major histocompatibility complex
MLIV	mucolipidosis type IV
MPSII	mucopolysaccharidosis type II
MPSIIIA	mucopolysaccharidosis type III A
MPSIIIC	mucopolysaccharidosis type III C
MSD	Multiple Sulfatase Deficiency
MTOC	microtubule-organizing centre
mTOR	mammalian target of rapamycin
mTORC1	mTOR complex 1
NCL	neuronal ceroid lipofuscinosis
NEPs	neuroepithelial progenitors
NPC	Niemann-Pick disease

NPC1	Niemann-Pick disease type 1
NSCs	Neural Stem Cells
PD	Parkinson's disease
PM	Plasma Membrane
Ptau	hyperphosphorylated tau
RG	radial-glia
RMS	rostral migratory stream
SGZ	subgranular zone
Shh	sonic hedgehog
SNAP-25	synaptosomal-associated protein 25
SNARE	soluble N-ethylmaleimide-sensitive factor attachment protein receptor
SRT	substrate reduction therapy
SUMF1	sulfatase modifying factor 1
SVZ	subventricular zone
SYT7	synaptotagmin 7
TA	transient amplifying
TFEB	Transcription Factor EB
TGN	*trans*-Golgi network
TRP	transient receptor potential
TRPML	mucolipins
TTX	tetrodotoxin
Tuj1	class III β-tubulin
UBA	ubiquitin-binding domain
v-ATPase	vacuolar H^+-adenosine triphosphatases
Wnt	wingless-type MMTV integration site family

ABSTRACT

Neural Stem Cells (NSCs) are self-renewing multipotent populations responsible for the generation of neurons and glial cells of the developing brain and account for the limited regenerative plasticity of the adult brain. In view of their reliable use as cellular model to study neurodegenerative diseases, and as potential donors in cell-based therapeutic approaches, we have isolated and characterized NSCs from a mouse model of a neurodegenerative Lysosomal Storage Disorder (LSD), the Multiple Sulfatase Deficiency (MSD), caused by mutations in the sulfatase modifying factor 1 (SUMF1) gene that encodes the enzyme responsible for sulfatase activation.

Isolated MSD-NSCs are phenotypically similar to wild-type precursors and are able to differentiate into neurons and astrocytes, although they show a progressive loss of their self-renewal capacity. Moreover, differentiated MSD cells recapitulate the main pathological features of the disease, such as progressive cell vacuolization, lysosomal accumulation of glycosaminoglycans (GAGs), altered autophagy with accumulation of poly-ubiquitinated proteins, and increased levels of apoptosis. Interestingly, glia-differentiated MSD cells display the tendency to form aggresomes, perinuclear aggregates of misfolded protein, which is a common feature to many neurodegenerative diseases.

We also showed that the overexpression of the Transcription Factor EB (TFEB), a master-gene that modulate lysosomal function and autophagy, induces lysosomal exocytosis through activation of mucolipin 1 (MCOLN1) and reduces

4

significantly primary and secondary pathologic storage, ameliorating the phenotype of MSD cells (Medina et al. 2011).

These results validate the use of NSCs isolated from LSD mouse models to study their neurodegenerative phenotype, and envisage their use to explore new therapeutic approaches by the modulation of TFEB expression in LSDs.

INTRODUCTION

The lysosome

Lysosomes are cellular organelles present in all cell types, which are primarily involved in degradation and recycling processes (Kornfeld and Mellman 1989). The lysosome was first described by de Duve in 1955 as an acidic organelle containing a set of lysosomal enzymes (De Duve et al. 1955), whose function is the degradation of other cellular components. The first morphological demonstration of lysosomes by electron microscopy of organelles prepared from rat liver was presented by Alex Novikoff in 1956 (Novikoff, Beaufay, and De Duve 1956). Since these early days, much has been learned about lysosomes and related organelles, about their diverse functions, biogenesis and failures in disease.

According to its physiological function at different stages, lysosome can be divided into the primary lysosome, the secondary lysosome and the residual body (Zhang et al 2009). Primary lysosomes are membrane-bound intracellular organelles that contain a variety of hydrolytic enzymes; they fuse with membrane-bound vacuoles that contain material to be digested, forming secondary lysosomes. Once in the lumen of lysosomes, substrates are then degraded via a finely orchestrated network of soluble lysosomal hydrolases (also referred to as acid hydrolases), integral lysosomal membrane proteins (LMPs), lysosomal related organelles (LROs) and other cellular constituents (Saftig and Klumperman 2009).

After digestion occurred, secondary lysosomes become residual bodies, containing only indigestible or slowly digestible materials and within which enzymatic activities have become virtually exhausted (Zhang et al 2009).

Macromolecules are delivered toward lysosomes for degradation from the extracellular space through endocytosis or phagocytosis, as well as from the cytoplasm through autophagy (Doherty and McMahon 2009; Kornfeld 1986; Ravikumar et al. 2009). Due to their crucial function, lysosomes are involved in various cellular processes, such as cholesterol homeostasis (Lange at al. 1998), membrane repair (Reddy et al. 2001), bone and tissue remodelling (Chapman et al. 1994), pathogen defence (Kanai et al. 1970), signal transduction (Mandeville et al. 1996), cell division (Allison and Mallucci 1964), neurotransmission (Holtzman 1977) and cell death (Guicciardi et al. 2004). These complex functions highlight the fact that the lysosome is a central organelle which is much more than just the wastebasket of the cell (Saftig 2006).

Components of lysosomes

As De Duve initially observed, lysosomes are limited by a single phospholipid bilayer. Their shape varies between globular and tubular (Saftig and Klumperman 2009) and their detailed structure differs depending on the cell type. In fact, lysosomes can be morphologically heterogeneous due to differences in their internalized content. This heterogeneity of the lysosomal content appears usually electron-dense but often includes irregularities and membrane sheets (Saftig and Klumperman 2009).

A common characteristic of lysosomal compartments is their acidic interior with a pH of 4.5–5, which is maintained by the vacuolar H^+-adenosine triphosphatases (v-ATPase), a transmembrane multiprotein complex (Schröder et al. 2010). Preservation of low pH is important for several lysosomal functions, such as cargo release, hydrolase maturation, vesicle maturation, autophagy and neurotransmitter loading into synaptic vesicles (Marshansky and Futai 2008; Mijaljica, Prescott, and Devenish 2011). Additionally, the pH gradient within the endomembrane system is required for intracellular trafficking and its loss can impair mannose-6-phosphate receptor (M6PR) recycling back to the Golgi for reloading of newly synthesized lysosomal hydrolases (Sobota et al. 2009).

Lysosomes contain more than 50 different acid hydrolases, such as proteinases, peptidases, phosphatases, nucleases, glycosidases, sulfatases and lipases, and several activator proteins that are localised mainly in the matrix. These hydrolases are then able to decompose macromolecules and even membranes into their monomeric constituents.

In other studies, it has been shown that cells can also contain lysosome-like organelles, such as melanosomes, lytic granules, major histocompatibility complex (MHC) class II compartments, platelet-dense granules and synaptic-like micro-vesicles (Dell'Angelica 2004). Nevertheless, acid hydrolases and LMPs are essential for the function of lysosomes.

Acid hydrolases

Each of the 50 known lysosomal acid hydrolases targets specific substrates for degradation, and their collective action is responsible for the total catabolic capacity of the lysosome (Saftig and Klumperman 2009). The breakdown products, such as amino acids, monosaccharides, oligosaccharides and nucleotides, are then transported back to the cytosol by specific transporter proteins residing in the lysosomal membrane (Saftig 2006). In addition to bulk degradation and pre-protein processing, lysosomal hydrolases are also involved in antigen processing, degradation of the extracellular matrix and initiation of apoptosis (Conus and Simon 2008).

Lysosomal targeting of newly synthesized lysosomal proteins can be (1) direct, from the *trans*-Golgi network (TGN) to the endosomal system, or (2) indirect, involving transport to the plasma membrane and subsequent endocytosis (Saftig and Klumperman 2009).

Most newly synthesized lysosomal hydrolases enter the lysosomal compartment directly via the biosynthetic route (Figure 1). They are synthesized in the rough endoplasmic reticulum (ER) and then packaged into vescicles in the

Golgi apparatus. In the TGN, acid hydrolases undergo a critical modification of one or several of their carbohydrates to mannose-6-phosphate (M6P) moieties by the enzyme N-acetylglucosamine (GlcNAc)-phosphotransferase (Hasilik, Waheed, and von Figura 1981; Sleat et al. 2005); then acid hydrolases bind the M6PR that delivers them to endosomes (Kornfeld and Mellman 1989). There are two types of M6PR, 300 kD cation-independent M6PR (CIM6PR; also known as IGF2R) and 46 kD cation-dependent M6PR (CDM6PR), both of which are ubiquitously expressed (Braulke and Bonifacino 2009). As the v-ATPase leads to acidification during the maturation of the endosomal compartment, the hydrolases dissociate from their receptors, which are then recycled back to the trans-Golgi network or to the cell surface (Saftig 2006) (Figure 1).

In the absence of a functional M6PR pathway, newly synthesized lysosomal hydrolases do not acquire M6P tags because of a deficiency in N-acetylglucosamine (GlcNAc)-phosphotransferase activity, also known as I-cell disease or mucolipidosis type II (Hasilik, Waheed, and von Figura 1981; Waheed et al. 1982; Reitman, Varki, and Kornfeld 1981). Nevertheless, in some I-cell diseased cells, a significant portion of newly synthesized lysosomal hydrolases do reach the lysosome (Waheed et al. 1982; Little et al. 1987; Owada and Neufeld 1982). In this condition, new hydrolases can follow the constitutive secretory pathway to the plasma membrane and after secretion might be taken up by endocytosis.

Lysosomal Membrane Proteins

Lysosomes are surrounded by a single phospholipid bilayer that serves as a limiting membrane. Such lysosomal membrane has several functions including sequestration of lysosomal enzymes, mediation of fusion events, acidification of the lysosomal lumen and transport of degradation products to the cytoplasm (Saftig 2006). In fact, the lysosomal membrane gathers the aggressive hydrolases to the organelle lumen, thus preventing undesired proteolytic damage to the surroundings; at the same time, it forms an impermeable barrier for the monomeric metabolites originating from the degradation of macromolecules, preventing their leakage in the cytoplasm. Therefore selective transport proteins are required in order to clear these compounds from the lysosomal lumen and facilitate their efflux into the cytosol.

LMPs are highly glycosylated proteins decorating the luminal surface of lysosomal membranes (Koike et al. 2005). The mammalian lysosome contains ~25 LMPs (Lübke, Lobel, and Sleat 2009), although additional LMPs are currently being revealed (Lübke, Lobel, and Sleat 2009; Schröder et al. 2007; Callahan, Bagshaw, and Mahuran 2009). The most abundant LMPs are lysosome associated membrane protein 1 and 2 (LAMP1 and LAMP2), lysosome integral membrane protein 2 (LIMP2, also known as SCARB2) and the tetraspanin CD63 (Saftig and Klumperman 2009). The sorting of most lysosomal membrane proteins depends on short sequence motifs within their cytoplasmic tails, which are necessary and sufficient to target them to lysosomes (Saftig 2006).

Furthermore, lysosomal membranes are characterized by a system of

transporters that play several important roles, such as establishing controlled acidic pH in the lysosome, absorption of the products of digestion, the release of Ca^{2+} from the lysosomal lumen that drives the fusion of lysosomes with late endosomes, and transport of the metals bound to endocytosed proteins across the lysosomal membrane into the cytoplasm (Ruivo et al. 2009; Jentsch 2007; Luzio et al. 2005). Among them, lysosomes contain also members of the transient receptor potential (TRP) superfamily; in particular, a key role is played by MCOLN1, an ion channel belonging to the subfamily of mucolipins (TRPMLs), which localizes also to late endosomes membrane. It has been shown that the channel is permeable to multiple ions including Ca^{2+}, Fe^{2+}, Na^+, K^+, Zn^{2+} and H^+ (X.-P. Dong et al. 2008; LaPlante et al. 2002; Raychowdhury et al. 2004) and therefore it appears to be a prominent lysosomal metal transporter. Although the exact function of MCOLN1 remains to be conclusively proven, it is becoming evident that it functions as a lysosomal Ca^{2+} release channel. Membrane trafficking deficits (Treusch et al. 2004), as well as impaired fusion of lysosomes with autophagosomes in MCOLN1 deficient cells (Vergarajauregui and Puertollano 2006) support the role of MCOLN1-mediated Ca^{2+} release in the Ca^{2+}-dependent membrane fusion along the endocytic pathway.

Besides transport proteins, the membrane also contains other enzymes, including the heparan-α-glucosaminide N-acetyltransferase (HGSNAT), which uses cytosolic acetyl coenzyme A for the acetylation of non-reducing terminal α-glucosaminyl residues of the heparan sulphate degradation intermediates in the lysosomal matrix (Fan et al. 2006; Hrebícek et al. 2006).
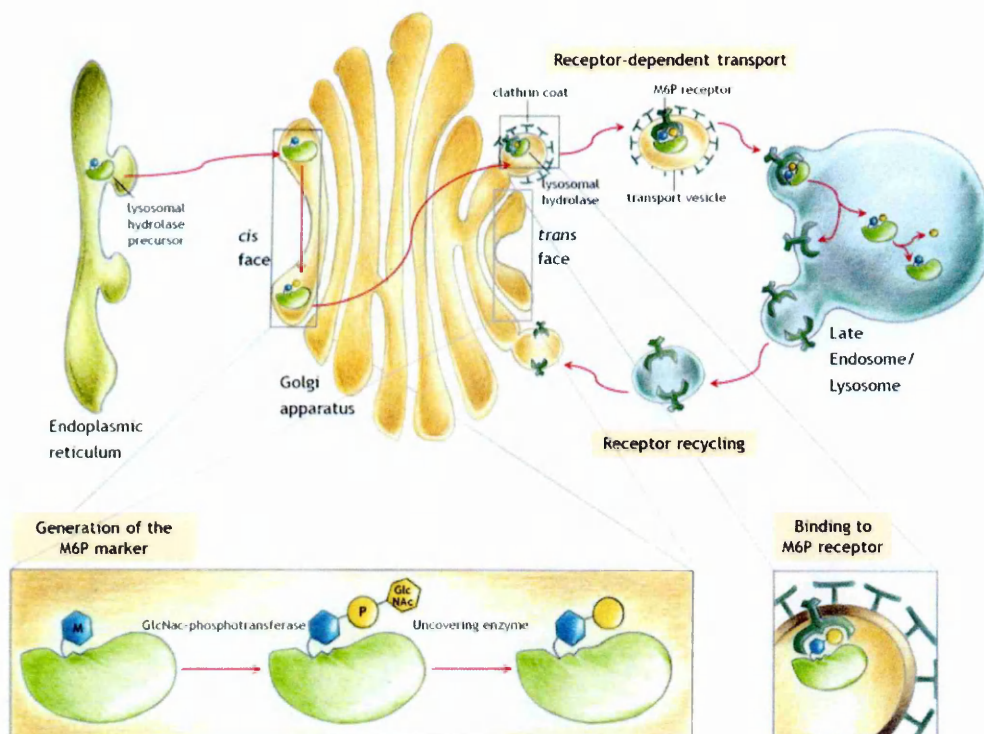
Figure 1. Transport of newly synthesized lysosomal hydrolases to lysosomes.

Lysosomal hydrolases are synthesized in the endoplasmic reticulum and move to the cis-Golgi network (CGN), were they are covalently modified by the addition of M6P groups. At the TGN, the M6P signal allows the segregation of lysosomal hydrolases from all other types of proteins through selective binding to the M6P receptors. The clathrin-coated vesicles produced bud off from TGN and fuse with late endosomes. At the low pH of the late endosome, the hydrolases dissociate from the M6PRs and the empty receptors are recycled to the Golgi apparatus for further rounds of transport.

Model taken from Coutinho et al. 2012

Lysosomal Related Organelles

LROs are specialised lysosomes with cell-type specific functions, such as melanosomes in melanocytes, Weibel–Palade bodies in endothelial cells, lamellar bodies in type II pneumocytes, dense bodies in plateles and granules in cytotoxic *T* cells and natural killer cells (Raposo, Marks, and Cutler 2007). LROs usually co-exist with normal lysosomes. They share features of late endosomes/lysosomes but are functionally, morphologically and compositionally distinct; they can also contain cell-type-specific proteins and might require additional cellular machinery for their biogenesis (Bonifacino 2004; Dell'Angelica 2004). Some LROs resemble lysosomes morphologically with electron-dense protein deposits and/or intralumenal membranes, and are accessible to endocytic traffic (Raposo, Marks, and Cutler 2007); other LROs present entirely novel morphological features as a product of their unique cargo, such as the proteinacious fibrils of melanosomes, the proteinacious tubules of Weibel-Palade bodies and the lipid swirls within lamellar bodies (Raposo and Marks 2002; Bonifacino 2004).

Together with lysosomes, LROs are involved in various physiological processes, such as cholesterol homeostasis, plasma membrane repair, bone and tissue remodelling, pathogen defence, cell death and cell signalling (Saftig and Klumperman 2009).

Function of lysosomes

Lysosomes are involved in several cellular processes including endocytosis, phagocytosis, autophagy and exocytosis (Figure 2).

Endocytosis and Lysosome Formation

One of the most important functions of lysosomes is the digestion of material taken up from outside the cell through endocytosis. However, this function is directly related also to lysosome formation within the cell. In other words, lysosome biogenesis represents an intersection between the secretory pathway, through which lysosomal proteins are processed, and the endocytic pathway, through which extracellular molecules are taken up at the cell surface. Specifically, lysosomes are formed by the fusion of transport vesicles budded from the TGN with endosomes, but they can also contain molecules taken up by endocytosis and coming from the plasma membrane. Material from outside the cell is taken up in clathrin-coated endocytic vesicles, which bud from the plasma membrane and then fuse with early endosomes. Membrane components are then recycled to the plasma membrane and the early endosomes gradually mature into late endosomes, which are the precursors to lysosomes. In addition, early endosomes receive also endogenous proteins from the TGN, such as M6PRs carrying acid hydrolases (Klumperman et al. 1993; Waguri et al. 2003) to be delivered to the lysosome; in this step, one of the important changes during endosome maturation is the lowering of the internal pH to about 5.5 (Figure 1).

15

Acid hydrolases are then targeted to lysosomes by mannose-6-phosphate residues, which are recognized by M6PRs in the TGN and packed into clathrin-coated vesicles (Rohrer and Kornfeld 2001). The clathrin coat is then removed and transport vesicles fuse with late endosomes, causing the acidification of the internal pH; this change in the internal pH causes the dissociation of hydrolases from the M6PR. The hydrolases are thus released into the lumen of the endosome, while M6PRs remain in the membrane and are eventually recycled to the Golgi (Figure 2). As late endosomes acquire a full complement of acid hydrolases they mature into lysosomes.

EXOCYTOSIS

ENDOCYTOSIS

residual body

primary lysosomes

secondary lysosomes

Golgi apparatus

AUTOPHAGY

autophagosomes

RER
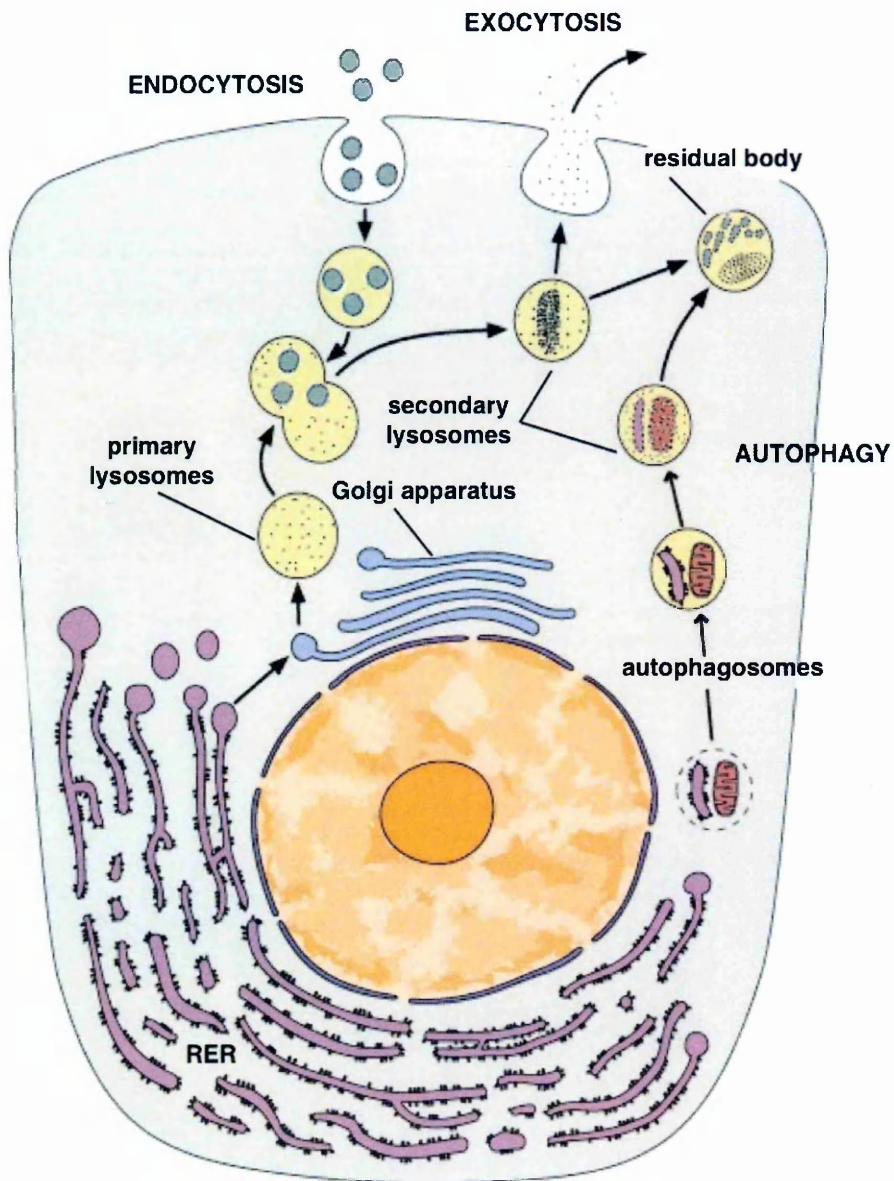
Figure 2. Lysosomes are involved in several cellular processes and undergo maturation.

Lysosomes participate in endocytosis, phagocytosis, autophagy and exocytosis. During their biogenesis and maturation they are subjected to a few steps of maturation. Primary lysosomes contain hydrolytic enzymes, they fuse with other endocytic vesicles that contain material to be digested, forming secondary lysosomes; once digestion occurred, secondary lysosomes become residual bodies, containing only indigestible or slowly digestible materials.

Modified from The McGraw-Hill Companies, Inc. 2006

Autophagy

The lysosome is the end-terminal for substrates coming from the autophagy pathway. Autophagy is a mechanism by which the cell digests its own intracellular components and other organelles, such as mitochondria (Figure 1 and 3). This process secures the cell's supply with macromolecules under conditions of starvation and allows the disposal of unneeded and non-functional organelles (Schröder et al. 2010).

Autophagy also was first observed by de Duve in 1963 (De Duve 1963); he identified membrane-bound vesicles containing endogenous organelles undergoing digestion; various stimuli, including starvation and glucagon administration, stimulate this process.

Autophagy is critical for growth, development and survival and therefore it is highly conserved among eukaryotic organisms, from yeasts to mammals (T Lang et al. 2000; Noda et al. 2000; Young et al. 2006).

There are various types of autophagy, differing in their mechanisms and functions (Klionsky 2005): microautophagy, macroautophagy and chaperone-mediated autophagy (CMA). Microautophagy is the non-selective internalization of small cytosolic portions via lysosomal invaginations. Macroautophagy (hereafter referred to as autophagy) is the selective sequestration of cytoplasm or organelles that are enclosed in the double-membrane structures known as autophagosomes, which subsequently fuses with lysosomes. CMA is the selective targeting of specific cytosolic soluble proteins to the lysosome via molecular chaperones. This latter process involves a direct translocation of the unfolded protein through the

lysosomal membrane by a mechanism not fully clarified, although critically depending on an isoform of the lysosome-associated membrane protein 2 (LAMP2A) as an essential component.

The first step of autophagy appears to be the formation of a cup-shaped structure, also referred to as an isolation membrane or pre-autophagosome, in the cytosol, where it gradually elongates to surround a portion of the cytoplasm and its constituents (Figure 3). Subsequently, the edges of the membrane fuse together to form a vesicle, which represents a double-membrane structure termed autophagosome (Juhasz and Neufeld 2006). Autophagosomes then undergo a maturation process consisting of multiple fusion events with both endosomes and lysosomes (Yoshimori 2004; Komatsu et al. 2006; Hara et al. 2006; Nakagawa et al. 2004; Ogawa et al. 2005; Ravikumar, Duden, and Rubinsztein 2002; Kamimoto et al. 2006; Eskelinen 2005) and their content is digested by lysosomal hydrolases. In fact, upon acquisition of lysosomal proteases and the v-ATPase, the interior of the autophagosome becomes acidified, and cytoplasmic materials are subjected to degradation (Kimura et al. 2007). Autophagosomes at this final stage, after fusion with lysosomes, are called autolysosomes (Figure 3).

Autophagy is regulated by more than 30 autophagy-related proteins (Atg), many of which have been discovered in yeast (Levine and Klionsky 2004). Most of the characterized *ATG* gene products, including Atg3, Atg5, Atg7, Atg10, Atg12, and LC3 (microtubule-associated protein 1 (MAP1) light chain 3), are involved in two ubiquitylation-like post-translational modifications of target proteins, which are the Atg12-conjugation and the LC3-modification (Atg8-lipidation in yeast), which are essential for the dynamic process of autophagosome formation (Kabeya et al.

19

2000; N Mizushima, Noda, et al. 1998; N Mizushima, Sugita, et al. 1998; Noboru Mizushima, Ohsumi, and Yoshimori 2002; I Tanida et al. 2001; Isei Tanida et al. 2002). Atg12-conjugation is essential for the formation of pre-autophagosomes, whereas LC3-modification is essential for the formation of autophagosomes (Kabeya et al. 2000; N Mizushima et al. 2001). Atg12 is activated by the E1-like enzyme Atg7, transferred to the E2-like enzyme Atg10, and conjugated to Atg5 to form an autophagosomal precursor (N Mizushima, Noda, et al. 1998; N Mizushima, Sugita, et al. 1998; N Mizushima et al. 2001; Noboru Mizushima, Ohsumi, and Yoshimori 2002; Nemoto et al. 2003; I Tanida et al. 2001). LC3 is the mammalian orthologue of yeast Atg8 and its nascent form, ProLC3, is processed: its carboxyl terminal region is cleaved off to become a soluble cytosolic form, LC3-I, exposing a carboxyl terminal glicine residue (Kabeya et al. 2000). LC3-I is then activated by Atg7, transferred to Atg3, a second E2-like enzyme, modified with phosphatidylethanolamine and becomes a membrane-bound form, LC3-II (Tanida et al. 2001; Tanida et al. 2002). LC3-II is localized to both the outer and the inner membrane of pre-autophagosomes and autophagosomes (Kabeya et al. 2000). Following the fusion of autophagosomes with lysosomes, intra-autophagosomal LC3-II is degraded by lysosomal hydrolytic enzymes (Kabeya et al. 2000). The formation of LC3-II is therefore a good marker to monitor the occurrence of autophagosome formation (Kabeya et al. 2000) and thus LC3 is commonly used as an autophagosomal marker.

In mammalian cells, autophagy is regulated by nutrient availability and hormones, and has been suggested to be essential for cellular homeostasis (Kimura et al. 2007). In addition to its homeostatic function, autophagy plays quite

important physiological roles. In fact, although autophagy is strongly induced under conditions of physiological stress, such as starvation, it also occurs at a basal level in normal conditions; for example, constitutive autophagy in nutrient-rich conditions is involved in intracellular protein quality control, in global turnover of cellular components (including organelles) (Komatsu et al. 2006; Hara et al. 2006) and as a defense mechanism against bacterial pathogens (Nakagawa et al. 2004; Ogawa et al. 2005) or the toxic effects of aggregate-prone proteins (Ravikumar, Duden, and Rubinsztein 2002; Kamimoto et al. 2006).
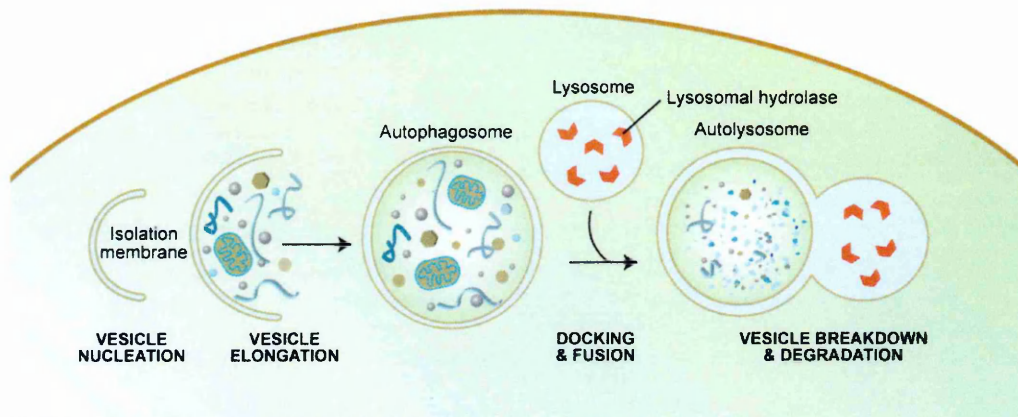
Figure 3: Schematic diagram of the steps of autophagy.

Autophagy begins with the formation of the phagophore or isolation membrane (vesicle nucleation step). The concerted action of the autophagy core machinery proteins at the phagophore assembly site is thought to lead to the expansion of the phagophore into an autophagosome (vesicle elongation). The autophagosome can engulf bulk cytoplasm in a non-specific manner, including entire organelles, or target cargos specifically. When the outer membrane of the autophagosome fuses with a lysosome (docking and fusion steps), it forms an autophagolysosome, or autolysosome. Finally, the sequestered material is degraded inside the autophagolysosome (vesicle breakdown and degradation) and recycled.

Model taken from Meléndez and Levine, 2009.

Exocytosis

Lysosomes are also involved in a secretory pathway known as lysosomal exocytosis (Figure 1). Exocytosis is the removal of cellular cargo by fusion of vesicles with the plasma membrane (PM) (Figure 4). In response to a transient increase of cytosolic Ca^{2+}, secretory vesicles move towards the PM, fuse with the membrane, and then expel the luminal contents into the external cellular environment. Initially, lysosomal exocytosis was considered to be limited to specialized secretory cells; in these cells, exocytosis has a housekeeping function responsible for the secretion of hormones, cytokines, and neurotransmitter. However, several studies indicate that this process occurs in all cell types (N W Andrews 2000; Rodríguez et al. 1997; Rodríguez et al. 1999). In fact, it has been demonstrated that in non-secretory cells exocytosis plays an important role in PM repair (Idone et al. 2008), bone resorption (Smit et al., 2000), cycling/recycling proteins to plasma membrane (Catala et al. 2009), pathogen invasion (Imai et al., 2003), neurite outgrowth (Seiler et al. 2008), and cellular clearance (Pawelek and Lerner 1978; Boissy, Zhao, and Gahl 1998). In fact, lysosomes have traditionally been viewed as terminal degradative compartments, but they are the most important exocytic organelle in non-secretory cells, behaving as Ca^{2+}-regulated exocytotic vesicles (Rodríguez et al. 1997).

Lysosomal exocytosis is an ATP- and temperature-dependent process, in a way similar to what is known for the classical secretory process (Rodríguez et al. 1997). Lysosomes have exocytic activity, a common feature with synaptic vesicles. In fact, they originate from a common early endosome and they also share

23

mechanisms of exocytic activation. During the process of the synaptic vesicle release, action potentials of neurons reach the presynaptic terminal and activate several voltage-regulated Ca^{2+} channels; this event induces an influx of Ca^{2+} into the presynaptic cytoplasm, the fusion of the synaptic vesicle with the PM and, eventually, the release of the neurotransmitter (Nizami et al. 2010). The Ca^{2+} sensor synaptotagmin and the soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) synaptobrevin-2 decorate the synaptic vesicles membrane and were found to bind the PM through the corresponding SNAREs, syntaxin-1A and synaptosomal-associated protein 25 (SNAP-25), respectively (Thorsten Lang and Jahn 2008). The influx of Ca^{2+} into the cell drives interactions between the vesicular and target SNAREs synaptotagmin 7 (SYT7) and syntaxin 4 (Thorsten Lang and Jahn 2008), thus inducing lysosomal exocytosis.

Moreover, lysosome exocytosis has emerged also as an important mechanism for propagating the Ca^{2+} wave in astrocytes to modulate synaptic transmission (Li et al. 2008; Z. Zhang et al. 2007), although on a timescale orders of magnitude slower than synaptic transmission.

Lysosomal exocytosis requires two sequential steps. (Figure 4). In the first Ca^{2+}-independent step (Jaiswal, Andrews, and Simon 2002), lysosomes are recruited to the close proximity of the cell surface. In the second step the pool of pre-docked lysosomes fuse with the PM in response to Ca^{2+} elevation, thus emptying their content outside the cell (N W Andrews 2000; Jaiswal, Andrews, and Simon 2002; Tucker, Weber, and Chapman 2004).

Taking advantage of exocytosis, lysosomes play a pivotal role in the

degradation of extracellular matrix (ECM) proteins, cell invasion, and cell migration into the ECM (Lakka et al. 2004; Tu et al. 2008).

Exocytosis of lysosomes in response to a transient increase in intracellular Ca^{2+} was first observed in eggs of sea urchin, in which, during the fertilization process, thousand of secretory granules are released in response to an increase in intracellular Ca^{2+} concentration (Baker and Whitaker 1980). Furthermore, exocytosis, was also observed during cell invasion of *Trypanosoma Cruzi*: binding of the parasite to the cell membrane triggers calcium influx and subsequent fusion of lysosomes with the region of the plasma membrane that surrounds the invading parasite (Norma W Andrews 2002).

Although the main steps of lysosomal exocytosis have been elucidated, little is known about its regulation and how this process is coordinated with lysosomal biogenesis.
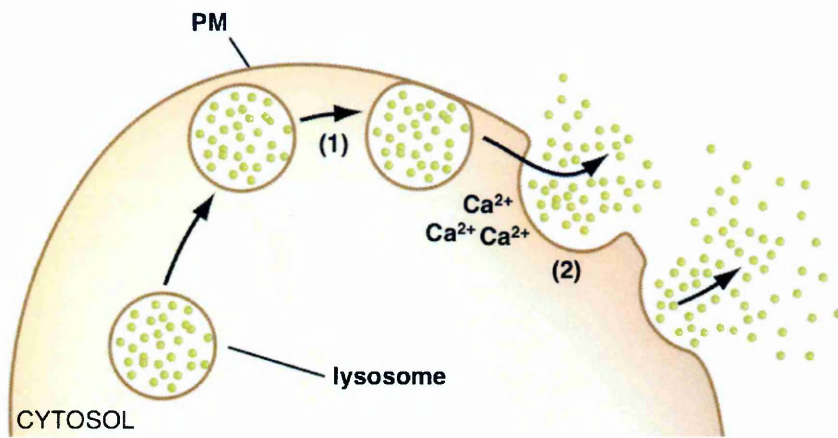
Figure 4: Schematic diagram of the steps of lysosomal exocytosis.

Exocytosis is the removal of cellular cargo by fusion of vesicles with the plasma membrane (PM). This process requires two sequential steps. (1) In the first Ca^{2+}-independent step, lysosomes are recruited to the close proximity of the cell surface. (2) In the second step the pool of pre-docked lysosomes fuse with the PM in response to Ca^{2+} elevation, thus emptying their content outside the cell.

Modified from Microbiology: An Evolving Science. Joan L. Slonczewski and John W. Foster.

Lysosomal Storage Diseases

In 1932, Pompe made the crucial observation of extensive glycogen accumulation, within membrane-bound vesicles in the heart and skeletal muscles of a 7 months old patient who had died from cardiac complications. Based on these findings, in 1963 Hers and coworkers deduced the link between the deposition of glycogen in Pompe patients and the inherited deficiency of the hitherto unknown lysosomal enzyme alfa-D-glucosidase (Hers 1963), identifying the first lysosomal disorder. The discovery of the involvement of lysosomes in glycogen degradation gave rise to the concept that also other lysosomal storage disorders could be explained by specific enzyme deficiencies (Parkinson-Lawrence et al. 2010).

Nowadays, LSDs are a class of metabolic disorders and comprise a group of more than 50 different genetic diseases (Wilcox 2004). Despite the large number and clinical diversity of lysosomal disorders, these diseases share some common features. First, they are typically inherited as autosomal recessive traits (only two are X-linked); second, they most commonly afflict infants and young children; third, most involve pathology of the brain; and fourth, when brain pathology is present they are untreatable (Jeyakumar et al. 2005). Although each of these conditions is rare, they exhibit a combined prevalence of 1:5,000 births; some of these disorders occur at higher frequency in geographically isolated populations owing to founder effects (Dahl, Hillborg, and Olofsson 1993), or in certain ethnic groups in which consanguineous marriages are common (Ozkara and Topçu 2004).

LSDs are caused by mutations in proteins critical for lysosomal function. They mostly involve the dysfunction of a specific soluble lysosomal hydrolases, which

result in impaired degradation, substrate accumulation and lysosomal storage (Figure 5).

However, lysosomal integral membrane proteins, proteins involved in post-translational modifications, in vesicular trafficking and in the biogenesis of lysosomal proteins have also been shown to cause storage disorder phenotypes. For example, over the last years a number of defects in lysosomal membrane proteins have been recognized as the primary cause of lysosomal diseases (Ruivo et al. 2009). In fact, lysosomal accumulation can also occur if the correctly degraded compound is not properly transported to the cytosol (like in Salla disease and in cystinosis).

Other causes of LSDs are deficiencies in membrane-associated enzymatic activities (like HSGNAT in mucopolysaccharidosis type III C, MPSIIIC) and impairments of ion translocation (like MCOLN1 in mucolipidosis type IV, MLIV). For a significant number of LSDs caused by defects in lysosomal membrane proteins, the pathogenetic events at a molecular level and also the function of the protein under physiological conditions is incompletely understood (Schröder et al. 2010). This applies to neuronal ceroid lipofuscinosis (NCL) variants caused by defects in the membrane proteins CLN3 (Jalanko and Braulke 2009) and CLN7 (Siintola et al. 2007).

However the biochemical nature of the defects resulting in lysosomal storage is very diverse and in many cases the sequence of events leading to lysosomal dysfunction is only incompletely understood (Schröder et al. 2010).

In general, LSDs are typically characterized by enlarged lysosomes that contain partially degraded material as a result of a deficit in either degradation of

specific compounds, such as glycosaminoglycans, lipids, or protein, or in transport across the lysosomal membrane or in endosome-lysosome trafficking. Any disruption of lysosomal function can lead to the accumulation of undegraded substrate(s) in endosomes and lysosomes, eventually compromising cellular function and ultimately resulting in a complex spectrum of clinical manifestations (Hopwood and Brooks 1997).

The accumulation of the primary storage material can cause a chain of secondary disruptions to other biochemical and cellular functions, which leads to the severe pathology observed in LSDs (Parkinson-Lawrence et al. 2010). Once material starts to accumulate, it builds up within lysosomes until the lysosomal burden of the cell reaches some maximum level, at which point storage material starts to accumulate in other parts of the cell (Jeyakumar et al. 2005). The accumulation of undigested molecules can subsequently alter many cellular processes, including lysosomal pH regulation, synaptic release, endocytosis, vesicle maturation, autophagy, exocytosis and Ca^{2+} homeostasis (Vitner, Platt, and Futerman 2010; Bellettato and Scarpa 2010; Ballabio and Gieselmann 2009; Bezprozvanny 2009). It is still undefined whether the storage material affects cell function only when it begins to accumulate in extra-lysosomal sites or if problems in cell homeostasis are triggered while the material is still confined to the lysosome (Jeyakumar et al. 2005).

The accumulation of the primary storage material can also have a functional impact on the cell, including the inhibition of other enzymatic processes, causing the accumulation of secondary undegraded substrates and the disruption of lysosomal biogenesis (Parkinson-Lawrence et al. 2010).

29

Clinically, LSDs are associated with a progressive phenotype involving multiple organs and tissues (Settembre et al. 2008).

Although lysosomal proteins are ubiquitously distributed, the accumulation of undegraded substrate(s) in LSDs patients is normally restricted to those cells, tissues, and organs in which substrate turnover is high (Parkinson-Lawrence et al. 2010). However, the Central Nervous System (CNS) is particularly affected in LSD patients; over two-thirds of LSDs involve CNS dysfunction, like progressive cognitive and motor decline, and these symptoms are often the most debilitating (Schultz et al. 2011). In the CNS, the progressive accumulation of undegraded material induces a series of secondary defects, eventually leading to severe neurodegeneration.

Storage may begin during early embryonic development, and the clinical presentation for LSDs can vary from an early and severe phenotype to late-onset mild disease (Filocamo and Marrone 2011). However, although individuals affected by LSDs can display early symptoms, many are clinically normal at birth and typically meet early developmental milestones, indicating that lysosomal storage does not affect neuronal function and maturation at early developmental stages. In general, this suggests that lysosomal dysfunction *per se* does not impact significantly the complex events of early brain development, such as neural induction, establishment of axis, neuronal differentiation and migration, and synapse formation (Schultz et al. 2011).

Several systems for the classification of LSDs have been proposed. Most simply the diseases are grouped according to the nature of the storage material – either regarding biochemical composition or morphological appearance – in

mucopolysaccharidoses, glycoproteinoses, (sphingo)lipidoses, glycogen storage diseases and neuronal ceroid lipofuscinoses.

Although individual LSDs are rare, as group they are one of the most common genetic disorders in children, affecting 1 out of every 7000-8000 live births.

Many of the phenotypes observed in LSDs can be markedly improved by substrate reduction therapy (SRT), enzyme replacement therapy (ERT) or gene therapy, whose efficacy can be monitored by concomitant resolution of storage material (Eng et al. 2001, 200; Liu et al. 2005). In fact, storage burden in tissue sections or body fluids can be used as a reliable indicator of therapeutic efficacy in emerging treatment strategies for some LSDs (Eng et al. 2001; Schultz et al. 2011).

However, the progressive lysosomal accumulation of undegraded metabolites results in generalized cell and tissue dysfunction, and, therefore, in a multi-systemic pathology (Filocamo and Morrone 2011); thus, such treatments are not yet available or feasible for most LSDs. Therefore, understanding how the affected cellular pathways interconnect and impact the viability of cells is critical for future therapeutic development (Schultz et al. 2011).

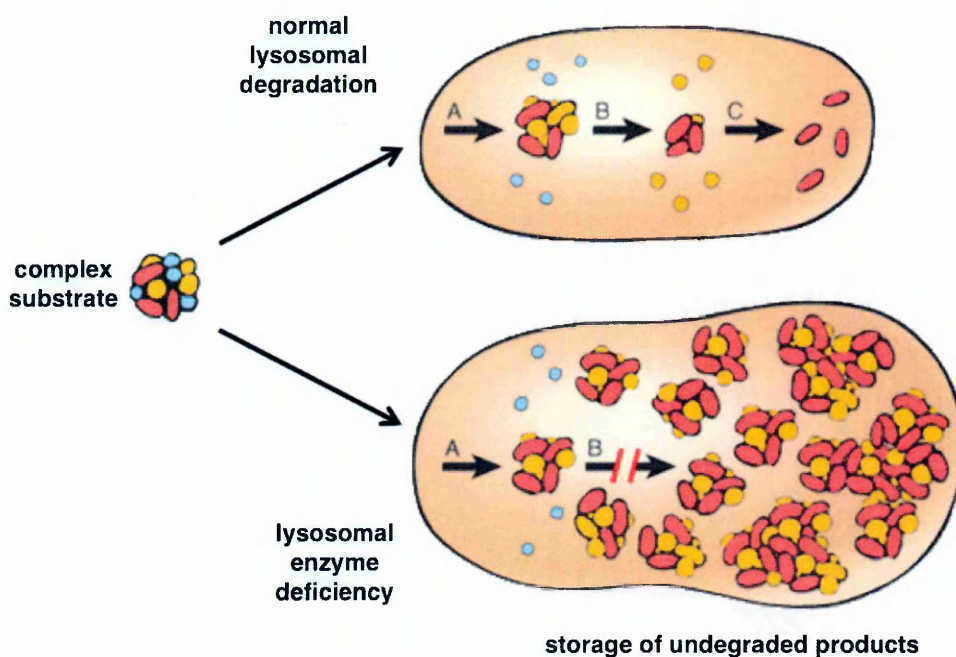**Figure 5. Schematic diagram illustrating the pathogenesis of lysosomal storage diseases.**

A complex substrate is normally degraded by a series of lysosomal enzymes (A, B, and C) into soluble end products. If there is a deficiency or malfunction of one of the enzymes (e.g., B), catabolism is incomplete and insoluble intermediates accumulate in the lysosomes.

Modified from Kumar: Robbins and Cotran Pathologic Basis of Disease

Multiple Sulfatase Deficiency

Among acid hydrolases acting within the lumen of lysosomes, sulfatases are a family of enzymes that share both structural and functional similarities. They are involved in a number of different biological functions as diverse as degradation of complex molecules, production of steroid hormones and cell signalling. In particular, they catalyze the hydrolysis of sulfate ester bonds from a wide variety of substrates, ranging from complex molecules, such as GAGs, to sulfolipids and steroid sulfates. These enzymes can be divided, at least in mammals, into two main categories based on their subcellular localization: those acting at an acidic pH, localized in the lysosomes, and those acting at a neutral pH that are found in the ER, in the Golgi apparatus, and at the cell surface (Hanson, Best, and Wong 2004; Parenti, Meroni, and Ballabio 1997) (Hopwood and Ballabio, 2001).

MSD is an extremely rare autosomal recessive disorder, with an incidence of 1:1,400,000 and characterized by a dramatic impairment of all sulfatase activities. Life expectancy for MSD patients is commonly under 10 years of age.

Almost two decades ago it was demonstrated that sulfatases undergo a unique post-translational modification, which is indispensable for their enzymatic activity (Schmidt et al. 1995). This modification involves a highly conserved cysteine residue (Cys), located within the active site of sulfatases, which is modified into a formylglycine residue (FGly). The gene encoding the enzyme involved in the post-translational modification of sulfatases was identified and found to be mutated in patients with MSD (Cosma et al., 2003; Dierks et al., 2003). The MSD causing gene is called SUMF1 and encodes a formylglycine-generating

enzyme (FGE) (Figure 6). SUMF1 exerts its activity within the ER; however, it can also be secreted and taken up by distant cells and tissues, where it relocalizes in the ER as an active enzyme (Zito et al. 2007). In MSD patients residual sulfatase activities are substantially reduced (Dierks et al. 2003; Cosma et al. 2003), but still detectable at variable levels, indicating that MSD is caused by hypomorphic mutations in SUMF1 gene and that the complete loss of SUMF1 function is likely to be lethal in humans (Cosma et al. 2003). Interestingly, the phenotype of MSD patients combines, with some phenotypical variability, all the clinical symptoms observed in each individual sulfatase deficiency (Bischel, Austin, and Kemeny 1966). As a major sign they show a neurodegenerative course of disease with loss of sensor and motor abilities and neurological deterioration. Mental retardation, hepatosplenomegaly, shortening of stature and corneal clouding appear like in different mucopolysaccharidoses.

In the past years, in our laboratory, a mouse line carrying a null mutation in the SUMF1 gene has been generated using a gene-trapping approach (Settembre et al. 2007). In these mice, the function of the entire sulfatase protein family has been completely abolished, mimicking the phenotype observed in MSD patients: massive accumulation of undegraded molecules, systemic inflammation and neurodegeneration. Moreover, the phenotype of these mice is severe and progressive; they display frequent early mortality (only the 10% reaches the age of 3 months), congenital growth retardation, skeletal abnormalities (including the typical flat facial appearance) and tremor and seizures due to defects of the CNS. Many tissues were examined for the presence of storage material, which was shown to increase with age; glycosaminoglycan accumulation was detected in liver,

34

kidney and, as a prime site, in macrophages which were massively present in all tissues. These macrophages and activated microglia in cerebellum and cortex, accompanied by neuronal cell loss and astroglyosis, indicate systemic neuroinflammation, which are thought to be key patho-physiological processes in MSD (Settembre et al. 2007).

In addition, nutrient-starved MSD mouse primary cells, as well as other monogenic LSDs, such as the Sanfilippo syndrome, or mucopolysaccharidosis type III A (MPSIIIA), present a block of autophagy as a consequence of decreased ability of lysosomes to fuse with autophagosomes; this leads to accumulation of toxic substrates, such as poly-ubiquitinated proteins and dysfunctional mitochondria, which are the putative mediators of cell death (Settembre et al. 2008).
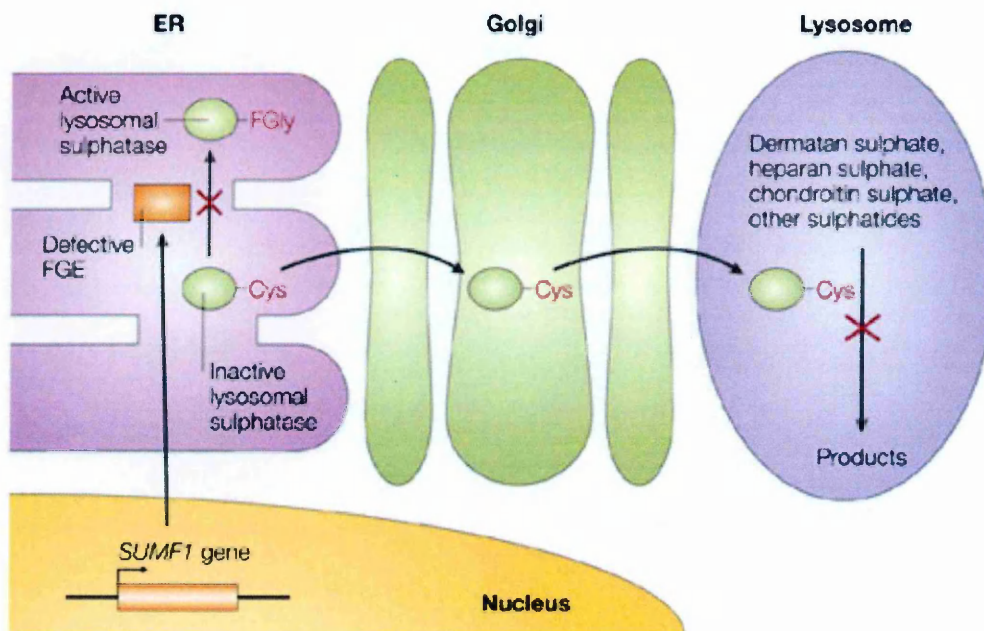
Figure 6. Mutations in the sulfatase-modifying factor-1 (SUMF1) gene result in the production of defective C-formylglycine-generating enzyme (FGE).

Defective FGE cannot convert the cysteine (Cys) residue in the active site of lysosomal sulfatases to formylglycine (FGly). As a consequence, inactive sulfatases are transported to the lysosome, where they are unable to degrade their substrate, leading to their accumulation and subsequently to MSD pathology.

Model taken from Futerman and van Meer, 2004

LSDs and autophagy

In the past years there has been an increased interest in investigating the possible involvement of autophagy in LSD pathogenesis (Cao et al. 2006; Koike et al. 2005; Fukuda et al. 2006; Jennings et al. 2006; Settembre et al. 2008). In our laboratory, we study two mouse models of neurodegenerative LSDs, MSD and MPSIIIA. Cells from both of these LSD models display: (1) increased numbers of autophagosomes, (2) reduced clearance of both endogenous and exogenous autophagic substrates and (3) defective organelle turnover (Settembre et al. 2008).

Interestingly, alterations in the autophagic/lysosomal pathway have been observed in more common multifactorial diseases (Levine and Kroemer 2008), such as Parkinson's disease (PD) (Pan et al. 2008; Ramirez et al. 2006) and Alzheimer's diseases (AD) (Nixon 2007), many forms of cancer (Kirkegaard 2009) and atherosclerosis (Martinet and De Meyer 2008). It is often not clear if the observed alterations represent causes or secondary consequences of the disease process.

Reports demonstrating that genetic disruption of autophagy causes neurodegeneration in mice (Hara et al. 2006; Komatsu et al. 2006) led to the hypothesis that the neurodegeneration in LSDs might be a consequence of impaired autophagy. Indeed, impaired autophagy has been reported also in other models of LSDs, including Pompe disease, Niemann-Pick disease (NPC), NCLs, MLIV, and GM1-gangliosidosis (Cao et al. 2006; Fukuda et al. 2006; Jennings et al. 2006; Pacheco, Kunkel, and Lieberman 2007; Venkatachalam et al. 2008).

LSDs and exocytosis

Lysosomal function is intimately linked to exocytosis, and several LSDs.

An example is MLIV; when human MLIV fibroblasts are treated with ionomycin to elevate intracellular levels of Ca^{2+} and induce lysosomal exocytosis, the release of soluble lysosomal enzymes in the extracellular space is strongly reduced compared to healthy fibroblasts, suggesting that MCOLN1 plays an important role in this process (LaPlante et al. 2006). Further, transfection with wild-type MCOLN1 cDNA rescues exocytosis, suggesting the possibility of treatments based on the restoration of this crucial cellular function (LaPlante et al. 2006).

Another example is Niemann-Pick disease type 1 (NPC1); in fibroblasts derived from mouse models, cholesterol accumulation inhibits Rab guanosine triphosphatases (GTPases), thus perturbing membrane recycling; overexpressing Rab4 in these cells led to an activation of exocytosis and reduced lysosomal accumulation (Choudhury et al. 2004). Thus, enhancing exocytosis can not only reduce the storage burden, but also improve secondary phenotypes.

Transcriptional regulation of lysosomal biogenesis

A commonly reported observation in several LSDs is a decrease in the activity of a disease-associated enzyme are concomitant with increases in other lysosomal enzymes, suggesting that gene expression required for their interdependence is similarly orchestrated (Schultz et al. 2011). On the other hand, it has been shown that expansion of the lysosomal compartment, which is a

38

common feature of all LSDs, is usually accompanied by increased activity (and release) of many lysosomal proteins (Moran et al. 2000). Thus, a coordinated control mechanism with enhanced transcriptional expression was long suspected.

Recently in our laboratory it was observed, by using the g:Profiler tool (Reimand et al. 2007), that genes encoding lysosomal proteins (hereafter referred to as lysosomal genes) tend to have coordinate expression. Furthermore, in most of them is present a common palindromic motif, which was named Coordinated Lysosomal Expression and Regulation (CLEAR) and which mediates their transcriptional activation (Sardiello et al. 2009). The CLEAR motif is a 10-base pair neucleotidic sequence (GTCACGTGAC), which is placed near the transcription start site of many lysosomal genes and is the target site of the bHLH transcription factor TFEB (Figure 7). It was found that most lysosomal genes share one or more copies of the regulatory motif in their promoters. The CLEAR network is comprised of several classes of genes, including genes that belong to the lysosomal complement (hydrolases, transporters, accessory proteins) and genes that participate in lysosomal biogenesis and function. Examples of the latter class are genes encoding subunits of the vacuolar proton pump, responsible for creating and maintaining the lysosomal acidic environment, and genes coding for the specialized transporters that import acid hydrolases into the lysosome.

It was observed that TFEB overexpression not only increases the number of lysosomes in the cell, but also improves cellular degradative capabilities. In fact, it was tested the clearing potential of TFEB on a neuronal cell model of Huntington's disease (HD), and found that TFEB transfection increased the clearance of the pathogenic polyglutamine-expanded huntingtin (Sardiello et al. 2009). These data

uncovered a regulatory network defining TFEB as a master regulator of lysosomal biogenesis. Furthermore, in cultured cell models of mucopolysaccharidosis type II (MPSII), MPSIIIA, and MSD, TFEB localizes primarily in the nucleus, but is cytoplasmic in control cells, suggesting that this pathway is activated under lysosomal storage conditions (Sardiello et al. 2009).

The observation that the process of lysosomal degradation is transcriptionally regulated raised the hypothesis that other lysosomal-related processes, like autophagy, might also be transcriptionally regulated by the same mechanism. In principle, enhancement of lysosomal function should result in a decrease in the number of autophagosomes due to increased degradation, whereas the opposite should occur in the presence of lysosomal inhibitors. However, it was observed that TFEB overexpression increases the number of autophagosomes and, conversely, RNA interference of TFEB decreases the number of autophagosomes, suggesting a role of TFEB in the regulation of the autophagic process as well (Settembre et al. 2011).

Interestingly, the fact that TFEB also regulates key autophagy switches indicates that the CLEAR network extends beyond the lysosomal complement to favor lysosome-dependent degradative pathways as a whole (Sardiello and Ballabio 2009).

Moreover, it was also hypothesized that TFEB may mediate starvation-induced autophagy. Interestingly, it was observed that upon starvation TFEB translocates from the cytoplasm (where it normally resides) to the nucleus where it is active and regulates the expression of its target genes (Settembre et al. 2011). This translocation occurs in a phosphorylation-dependent manner. In fact, it was

demonstrated that TFEB is maintained inactive in the cytoplasm through its phosphorylation on a critical serine residue (Ser142), and then it translocates to the nucleus when dephosphorylated (Settembre et al. 2012).

In particular, it was also shown that TFEB phosphorylation occurs on the lysosomal membrane by the master growth regulator, mammalian target of rapamycin (mTOR) complex 1 (mTORC1) (Settembre et al. 2012). Therefore, it was proposed the hypothesis that under full nutrients and in the absence of lysosomal stress the interaction between the lysosomal amino acid content and the v-ATPase complex, involved in lysosomal acidification, regulates Rag GTPases, which in turn activate mTORC1 by translocating it to the lysosomal surface (Sancak et al. 2008; Zoncu et al. 2011). At the lysosome, mTORC1 binds and phosphorylates TFEB, therfore controlling its subcellular localization and its inactive state. Indeed, phosphorylation by mTORC1 maintains TFEB in the cytoplasm and prevents it from translocating to the nucleus. Starvation, v-ATPase inhibition, or lysosomal stress switch the Rags off, leading to mTORC1 detachment from the lysosome and to its inactivation. TFEB can no longer be phosphorylated and thus it translocates to the nucleus, where it activates gene expression programs that boost lysosomal function and autophagy. These data demonstrated that TFEB mediates starvation-induced autophagy (Settembre et al. 2012).

Therefore, TFEB acts both as a sensor of lysosomal state, when on the lysosomal surface, and as an effector of lysosomal function when in the nucleus. This unique lysosome-to-nucleus signalling mechanism allows the lysosome to regulate its own function (Settembre et al. 2012).
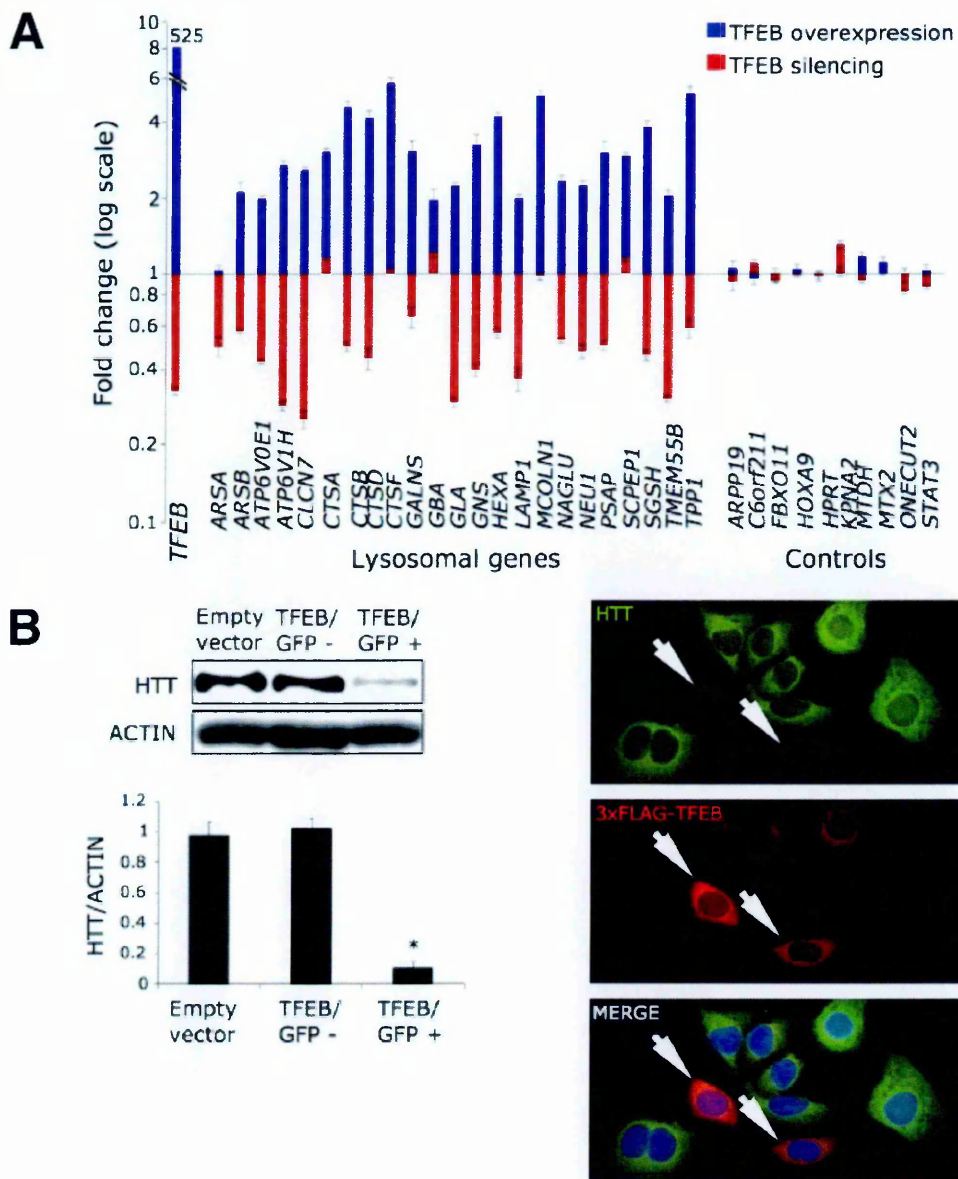
Figure 7. TFEB controls the expression of lysosomal genes and enhances cellular clearance.

(A) Expression analysis of lysosomal genes following TFEB overexpression and silencing. Blue bars show the fold change of the mRNA levels of lysosomal genes in TFEB- vs. pcDNA3-transfected cells. Red bars show the fold change of mRNA levels in mimic-miR-128-transfected cells vs. cells transfected with a standard control microRNA (mimic-miR-cel-67). (B) Immunoblot analysis of TFEBEGFP-positive (+) and TFEB-EGFP-negative (-) HD43 cells (left panel) and immunofluorescence analysis of TFEB and HTT in HD43(Q105) cells transfected with 3xFLAG-TFEB construct (right panel).

Taken from Sardiello et al., 2009

Neural Stem Cells

Stem cells are undifferentiated cells that are capable of giving rise to indefinitely more cells of the same type, and from which certain other types of cell arise by differentiation. Indeed, stem cells exhibit two defining characteristics: the self-renew, which is the ability to go through numerous cycles of cell division while maintaining the undifferentiated state, and the potency, which is the capacity to generate a diverse range of specialized cell types through differentiation (Gage 2000).

There are two types of mammalian stem cells: embryonic and adult. Embryonic tem cells (ESCs) derive from the inner cell mass of the blastocyst and are totipotent, which means that they can differentiate into all of the specialized embryonic tissues. Adult stem cells are found in adult tissues and act as a repair system for the body, replenishing specialized cells, but also maintaining the normal turnover of regenerative organs; unlike ESCs, they are often restricted to certain lineages (for exemple hematopoietic or neural fate) (Figure 8).

NSCs are self-renewing multipotent populations present in the developing and adult mammalian CNS (Chojnacki and Weiss 2008; Temple 2001). During the process of neurogenesis, NSCs generate the neurons and glia of the developing brain and also account for the limited regenerative potential of the adult brain.

Neurogenesis is the process of generating functional neurons from precursors and in mammals it begins with the induction of the neuroectoderm, which forms the neural plate (at embryonic day 7.5 (E7.5) in mice) and then folds to give rise to the

43

neural tube (at E8.5 in mice). These structures are made up by a layer of so-called neuroepithelial progenitors (NEPs) (Götz and Barde 2005), which are probably a complex and heterogeneous population. During this neural differentiation, ESCs undergo progressive lineage restrictions, leading to the generation of a range of distinct neural precursor populations that can be used to study the molecular and cellular events that occur during stage-specific transitions between different populations (Nishikawa, Jakt, and Era 2007; S.-C. Zhang 2006, 200).

Neurogenesis was traditionally viewed to occur only during embryonic and perinatal stages in mammals (Ming and Song 2005). Nevertheless, Altman's pioneering studies decades ago provided the first anatomical evidence for the presence of newly generated dentate granule cells in the postnatal rat hippocampus (Altman and Das 1965). Multipotent neural stem cells were later derived from the adult mammalian brain (Reynolds and Weiss 1992; Richards, Kilpatrick, and Bartlett 1992). Since then, significant progress has been made over the past decades in the study of almost every aspect of adult neurogenesis in the mammalian CNS.

In general, adult neurogenesis recapitulates the complete process of neuronal development in embryonic stages, but is spatially restricted under normal conditions to two specific "neurogenic" brain regions: the subgranular zone (SGZ) in the dentate gyrus of the hippocampus, where new dentate granule cells are generated, and the subventricular zone (SVZ) of the lateral ventricles in the forebrain, where new neurons are generated and then migrate through the rostral migratory stream (RMS) to the olfactory bulb to become interneurons (Gage 2000; Ming and Song 2011).

In the adult SGZ, proliferating radial and non-radial precursors give rise to intermediate progenitors, which in turn generate neuroblasts. Immature neurons migrate into the inner granule cell layer and differentiate into dentate granule cells in the hippocampus (Ming and Song 2012).

In the adult SVZ, proliferating radial glia-like cells give rise to transient amplifying (TA) cells, which in turn generate neuroblasts. In the RMS, neuroblasts form a chain and migrate toward the olfactory bulb through a tube formed by astrocytes (Lois, García-Verdugo, and Alvarez-Buylla 1996). Once reaching the core of the olfactory bulb, immature neurons detach from the RMS and migrate radially toward glomeruli where they differentiate into different subtypes of interneurons (Lledo, Alonso, and Grubb 2006).

Therefore, the SGZ and the SVZ are the two main niches that *in vivo* support self-renewal and regulate the balance between symmetrical self-renewal, by which NSCs proliferate, and fate-committed asymmetrical division, by which NSCs generate differentiated cells (Alvarez-Buylla and Lim 2004; Garcion et al. 2004; Shen et al. 2004; Shen et al. 2008). In fact, niches are defined as microenvironments that anatomically house stem cells and functionally control their development *in vivo*.

The size of the NSC pool in the SVZ is much larger than that in the SGZ (Lois and Alvarez-Buylla 1993; Morshead et al. 1994).

Due to the constitutive migration and high proliferation rate of neural progenitor cells in the SVZ (Lucassen et al. 2010; Curtis, Kam, and Faull 2011), it has been proposed that the neuronal differentiation of NSCs in the SVZ might be particularly important for the autonomous repair of the brain during the

pathogenesis of a disease. In fact, studies based on animal disease models revealed that neural progenitor cells in the SVZ can also migrate to regions where neuronal disorders occur to compensate for the loss of living neurons (Arvidsson et al. 2002; Tattersfield et al. 2004).

The proliferation and differentiation of NSCs are strictly regulated by a complex regulatory system, including a variety of intrinsic and extrinsic mechanisms, such as morphogens and growth factors, as well as transcription factors and epigenetic mechanisms. Morphogenic proteins and transcription factors are the fundamental forces that determine the fate of NSCs and the process of neurogenesis. Morphogens are a group of proteins that not only are vital for the embryonic development and patterning of the brain, but also function to regulate the self-renewal and differentiation of NSCs in the adult brain. This group of proteins includes Notch, sonic hedgehog (Shh), wingless-type MMTV integration site family (Wnt), fibroblast growth factor (FGF), and bone morphogenetic proteins (BMPs). During the process of embryonic development, ESCs in the ectoderm differentiate into excitatory and inhibitory neurons. These morphogenetic proteins, combined at different concentrations, act to pattern the brain along the anterior-posterior and dorsal-ventral axis into different regions. For instance, FGF is responsible for the anterior-posterior patterning and BMP and Shh are specific for the dorsal-ventral patterning; Wnt contributes to both processes. In the adult brain, the morphogenic proteins continue to subtly modulate the number and differentiation of neural precursor cells. In both the SGZ and the SVZ, Shh is essential for the maintenance of radial glia-like cells (Ahn and Joyner 2005; Balordi and Fishell 2007; Han et al. 2008), whereas Notch is fundamental for the neuronal

46

differentiation of NSCs (Imayoshi et al. 2010; Pierfelice, Alberi, and Gaiano 2011). Wnt can also promote the neuronal differentiation of NSCs in the SGZ (Lie et al. 2002), whereas BMP determines their glial fate (Lim et al. 2000; Bonaguidi et al. 2005; Mira et al. 2010).

The concurrent action of these morphogens via a concentration gradient leads to the differentiated expression of hundreds of transcription factors to determine the fate of newborn neurons. However, limited information is available on the roles that transcription factors play in NSC differentiation in the adult brain, and only a handful of transcription factors have been extensively studied. One such factor is Sox2, which is present in both the SGZ and SVZ, and colocalizes with NSC markers glial fibrillary acidic protein (GFAP), nestin, brain lipid-binding protein (BLBP), and Musashi-1 (Ferri et al. 2004, 200; Komitova and Eriksson 2004; Suh et al. 2007; Lugert et al. 2010). Evidence based on transgenic mice revealed that Sox2 acts probably through an interactive regulation with Notch signaling (Taranova et al. 2006; Ehm et al. 2010). Another well-studied transcription factor is Pax6, which is expressed in NSCs in the SGZ (Maekawa et al. 2005; Nacher et al. 2005; Hodge et al. 2008; Roybon et al. 2009) and in neuroblasts in the SVZ (Herold et al. 2011; Jones and Connor 2011) and functions to promote the dopaminergic fate determination of NSCs (Kohwi et al. 2005; Brill and Huguenard 2008; Spitere et al. 2008).

On the other hand, abundant evidence supports the finding that NSC differentiation and proliferation in the adult SVZ and SGZ is closely regulated by the local environmental factors, such as surrounding neurons, astrocytes, and other non-neuronal cells (Suh, Deng, and Gage 2009), either in an activity relevant

47

manner or through growth factor release.

In general, endogenous NSCs in their undifferentiated state are recognized for the expression of the nestin marker. The neural specific intermediate filament nestin, in fact, is utilized to visualize the regions of neurogenesis throughout the life of the animals. Cells that are immunoreactive for nestin are thought to be involved in neurogenesis (Doyle, Khan, and Cunningham 2001; Yue et al. 2006), and therefore to differentiate into neurons and glial cells (Itoh et al. 2006). During brain development, nestin is expressed by radial glia cells, and nestin expression starts to disappear around postnatal day 11 (P11) in the rat cortex (Kálmán and Ajtai 2001). Based on these data, nestin might provide an ideal marker to examine neurogenesis within the adult brain.

During the development of the CNS, NSCs are precursors of glia and neurons, both characterized by the expression of their own specific markers, GFAP and neuron specific class III β-tubulin (Tuj1), respectively.

GFAP is widely known as a marker for mature astrocytes in the adult brain. A large proportion of the newborn cells in the SGZ of the hippocampal region have also been found to be GFAP-positive (Eckenhoff and Rakic 1988; Maslov et al. 2004). However, the use of GFAP as a marker for neurogenesis is hampered by the finding that the glial cell lineage and mature astrocytes are also labeled. Thus, the nestin-positive, but not the GFAP-positive, precursors are the precursors involved in neurogenesis (Cao et al. 2006).

Tuj1 expression starts as early as embryonic day 8.5 (Easter, Ross, and Frankfurter 1993) and can be detected throughout brain development (Menezes and Luskin 1994). Tuj1 has been found to label newly generated immature

postmitotic and differentiated neurons (Menezes and Luskin 1994). With respect to adult neurogenesis, Tuj1 is used as a neuron-specific marker of newly generated cells (F Doetsch, García-Verdugo, and Alvarez-Buylla 1997; Gould et al. 2001).
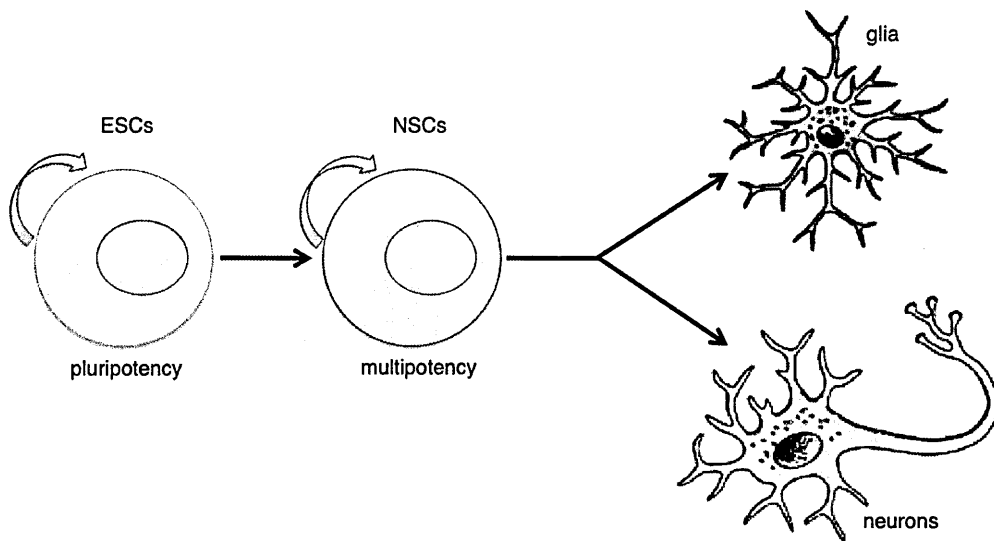

Figure 8. Schematic diagram illustrating lineage commitment of adult neural stem cells.

Pluripotent ESCs can form any body tissue (except for the placenta). During development, cells derived from these stem cells become progressively more specialized, like NSCs that are multipotent precursors of the two main cell type of CNS, glia and neurons.

Isolation of adult Neural Stem Cells

Isolation of NSCs from their adult natural niche and their purification and expansion have been problematic, as the factors and cell contacts required to maintain these cells in their physiological state are poorly understood (Conti and Cattaneo 2010). However, epidermal growth factor (EGF) and FGF2 have been key players in the identification of cell culture conditions that sustain prolonged cell division of cells with NSC properties (Reynolds and Weiss 1992; Reynolds and Weiss 1992; Laywell, Kukekov, and Steindler 1999).

Two main strategies have been developed for NSC isolation and *in vitro* long-term propagation, the neurosphere system and the monolayer system.

Neurosphere system

Neurospheres are free-floating aggregates of neural progenitors, each potentially derived from a single NSC (Reynolds and Weiss 1992; Reynolds, Tetzlaff, and Weiss 1992; Laywell, Kukekov, and Steindler 1999). Their generation relies on tissue microdissection followed by exposure to mitogens (Chojnacki et al. 2008). Commonly, mouse and rat neurospheres are harvested from neural tissue at E10.5-E18.5 or from the adult SVZ (F Ciccolini 2001; Francesca Ciccolini et al. 2005; F Ciccolini and Svendsen 1998; Gritti et al. 1995; Louis and Reynolds 2005; Svendsen et al. 1998; Tropepe et al. 1999; Uchida et al. 2000). For their expansion, cells are plated in low-attachment tissue culture plastic dishes in serum-free media supplemented with EGF (10-20 ng per ml) and/or FGF2 (10-20 ng per

ml) (Singec et al. 2006). In these conditions, most differentiating or differentiated cells are expected to die, whereas the NSCs respond to the mitogens, divide and form floating aggregates (primary neurospheres) that can be dissociated and re-plated to generate secondary neurospheres. This procedure can be repeated several times to expand a NSC population.

Neurospheres have been used *in vitro* for defining, by extrapolation, the persistence and properties of NSCs *in vivo* (Golmohammadi et al. 2008; Marshall, Reynolds, and Laywell 2007). In fact, the cellular milieu of the neurosphere has been suggested to provide an *in vitro* counterpart to the *in vivo* neurogenic compartment, a microenvironment that is relevant for NSC maintenance, proliferation and differentiation. The concept of a neurosphere as an *in vitro* recapitulation of a niche-like structure has become extremely popular in the NSC field (Conti and Cattaneo 2010). The regulation of stem cell features in the niche requires both interactions between stem cells and interactions between stem cells and neighbouring differentiated cells, mediated by soluble and adhesion molecules and extracellular matrix components (Conti and Cattaneo 2010).

However, it has been demonstrated the tendency of neurospheres to generate differentiated cells in their core (Campos 2004), since different cells in the sphere can be exposed to suboptimal conditions due to their three-dimensional structure. Consequently, the interaction between differentiating cells and precursor cells may expose the NSCs to paracrine factors that promote differentiation. Therefore, the maintenance of the neurogenic versus gliogenic potential gradually declines with *in vitro* passages (Conti and Cattaneo 2010). Hence, neurospheres can be considered as the *in vitro* counterpart of the *in vivo* niche structure only for a

limited time after brain dissection.

Different neuronal differentiation protocols based on mitogen removal and exposure to fetal bovine serum and/or to specific substrates and cytokines have been developed (Garcion et al. 2004; Chojnacki and Weiss 2008; Tropepe et al. 1999; Grandbarbe et al. 2003; Weiss et al. 1996), but none of them generates cells that are positive for the early neuronal marker Tuj1 at a proportion greater than 20%. On the whole, this suggests that the neurosphere system is not particularly efficient in terms of neurogenic competence, but it can be useful for studying self-renew abilities and proliferation capacities of NSCs after tissue dissociation.

Monolayer system

Early attempts to culture NSCs in monolayer conditions relied on plating them on polyornithine-, laminin- or fibronectin-coated dishes in serum-free media (Johe et al. 1996) and in the presence of morphogens (Palmer, Takahashi, and Gage 1997). These cells show homogeneity for nestin and Sox2 expression, and symmetrical cell division continuously replenishes the supply of multipotent progenitors.

More recently, other strategies for the derivation and stable long-term propagation of NSC lines from different sources of rodent (Conti et al. 2005; Pollard et al. 2006, 200) and human (Sun et al. 2008) origin have been described. According to these procedures, neural precursors can be competently expanded as adherent, clonal, uniform NSC lines by exposure to EGF and FGF2 (Elkabetz et al. 2008; Koch et al. 2009; Conti et al. 2005). Under these conditions cells divide

symmetrically, retaining their tripotential differentiation capacity, indicating that monolayer culture systems can maintain almost pure NSC populations (Conti et al. 2005), with a negligible differentiated component. The key aspect of the NSC culture system lies in the combination of EGF and FGF2 used and the focus on cells that grow adherently. The continuous provision of EGF together with FGF2 seems to be essential for the derivation and propagation of these monolayer-growing NSCs (Palmer, Takahashi, and Gage 1997). When grown in these conditions, NSC population shows a remarkable antigenic similarity to forebrain neurogenic radial-glia (RG) (Conti et al. 2005; Pollard et al. 2006). The fact that NSCs can also be established from long-term expanded neurospheres indicates that RG-like cells might be the NSC fraction in neurospheres and that monolayer growth conditions may allow their enrichment and subsequent expansion (Conti et al. 2005).

Interestingly, cells in these EGF- and FGF2-dependent monolayers retain multipotency and neurogenic efficiency also after prolonged *in vitro* expansion and show a high competence to efficiently originate antigenically and electrophysiologically mature neurons on exposure to optimized differentiating conditions (Koch et al. 2009; Conti et al. 2005; Spiliotopoulos et al. 2009; Goffredo et al. 2008). This capacity can probably be interpreted as a consequence of the homogeneity of the starting population.

AIM

The main objective of the research described in this thesis is to establish a cellular system to study the mechanisms of neurodegeneration in MSD pathology. This *in vitro* system must resemble the main hallmarks of the disease, such as the primary accumulation of undegraded substrates, secondary accumulation of undigested substrates, impaired autophagy and increased apoptosis.

Although cell death characterizes the later stages of the disease, for most of the clinical course the pathology mainly involves neuronal dysfunction rather than loss. Therefore, the ultimate goal will be to elucidate the intracellular mechanisms that lead to neuronal dysfunction after the pathologic accumulation of undegraded substrates.

Therefore, my thesis project aimed firstly at the isolation and characterization of NSCs from MSD and wild-type adult mice. For this purpose, I successfully built a robust protocol to isolate NSCs from postnatal brain tissue; once established in culture, I defined the progenitor identity of these cells. In order to assess whether NSCs could be a reliable tool to clarify the mechanisms of neurodegeneration in MSD pathology, I focused on the characterization of MSD-NSCs and on investigating whether they also reflect the main hallmarks of the disease, such as the progressive accumulation of undegraded GAGs, impaired autophagy and high levels of apoptosis, as seen in adult mice.

Furthermore, establishing an *in vitro* system that recapitulates the main hallmarks of LSDs would be useful also to identify relevant pathogenic intracellular

pathways leading to the developing of new therapeutics strategies for the treatment of MSD, as well as other neurodegenerative LSDs. An intriguing therapeutic strategy that allows us to test our *in vitro* system came from studies carried out in our laboratory on the transcription factor TFEB, a master gene for lysosomal biogenesis and function. TFEB overexpression is able to reduce mutant huntingtin in a cellular model of HD (Sardiello et al. 2009). It was shown that TFEB is able to increase the capacity of the lysosome to degrade by increasing the bulk of lysosomal components. Therefore, we tested the role of TFEB in the clearance of pathologic undegraded substrates in isolated MSD-NSCs. Surprisingly, we found that TFEB overexpression is able to reduce primary accumulation of GAGs in differentiated MSD-NSCs by inducing lysosomal exocytosis (Medina et al. 2011). Therefore, our studies envisage a novel tool, by targeting lysosomal exocytosis mechanism, to reduce the lysosomal burden in storage diseases.

To achieve all these objectives, I selected the following main goals:

(1)   isolation and characterization of NSCs from MSD mice;

(2)   phenotyping of MSD-NSCs to investigate the intracellular cascades involved in neurodegeneration;

(3)   modulating lysosomal function as a novel tool to treat LSDs.

RESULTS

Isolation of NSCs from post-natal brain of wild-type and MSD mice

The crucial point of this research project is the isolation of NSCs from the brain of postnatal wild-type and MSD mice. Recently, it has been established a methodology that allows for the isolation and continuous *ex vivo* expansion of NSCs from mouse brain tissue that can be easily cryopreserved and readily differentiate into neurons and glial cells (Conti et al. 2005).

Two steps basically constitute the NSCs isolation protocol carried out in this project: (1) neural tissue dissociation and (2) positive selection of NSCs with anti-prominin-1 MicroBeads (MACS, Miltenyi Biotec) (Peh et al. 2009) (Figure 9). In the first step, after brain tissue micro-dissection the cortical hemispheres dissociation is achieved with an enzymatic degradation using papain, a protease able to breaks the extracellular adhesion proteins holding the cells together; in this way, the tissue can be dissociated to single-cell suspensions. Then, the second step leads to an enrichment of the cell population in NSCs. In order to do that, I incubated the cell suspension with anti-prominin-1 antibody. Prominin-1 is a transmembrane glycoprotein expressed in various stem cells, including those from postnatal CNS (Peh et al. 2009). Therefore, NSCs were magnetically labeled with anti-prominin-1 MicroBeads and loaded onto a MACS® Column, which is placed in the magnetic field of a MACS Separator. The magnetically labeled prominin-1+ cells are retained within the column whereas the unlabeled cells run through. After removal of the

column from the magnetic field, the magnetically retained prominin-1+ cells can be eluted as the positively selected cell fraction.

As reported in literature (Conti et al. 2005), *in vitro* NSCs grow in so-called neurospheres, floating heterogeneous aggregates of cells, containing a large proportion of stem cells. Isolated neurospheres were propagated in culture at clonal density on uncoated plates in medium containing EGF and FGF2 and with penicillin/streptomycin (NS expansion medium). Over 3-5 days, cells formed aggregates that, after harvesting and sedimentation to remove debris, subsequently outgrew NSCs.

From neurospheres, NSCs were conveniently dissociated to single cells and plated directly on coated plates, to finally obtain a monolayer cell culture. These cells were propagated in culture with NS expansion medium. The entire isolation protocol was carried out for three wild-type and three Sumf1 -/- mice.

(1) Neural tissue dissociation with papain enzymatic activity

Dissection of both hemispheres (cortical regions) from postnatal pups

Place brain on dish, cut into small pieces and collect pieces

Add enzyme and incubate at 37°C

Dissociate mechanically and incubate at 37°C

Apply to a cell strainer, wash and centrifuge

Add anti-prominin 1 Micro Beads

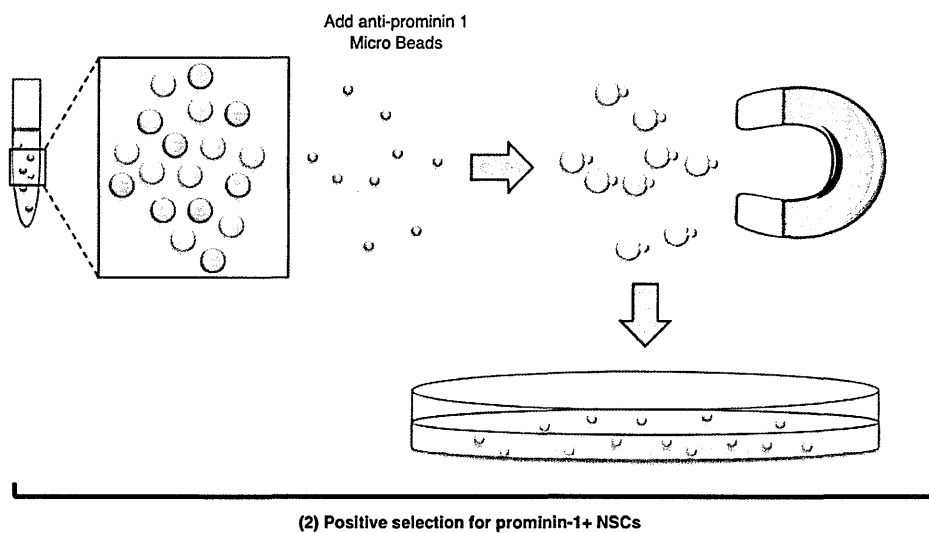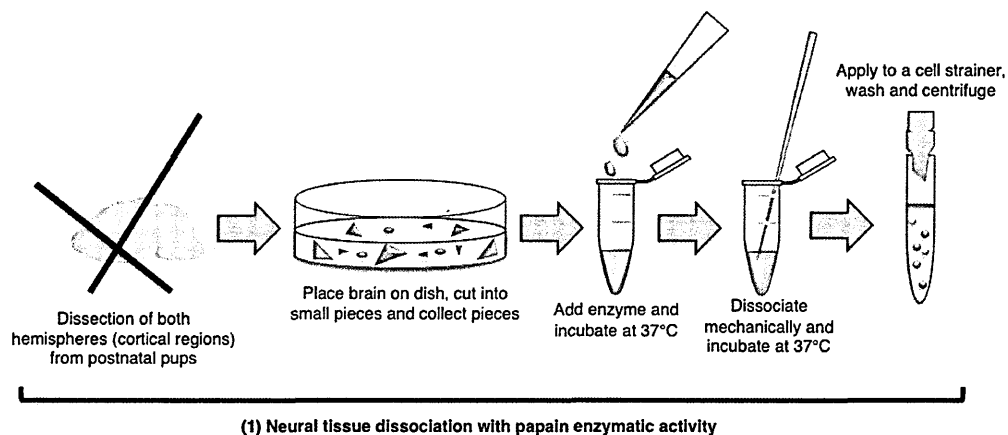**(2) Positive selection for prominin-1+ NSCs**

Figure 9. NSCs isolation protocol

NSCs are isolated following a two step protocol: (1) brain tissue micro-dissection and neural tissue dissociation with papain, and (2) labeling of NSCs with magnetic anti-prominin-1 MicroBeads (MACS, Miltenyi Biotec) and positive selection through the magnetic field of a MACS Separator (MACS, Miltenyi Biotec).

58

NSCs uniformly express morphological and molecular features of radial glia progenitors

Once established in culture, NSCs showed the typical elongated bipolar morphology with lamellate extensions, end-feet and oval nuclei anticipated for radial glia, with no substantial differences between MSD cells and their wild-type counterpart (Figure 10-A). In these conditions, NSCs proliferate continuously, with a doubling time of around 25 hours.

By flow cytometry analysis I observed that around 99.3% of the wild-type cells and 98.8% of MSD cells expressed the NSC marker nestin, suggesting that isolated NSCs have a defined progenitor identity (Figure 10-B).

Therefore, I analyzed their progenitor signature at a molecular level, testing a set of markers commonly used to define this cell population (Figure 11). By RT-PCR, I found that both wild-type and MSD-NSCs lacked the pluripotency marker genes typical of embryonic stem cells such as *oct4* and *nanog* (Schöler et al. 1990; Cavaleri and Schöler 2003), whereas they expressed Pax6, and BLBP mRNAs (Hack et al. 2004; Feng, Hatten, and Heintz 1994, 199). In addition, NSCs expressed the neural precursor markers *olig2, Sox2* and *mash1* (Gabay et al. 2003; Lo et al. 1991) and lacked expression of *dlx2*, a marker of transient amplifying neuroblasts but not of NSCs (Fiona Doetsch et al. 2002) and, as expected, they did not express the marker of neuronal differentiation NF-L (Schimmelpfeng, Weibezahn, and Dertinger 2004). This set of markers is considered diagnostic for neurogenic radial glia, precursors of both neurons and astrocytes during development of the nervous system (Campbell and Götz 2002;

59

Hartfuss et al. 2001; Noctor et al. 2001).

Therefore, NSCs isolated from wild-type as well as those isolated from MSD affected littermates uniformly express morphological and molecular features of radial glia progenitors.
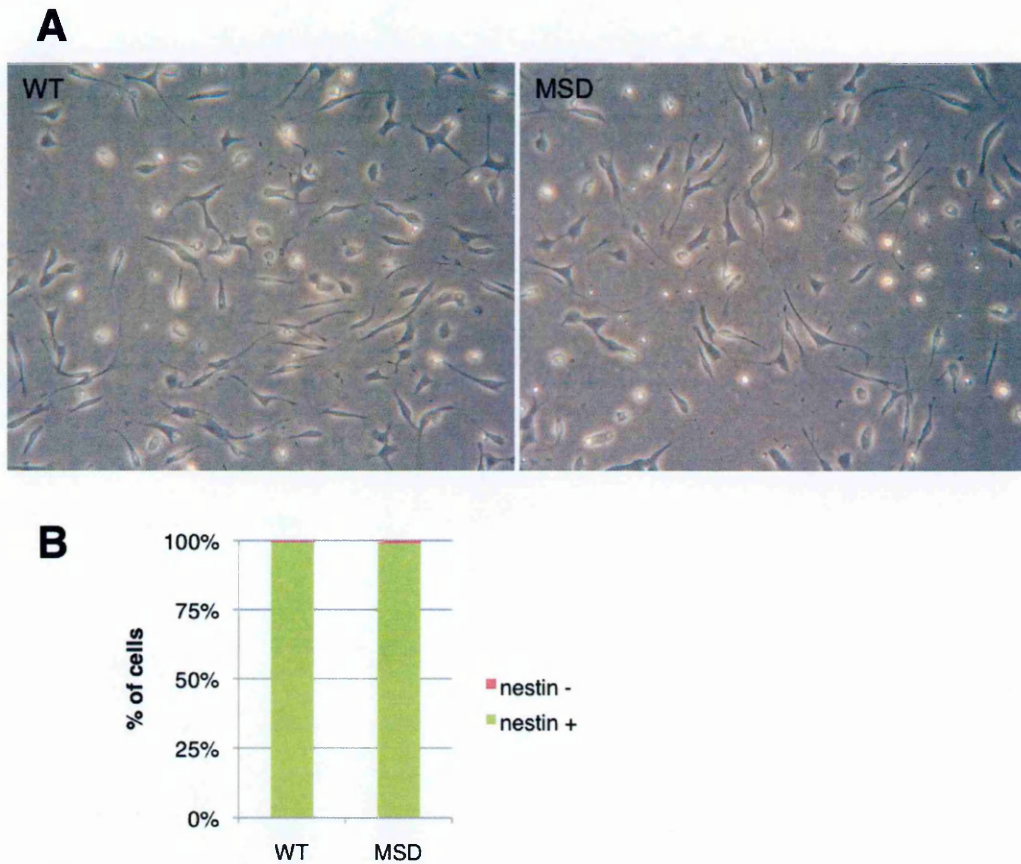
Figure 10. NSCs exhibit morphologic similarities to radial glia

(A) Bright field pictures of isolated wild-type (WT) and MSD-NSCs showed elongated bipolar morphology with lamellate extensions, end-feet and oval nuclei anticipated for radial glia, and (B) Flow cytometry analysis revealed a high expression of the neurogenesis marker nestin in WT- (99.3 %) and MSD-NSCs (98.8 %).
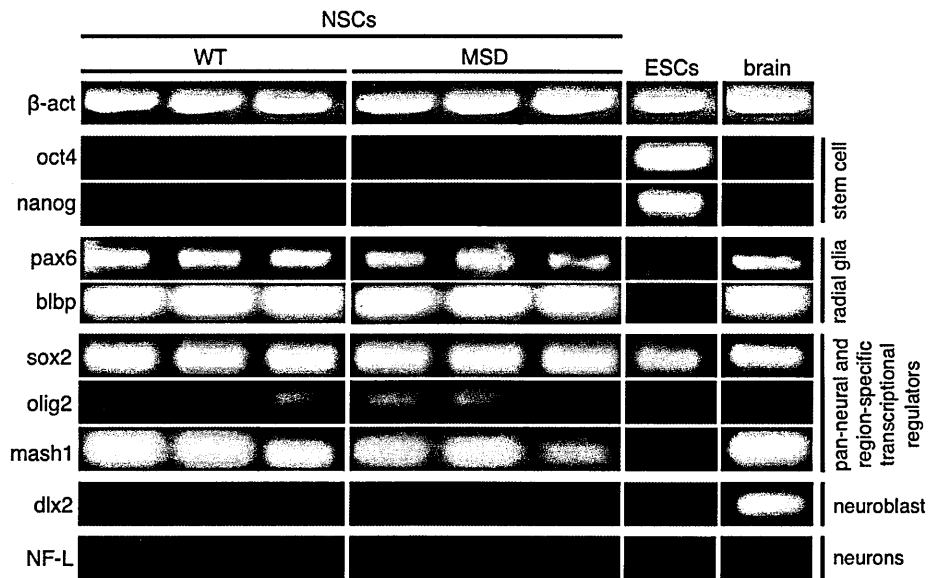
Figure 11. NSCs exhibit phenotypic similarities to radial glia

Expression pattern of a set of markers by RT-PCR on cDNA obtained by RNA retrotrnscription. RNA extraction was obtained from wild-type (WT) and MSD undifferentiated NSCs, from wild-type ESCs, and from wild-type mouse brain. Genes tested were divided in five different groups: stem cell markers (oct4, nanog), radial glia markers (pax6, blbp), pan-neuronal and region specific transcriptional regulators (sox2, olig2, mash1), a neuroblast marker (dlx2), and a differentiated neuron marker (NF-L).

MSD-NSCs present a progressive loss of self-renewal

To assess whether in absence of SUMF1 protein NSCs display a defect in their ability to self-renew, I tested them with the neurosphere assay (Figure 12). Cells were plated as single cells at a density of $5x10^4$ cells/ml in medium containing FGF2 and EGF. After incubation for 7 days, primary neurospheres were counted and then dissociated and re-plated in the same conditions; similarly, I generated secondary and tertiary neurospheres. I observed a significant decrease in the number of MSD tertiary neurospheres compared to wild-type counterpart, thus suggesting that accumulation of undegraded substrates is likely to impact on the ability of NSC population to self-renew. Interestingly, it was demonstrated that NSCs isolated from a mouse model of another neurodegenerative lysosomal disorder, Niemann-Pick disease type 1 (NPC1) show a similar defect in the self-renew (Yang et al. 2006); although a greater understanding of the machanisms involved in this defect is needed, this observation raises the possibility that neurodegeneration observed in LSDs may be characterized by a general reduction in the NSCs population, due to a defect in their self-renew ability.
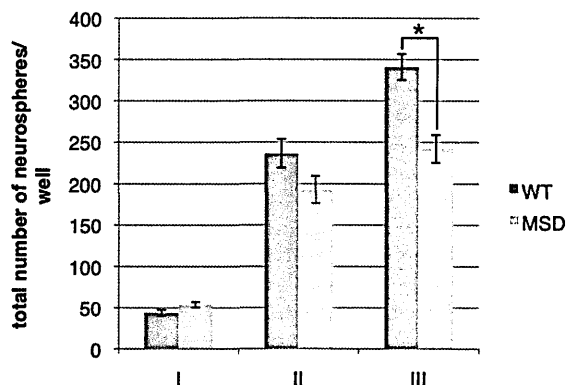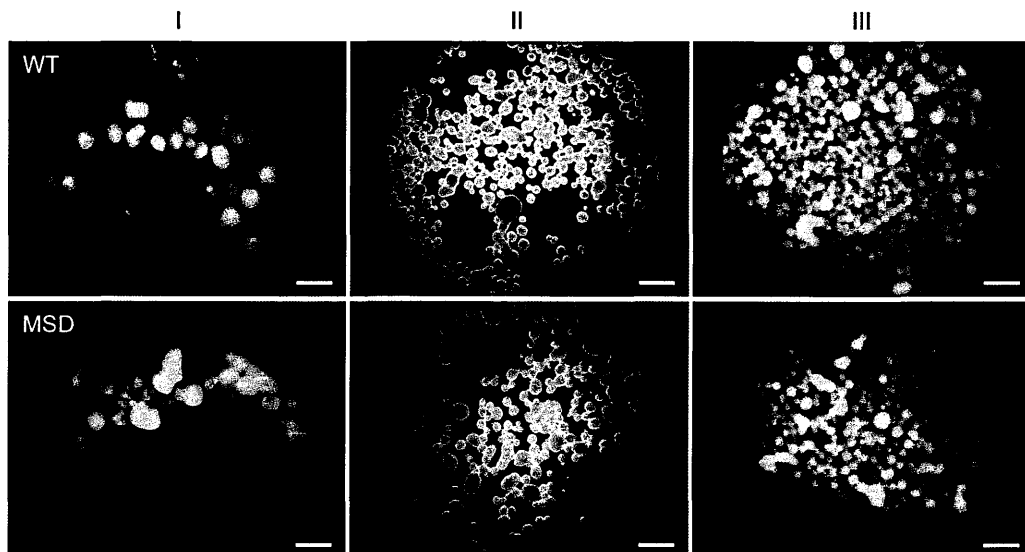
Figure 12. MSD neurospheres show an impaired self-renewal

Bright field images of neurospheres assay. $5x10^4$ cells/ml were plated in NS expansion medium and formed primary neurospheres (I); after 7 days primary neurospheres were counted and then dissociated and re-plated in the same conditions, generating secondary neurospheres (II); similarly, tertiary neurospheres (III) were generated. Scale bars represent 200 μM. Data are mean of replicates (n=30) ± SEM; *p < 0.05 by Student's t-test.

MSD-NSCs are able to differentiate into astrocytes and neurons

Neural progenitors are committed cells with multipotential fate. To confirm the nature of isolated NSCs, I tested their ability to give rise to the two main cell types of the CNS: astrocytes and neurons. Thus, cell differentiation was studied at molecular level by immunofluorescence and immunoblotting using specific antibodies for differentiation markers, such as GFAP for glial differentiation, and Tuj1 for neuronal cells. Fully differentiation capacity was assessed by immunofluorescence looking at the absence of nestin expression after glial or neuronal differentiation protocols (Figure 13-A).

As expected, both wild-type and MSD-NSCs showed uniform expression of the progenitor marker protein nestin when kept in the undifferentiated state. Moreover, wild-type differentiated cells showed a uniform expression of GFAP or Tuj1, after glial or neuronal differentiation, respectively; at the same time, they did not show expression of nestin. In a similar way, MSD-NSCs were able to differentiate into glia and neurons, showing the same uniform expression of GFAP and Tuj1, respectively, and no expression of nestin. The same results were obtained by western blot analysis of protein homogenates from wild-type and MSD-NSCs, both undifferentiated and differentiated to glial and neural cells (Figure 13-B).

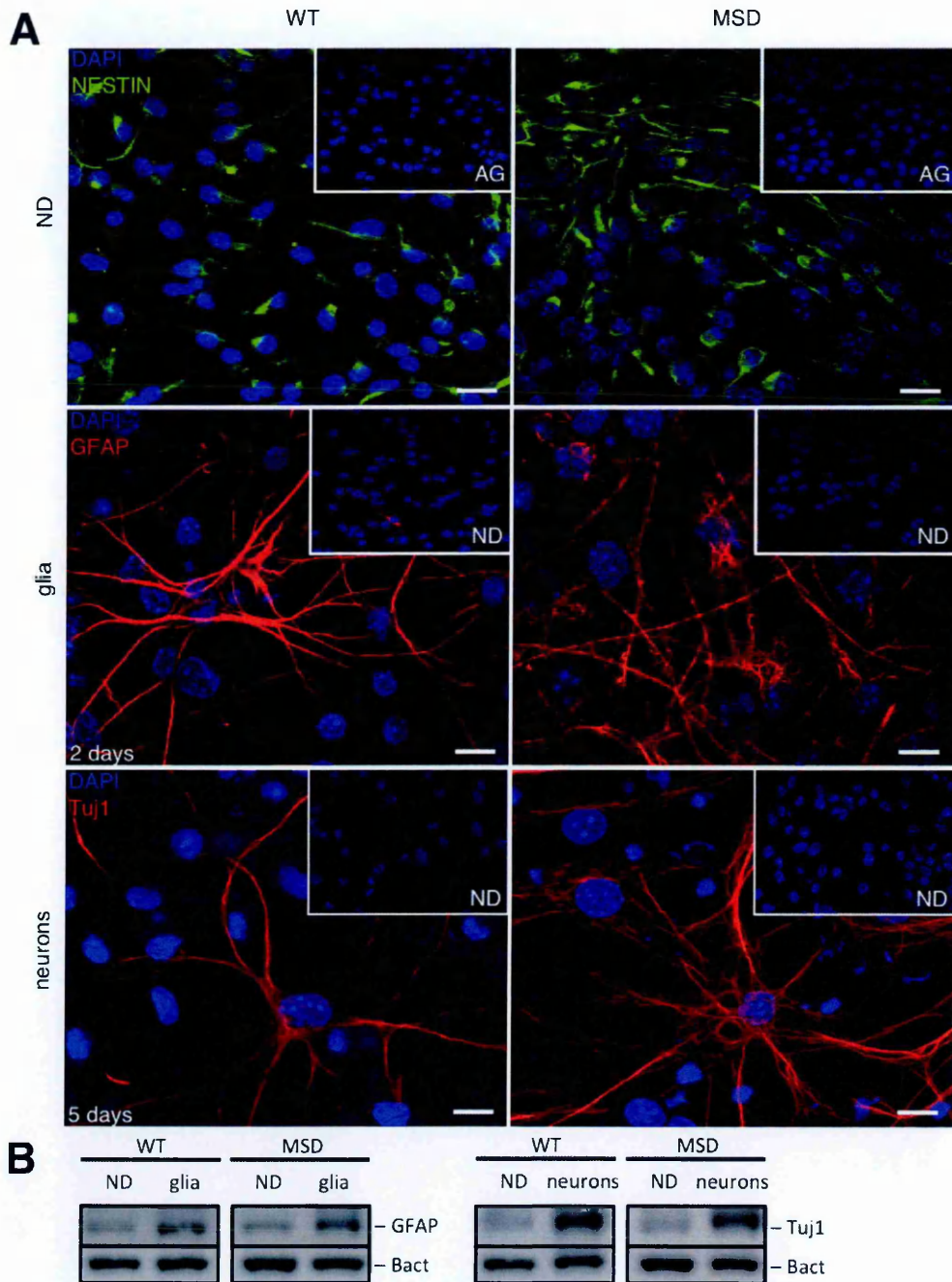**Figure 13. MSD-NSCs correctly express differentiation markers**

(A) Confocal microscopy image of wild-type (WT) and MSD undifferentiated NSCs (ND), glia (AG) and neurons stained with immunofluorescence detecting nestin (upper panel), GFAP (middle panel) and Tuj1 (lower panel). Scale bars represent 50 μM. (B) Immunoblotting detecting GFAP (left panel) and Tuj1 (right panel) in protein extracts from NSCs, glia and neurons.

MSD neurons develop neurites and are electrophysiologically active

During differentiation, neurons extend numerous processes that develop into dendrites and axons. These processes, also termed neurites, are critical for communication between neurons through interconnection of neuronal cell bodies. Moreover, neurite development was studied in another LSD, Sandhoff disease, which is a neurodegenerative disorder characterized by the deficiency in the hexosaminidase B (HEXB) gene; in particular in explant culture of retinal tissue from HexB -/- mice it was observed an impaired neurite outgrowth (Sango et al. 2005), suggesting that lack of a lysosomal enzyme has an effect on neuronal branching. Therefore I performed the neurite assay in order to establish whether the absence of SUMF1 protein could affect neurite outgrowth of 3- and 7-days differentiating neurons (Figure 14-A). Nevertheless, I observed no differences in neurite outgrowth between MSD neurons and their wild-type counterpart, suggesting once again that neuronal differentiation and processes extension is not affected in MSD cells.

For unambiguous assignment of neuronal identity, we investigated the electrophysiological properties of neural differentiated NSCs. Toward this goal, in collaboration with Dr. Francesco Miceli and Prof. Maurizio Taglialatela from University of Naples "Federico II", we recorded currents from wild-type and MSD-derived neurons using the whole-cell configuration of the patch-clamp technique. During depolarizing test potentials, MSD neurons and their wild-type counterpart, showed an inward current followed by a sizeable outward voltage-gated current, with features of a delayed-rectifier K^+ current (Figure 14-B, upper panel).

Furthermore, in MSD neurons the fast inactivating inward current was blocked by the selective Na^+ channel blocker tetrodotoxin (TTX) (1 μM) and peaked at a test potential of around -20 mV, typical features of voltage-gated Na^+ currents in neurons (Figure 14-B, lower panel). These preliminary electrophysiological data suggest that isolated NSCs are capable to give rise to electrophysiologically active neurons, exhibiting excitability properties and underlying conductances typical of maturing nerve cells. Most importantly, we did not find any differences in the electrophysiological properties between MSD derived neurons and their wild-type counterpart.

Taken together, these observations suggest that MSD pathology does not affect the ability of NSCs to differentiate into astrocytes and functional neurons.
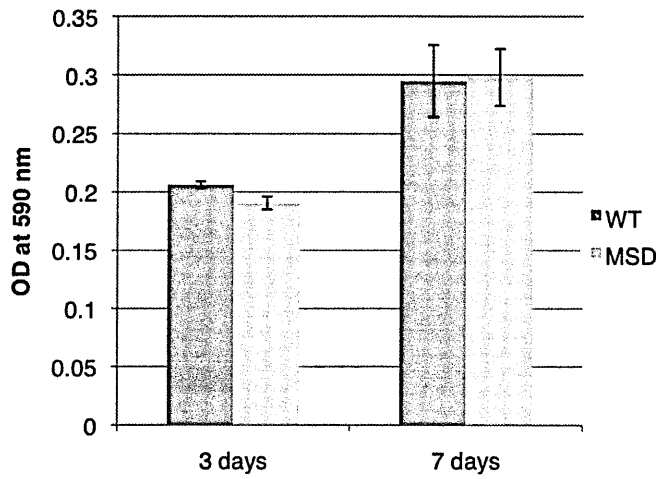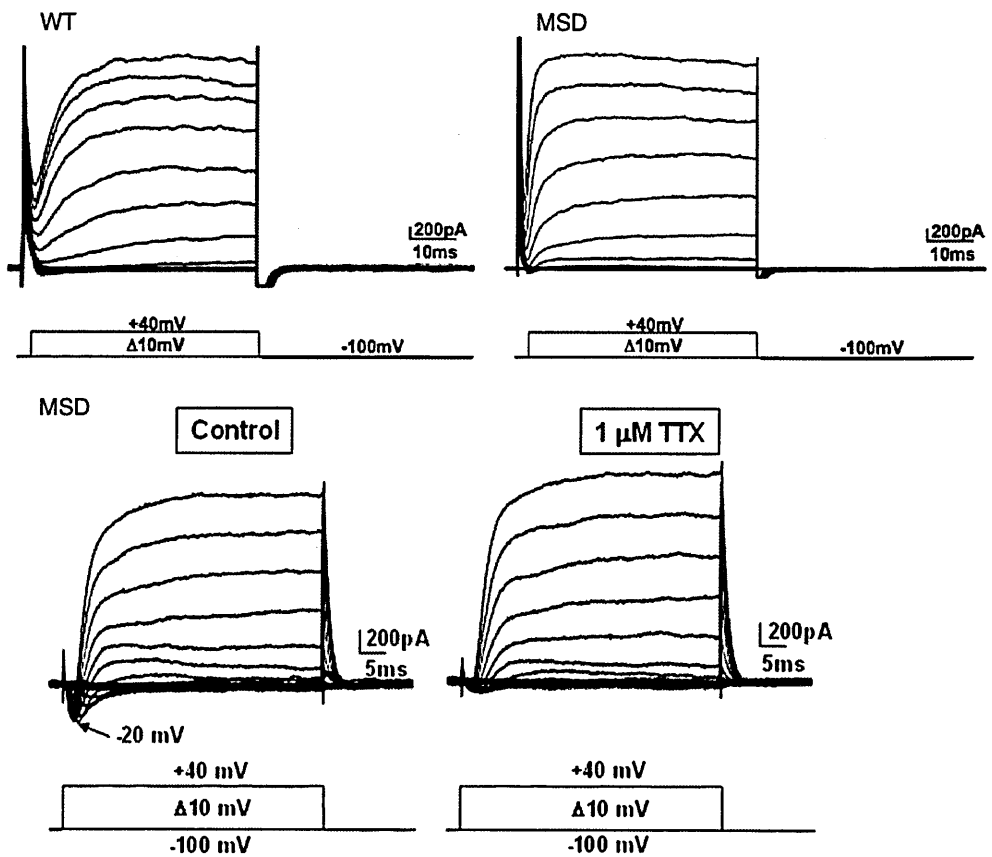
Figure 14. MSD differentiated neurons develop proper neurites and are electrophysiologically active

(A) Neurite outgrowth assay and (B) electrophysiological test on NSC-derived neurons; superimposed inward and outward current tracings were obtained using the indicated electrophysiological protocol. Data are mean of replicates (n=2) ± SEM.

MSD-NSCs recapitulate the progressive GAG accumulation

MSD-affected mice present a massive accumulation of undegraded GAGs in brain tissue, vacuolization and increased neurodegeneration (Settembre et al. 2007). Thus, after the isolation and characterization of MSD NSCs, I started to study these pathologic features in differentiating cells. Firstly I observed that during both astrocyte and neuronal differentiation, MSD cells display a progressive and massive perinuclear vacuolization compared to their wild-type counterpart (Figure 15-A). In particular, MSD cells display a thick cell body with enlarged extensions, whilst wild-type cells acquired the typical morphology of terminally differentiated CNS cells. In addition, MSD cells vacuoles have increasing dimensions during the course of the differentiation process, eventually reaching, and in some cases exceeding, the diameter of the nucleus. Electron microscopy analysis confirmed the presence of vacuole structures containing heterogeneous undegraded material (Figure 15-B).

Then I investigated whether MSD cells reflect also the main hallmark of the disease, which is the progressive accumulation of undegraded GAGs (Figure 16). By alcian blue staining (Figure 16-A) and GAG colorimetric assay (Figure 16-B) I detected increased levels of GAGs in MSD not-differentiated cells and in both glial and neuronal fate, reflecting the GAG storage phenotype observed *in vivo*. In fact, MSD-NSCs showed massive accumulation of GAGs even from the undifferentiated state; the storage then becomes prominent during both glial and neuronal differentiation and also increases as the differentiation proceeds, thus recapitulating the progressive accumulation seen *in vivo*. Pulse-and-chase

experiments using H^3-glucosamine to label GAGs confirmed that MSD differentiated cells accumulate GAGs (Figure 16-C).

The hypothesis is that during the differentiation process cells arrest their proliferation rate thus not dividing themselves anymore, and non-mitotic cells cannot dilute accumulating cytosolic content by cell division; in the case of MSD, due to the enzymatic inactivity of sulfatases, cells are not able to burn off the undegraded substrates that subsequently accumulate. In this condition, MSD cells accumulate all the undegraded material in a progressive manner as the differentiation proceeds.

Interestingly, also in a neuronal model of mucopolysaccharidosis type VII it was found a massive accumulation of abnormally high levels of GAGs, thus giving confidence to my model (Heuer et al. 2001).

These results suggest that long-term differentiated MSD-NSCs are able to recapitulate the main hallmarks of the disease, which is the progressive storage of GAGs.
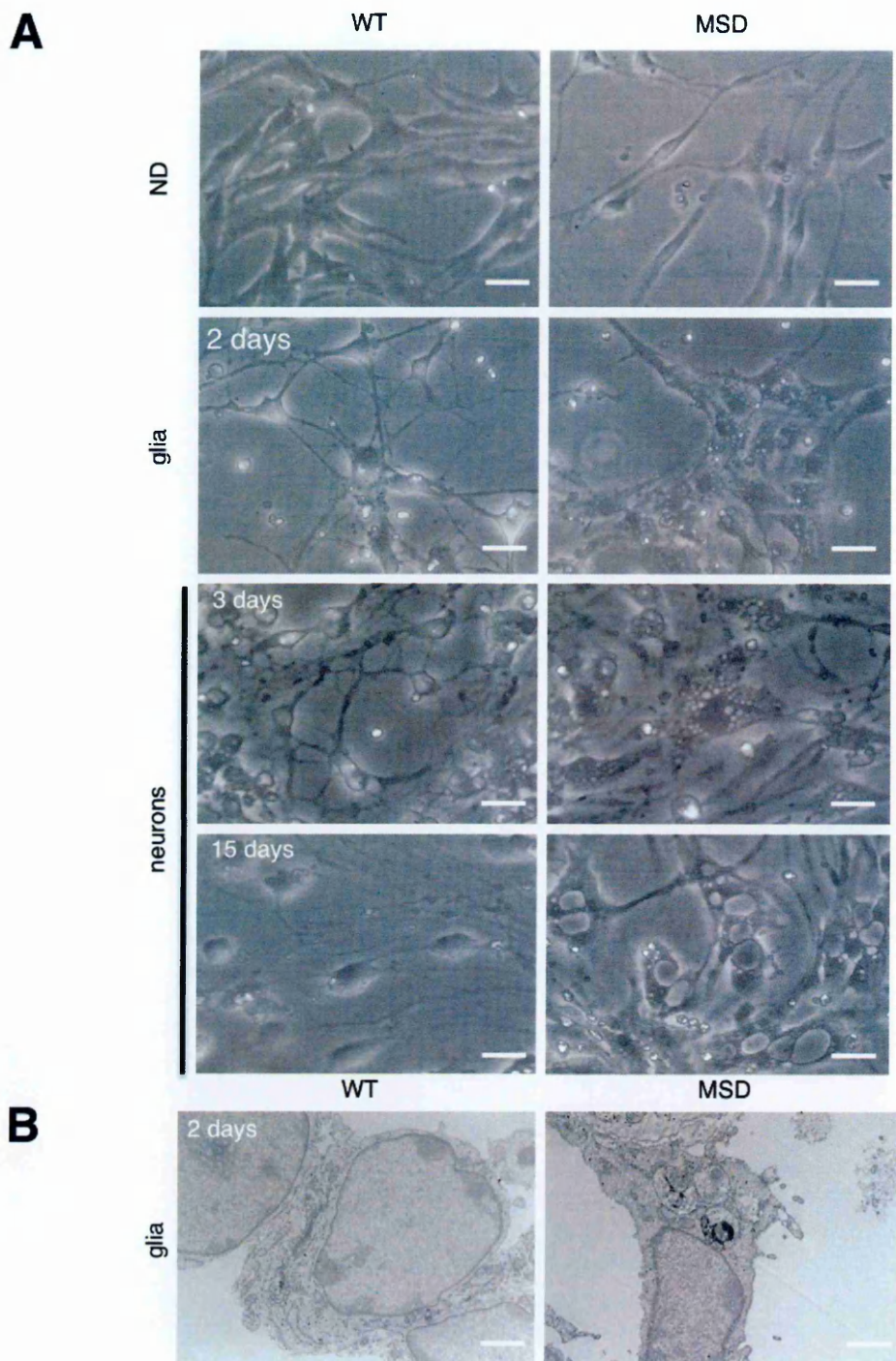
Figure 15. Differentiating MSD-NSCs display progressive vacuolization and undegraded material

(A) Bright field images of wild-type (WT) and MSD-NSCs, glia and neurons. (B) Electron Microscopy images of WT and MSD-derived glia cells. Scale bars represent 50 µM (A) and 1 µM (B).
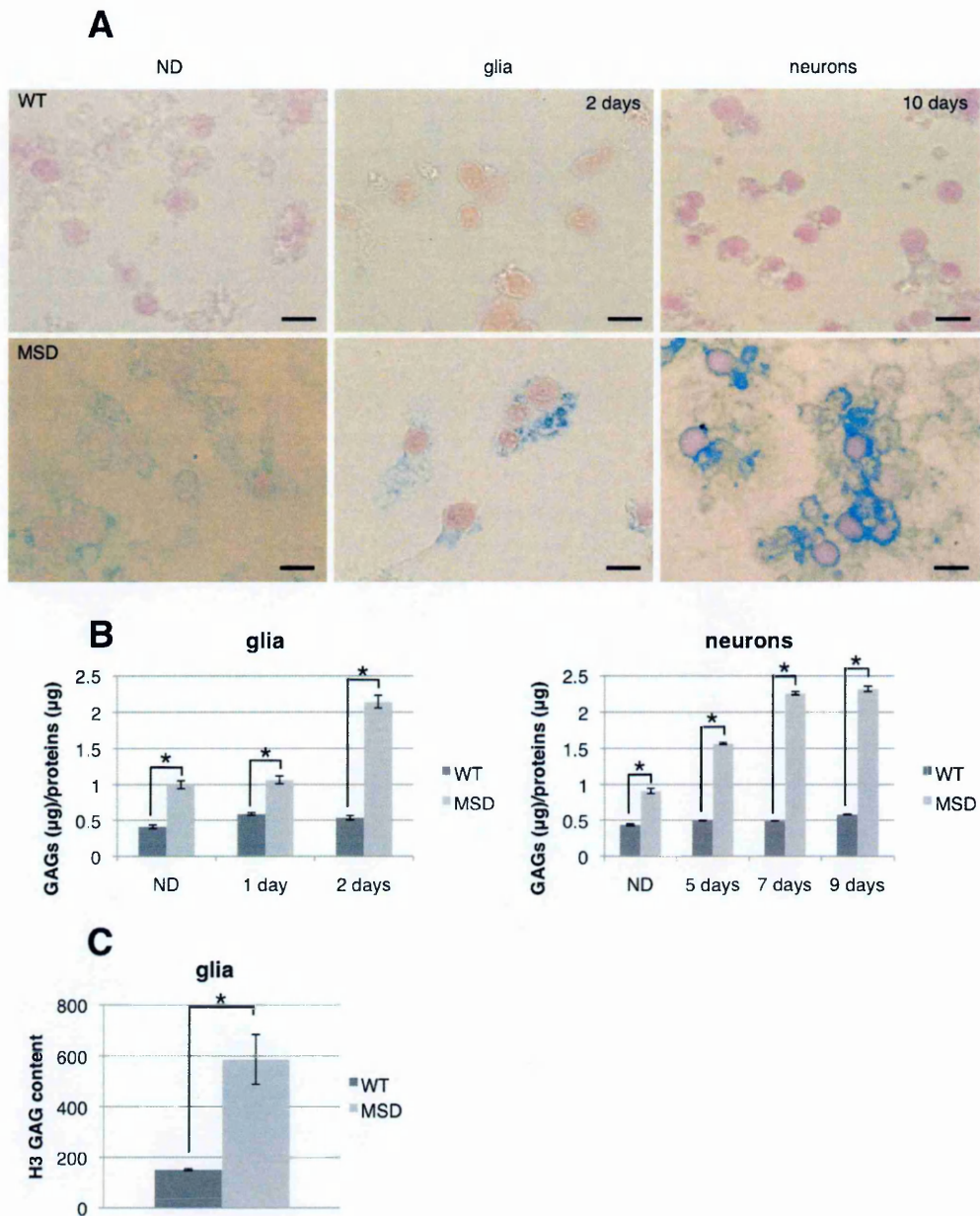
Figure 16. Differentiated MSD-NSCs display progressive accumulation of GAGs

*(A) Alcian-blu staining of GAGs on wild-type (WT) and MSD-NSCs, glia and neurons. Scale bars represent 50 μM. (B) GAG quantitative assay on wild-type and MSD-NSCs, glia (left panel) and neurons (right panel). (C) Pulse-and-chase incorporation of H^3-glucosamine in wild-type and MSD-derived glia cells. Data are mean of replicates (n=5) ± SEM; *p < 0.05 by Student's t-test (B and C).*

MSD differentiated cells suffer from impaired autophagy

In a previous work from our laboratory, it was shown that LSDs are associated with a lysosomal dysfunction that impairs the autophagic pathway (Settembre et al. 2008). This impairment ultimately leads to cell death, although the detailed mechanism has not been described yet. In particular, it was demonstrated that in MSD pathology a block of autophagic pathway occurs as a consequence of decreased ability of lysosomes to fuse with autophagosomes. Accordingly to this previous *in vivo* data, I observed an increase of LC3-II levels in cellular extracts from glial and neuronal differentiated MSD-NSCs (Figure 17-A), thus indicating autophagic activation. However, the autophagy process requires the fusion of newly formed autophagosomes with lysosomes, in order to digest autophagosome content; in glia differentiated MSD-NSCs I observed a decreased intracellular co-localization of autophagosome punctae containing LC3-II molecules with the lysosomal membrane protein marker cathepsin D (CatD) (Figure 17-B), suggesting an impaired fusion of autophagosomes with lysosomes and a block of the autophagy process.

The consequential effect of defective autophagy is the accumulation of poly-ubiquitinated proteins normally destined for recycling in the lysosomal compartment; in fact, poli-ubiquitin is a well-known signal for protein degradation. As expected, I detected a massive accumulation of poly-ubiquitinated proteins in MSD not differentiated cells, but also in glia and neurons, by immunofluorescence (Figure 18-A, left panel) and immunoblotting (Figure 18-B, left panel). These results confirm those found in the previous *in vivo* work (Settembre et al. 2008), in which a

massive and progressive accumulation of ubiquitin-positive inclusions were observed in the cerebral cortex as well as in other brain regions of MSD mice.

In addition, I found that p62/SQSTM1, a protein involved in targeting poly-ubiquitinated proteins to the autophagosomes (Pankiv et al. 2007), significantly accumulates in differentiated MSD-NSCs, both glia and neurons (Figure 18-A and B, right panels). The p62/SQSTM1 protein is known to be a common component of ubiquitin-positive protein aggregates in neurodegenerative diseases, being involved in targeting poly-ubiquitinated proteins to the autophagosomes, where they are selectively degraded via the autophagic pathway.
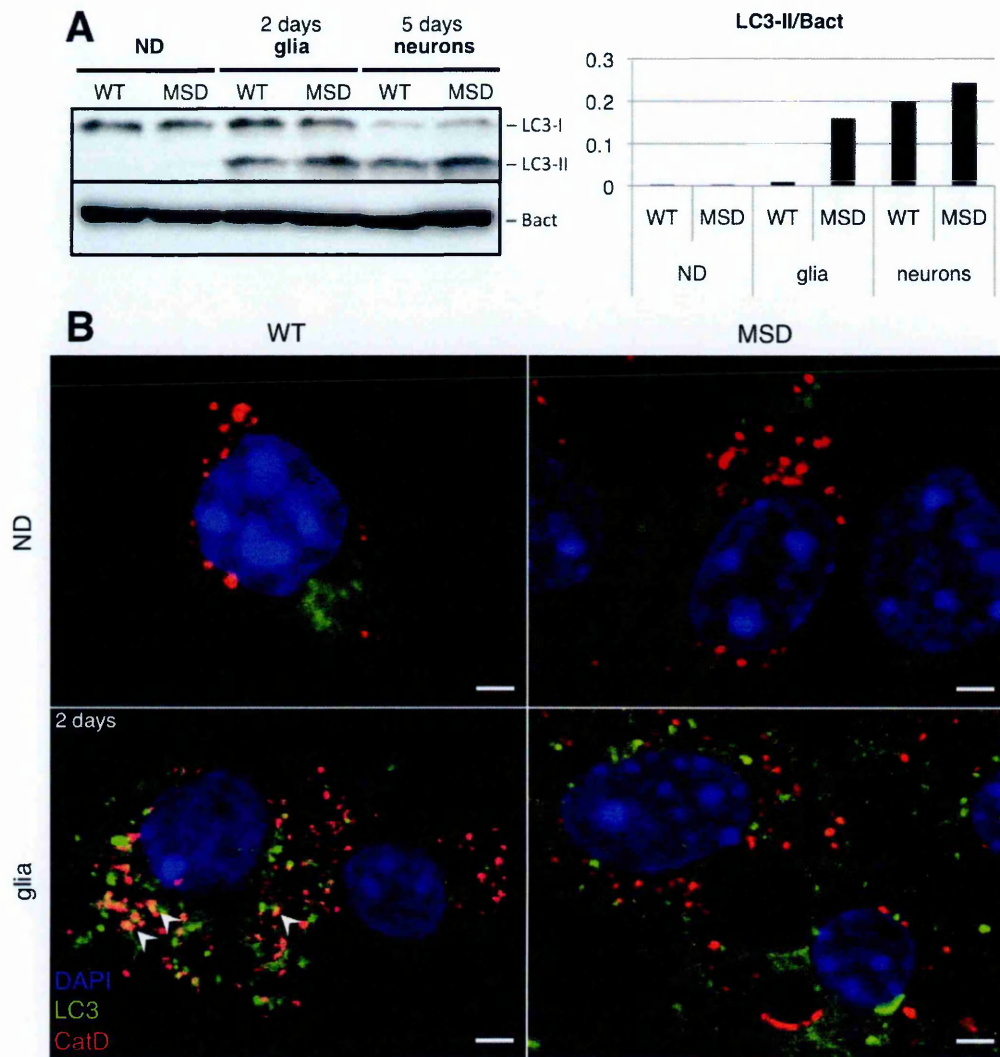
Figure 17. Differentiating MSD-NSCs display impaired autophagy

(A) Immunoblotting of LC3-I and –II in wild-type (WT) and MSD undifferentiated cells (ND), glia and neurons (left panel). LC3-II/Bact ratio (right panel). (B) Confocal microscopy image of immunofluorescence detecting LC3 and CatD in WT and MSD-NSCs, undifferentiated and differentiated to glia. Scale bars represent 10 µM. Arrows indicate colocalization between autophagosomes (marked with LC3) and lysosomes (marked with CatD), suggesting that in glia-differentiated WT cells autophagosomes properly fuse with lysosomes and, therefore, the autophagy process is working. Such colocalization is not present in glia-differentiated MSD cells, suggesting a block of the autophagy process.

75
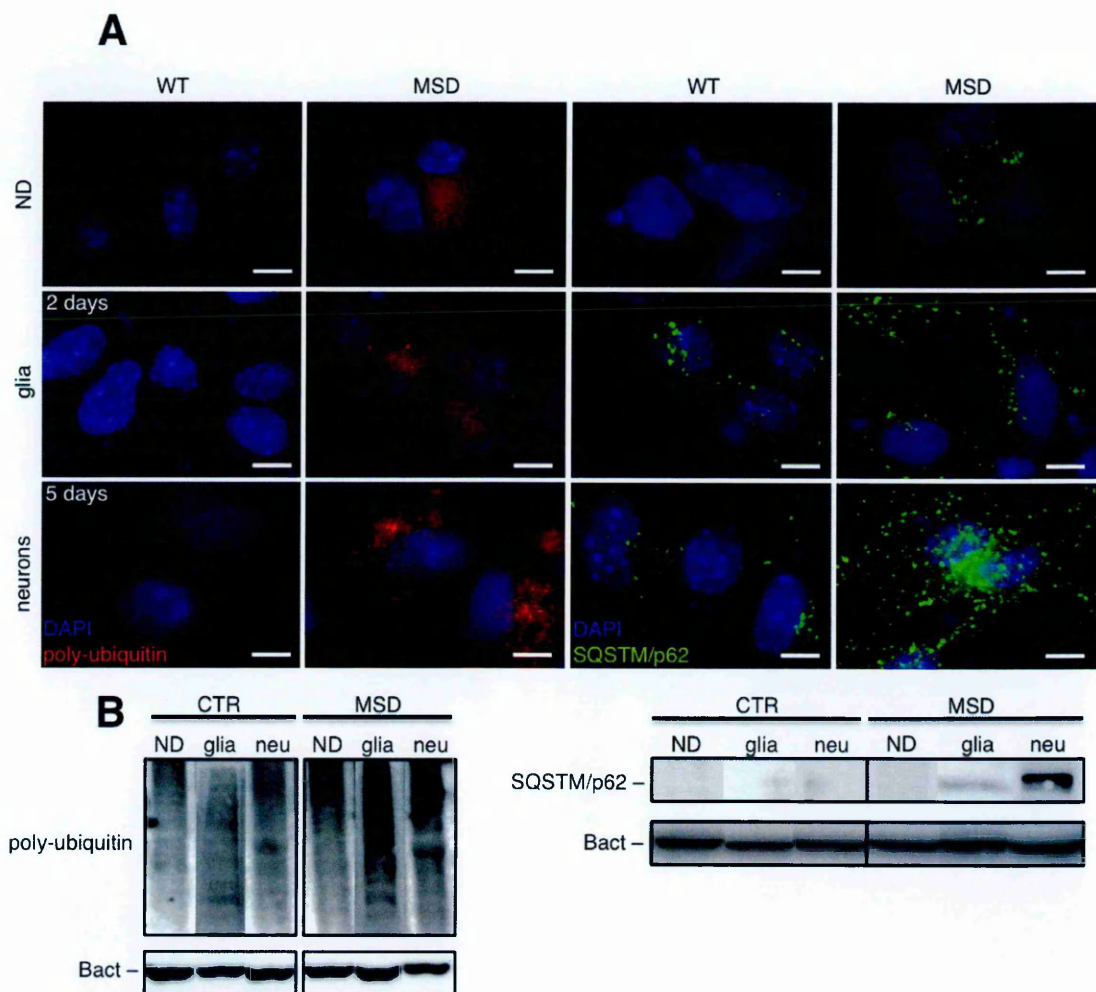
Figure 18. Differentiating MSD-NSCs display accumulation of poly-ubiquitinated proteins and p62/SQSTM1

(A) Epifluorescence microscopy image of immunofluorescence detecting poly-ubiquitin (left panel) and p62/SQSTM1 (right panel) in wild-type (WT) and MSD undifferentiated cells (ND), glia and neurons. Scale bars represent 10 µM. (B) Immunoblotting of poly-ubiquitin and p62/SQSTM1 in WT and MSD ND cells, glia and neurons.

MSD differentiated cells display the tendency to form aggresomes

It has been demonstrated that when poly-ubiquitinated misfolded proteins cannot be properly cleared, they accumulate into the aggresome (Goldberg 2003; Kawaguchi et al. 2003), an inclusion body localized in the proximity of the microtubule-organizing centre (MTOC) (Iwata et al. 2005; Pandey et al. 2007), where protein aggregates are ensheathed by the intermediate filament protein vimentin (Johnston, Ward, and Kopito 1998). Microtubule-associated histone deacetylase 6 (HDAC6) mediates this process (Matthias, Yoshida, and Khochbin 2008) through its ubiquitin-binding domain (UBA): HDAC6 binds to and facilitates the transport of poly-ubiquitinated misfolded proteins along microtubules to aggresome (Kawaguchi et al. 2003).

In wild type NSCs differentiated to glia we observed a typical cytoscheleton-associated localization of HDAC6, while in glia-differentiated MSD-NSCs we found an increase in the amount of HDAC6 detected by immunoblotting analysis (Figure 19-A, left panel), as well as an altered punctate cytoplasmic staining, occasionally colocalizing with poly-ubiquitinated proteins, as shown by immunofluorescence on wild-type and MSD glia cells (Figure 19-B, upper panel). Immunoblotting analysis showed also a consistent increase in vimentin expression (Figure 19-A, right panel). These observations suggest that, as a consequence of impaired autophagy, the presence of poly-ubiquitinated proteins produce an elevation in the levels of HDAC6, whose role is to target them to the aggresome.

Aggresome clearance is then mediated by ubiquitin-binding proteins like p62/SQSTM1 and neighbor of BRCA1 gene 1 (NBR1) (Kirkin et al. 2009), and in

fact we observed a higher co-localization of p62/SQSTM1 and HDAC6 in glia differentiated MSD-NSCs compared to wild type cells (Figure 19-B, lower panel), suggesting an increased aggresome formation. As an adaptor protein, p62/SQSTM1 is then responsible for misfolded protein degradation through autophagy pathway (Kirkin et al. 2009; Komatsu et al. 2010; Komatsu et al. 2007), which is impaired in MSD pathology. In fact, we did not observed co-localization between poly-ubiquitinated protein aggregates and the autophagy marker LC3, suggesting that the block of the autophagy process observed *in vivo* impairs not only the fusion of lysosomes with autophagosomes, but also the sequestration of toxic protein aggregates within the cytoplasm. In fact, even when we induced autophagy activation through starvation of undifferentiated cells we still did not observe the incorporation of poly-ubiquitinated proteins inside autphagosomes, rather producing as the only effect an increase in the size of aggregates (Figure 20).

As far as we known this is the first time that aggresome has been described in brain-derived cells from mice affected of MSD. Therefore, more experiments are needed to assess the contribution of aggresome formation in MSD pathology, and in particular its role in neurodegeneration.
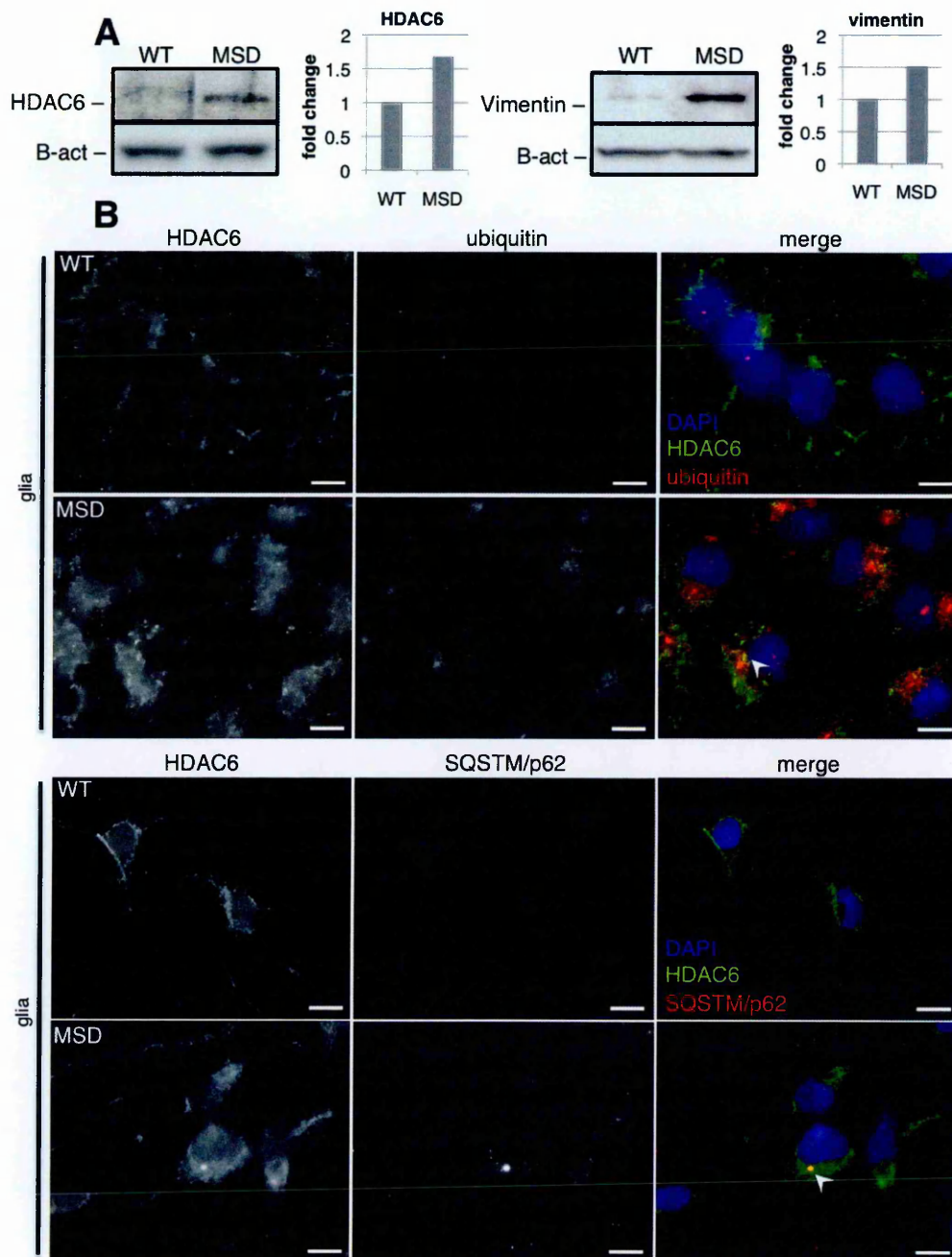
Figure 19. Differentiating MSD-NSCs display the tendency to form aggresomes

(A) Immunoblotting of HDAC6 and vimentin in wild-type (WT) and MSD-glia. (B) Epifluorescence microscopy image of immunofluorescence detecting poly-ubiquitin (upper panel) and p62/SQSTM (lower panel) in WT and MSD cells, glia and neurons. Scale bars represent 10 μM.
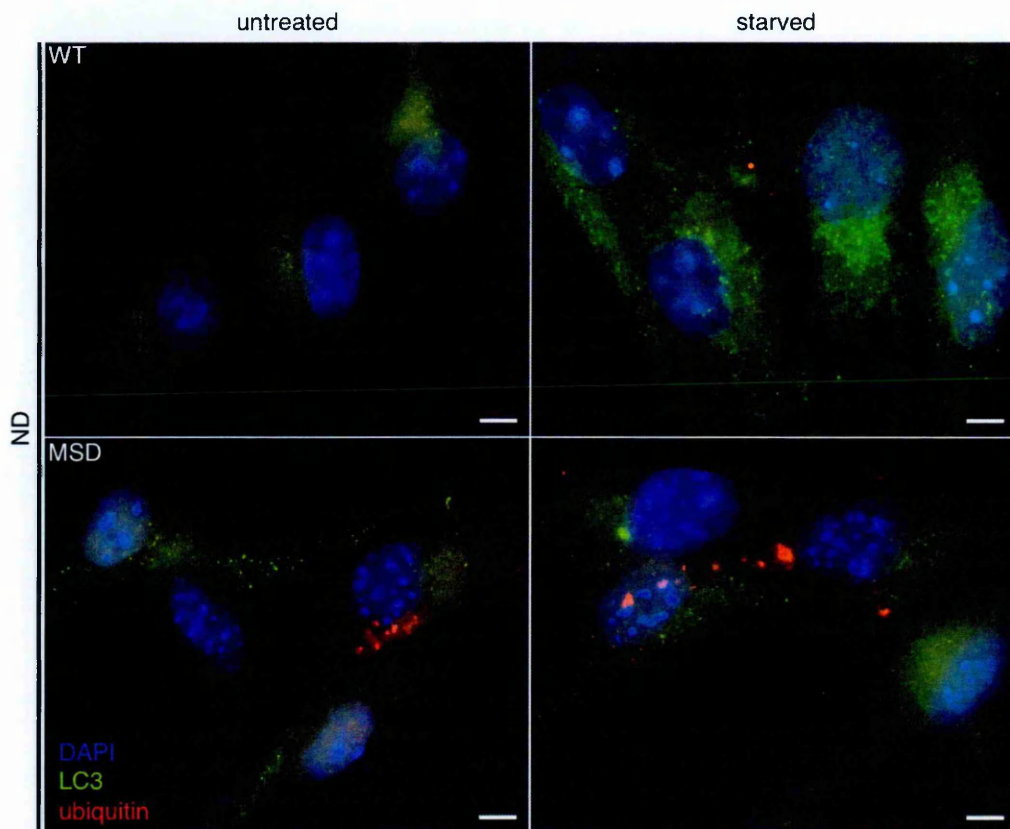
Figure 20. Defects in the autophagy pathway impairs toxic protein aggregates sequestration into autophagosomes.

Epifluorescence microscopy image of immunofluorescence detecting poly-ubiquitin and LC3 in wild-type (WT) and MSD undifferentiated cells (ND). Scale bars represent 10 μM. Incorporation of poly-ubiquitinated proteins inside autphagosomes in MSD ND cells was not observed upon starvation-induced autophagy.

MSD differentiated cells show increased apoptosis

To determine whether the accumulation of GAGs, poly-ubiquitinated proteins, p62/SQSTM1 and the block of autophagy are likely to sensitize NSCs to cell death, I analyzed the levels of apoptosis in MSD-NSCs (Figure 21). I tested their viability in the undifferentiated state and also in the two main differentiation programs, glia and neurons. Apoptosis was measured by TUNEL (Terminal deoxynucleotidyl transferase dUTP nick end labeling) assay, a method for detecting DNA fragmentation resulting from apoptotic signaling cascades, by labeling the terminal end of nucleic acids. I observed that in undifferentiated conditions or during the differentiation programs MSD cells suffer an increased level of apoptosis. These findings well correlated with previous *in vivo* data, showing elevated apoptosis in brain cortex of MSD mice. These results suggest that brain progenitor cells from MSD brain are sensitized to apoptosis and, together with the reduced self-renew ability, this might count for the reduced brain size observed in early MSD pups (Settembre et al. 2007). In addition, after differentiation the cells are even more sensitized to apoptosis *in vitro* suggesting that the progression of lysosomal accumulation is even deleterious for post-mitotic NSCs.

In summary, MSD-NSCs represent a confident model to recapitulate *in vitro* the basic features of the disease, and that represent a useful tool to investigate the mechanisms of neurodegeneration.
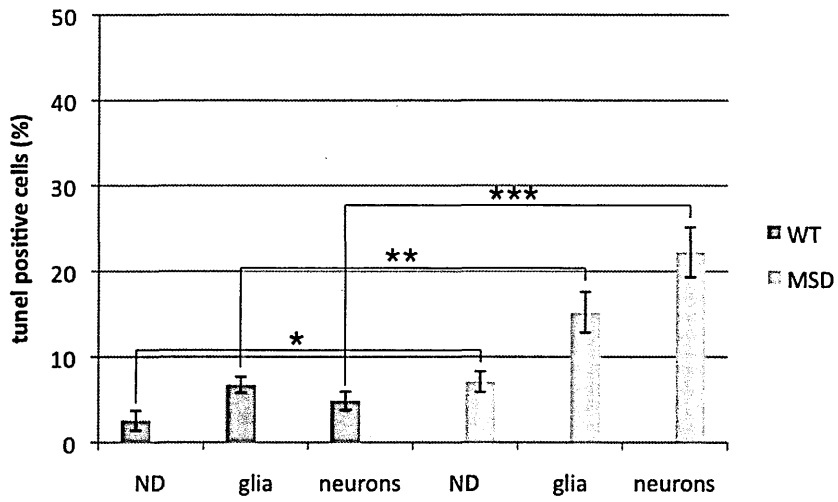
Figure 21. MSD-NSCs suffer increased apoptosis

Tunel assay was performed following the manufacturer's manual (Roche) on NSCs, glia and neurons derived from wild-type (WT) and MSD cells. Data are mean of replicates (n=20) ± SEM; *p < 0.05, **p < 0.005, *p < 0.0005 by Student's t-test.

TFEB induces lysosomal biogenesis in NSCs

Lysosomal biogenesis and function are transcriptionally regulated by TFEB (Sardiello et al. 2009). Moreover, TFEB overexpression not only increases the number of lysosomes in the cell, but it is also able to improve the degradative capability of the cell, as showed by the reduction of accumulated expanded huntingtin in a neuronal cell model of HD (Sardiello et al. 2009).

Therefore we questioned whether TFEB could mediate the clearance of accumulated material also in a cellular model of LSD. In particular, we investigated whether TFEB overexpression could reduce GAG accumulation in MSD-NSCs.

Firstly we observed that TFEB overexpression in NSCs increased lysosomal compartment (Figure 22). We nucleofected wild-type and MSD-NSCs with a bicystronic plasmid expressing TFEB-GFP and performed an immuno-fluorescence against LAMP1, a lysosomal membrane protein, in order to visualize the lysosomal compartment. Cells overexpressing TFEB always showed an increased LAMP1 compared to non-transfected cells in their proximity, with a slightly enhanced signal in MSD cells.
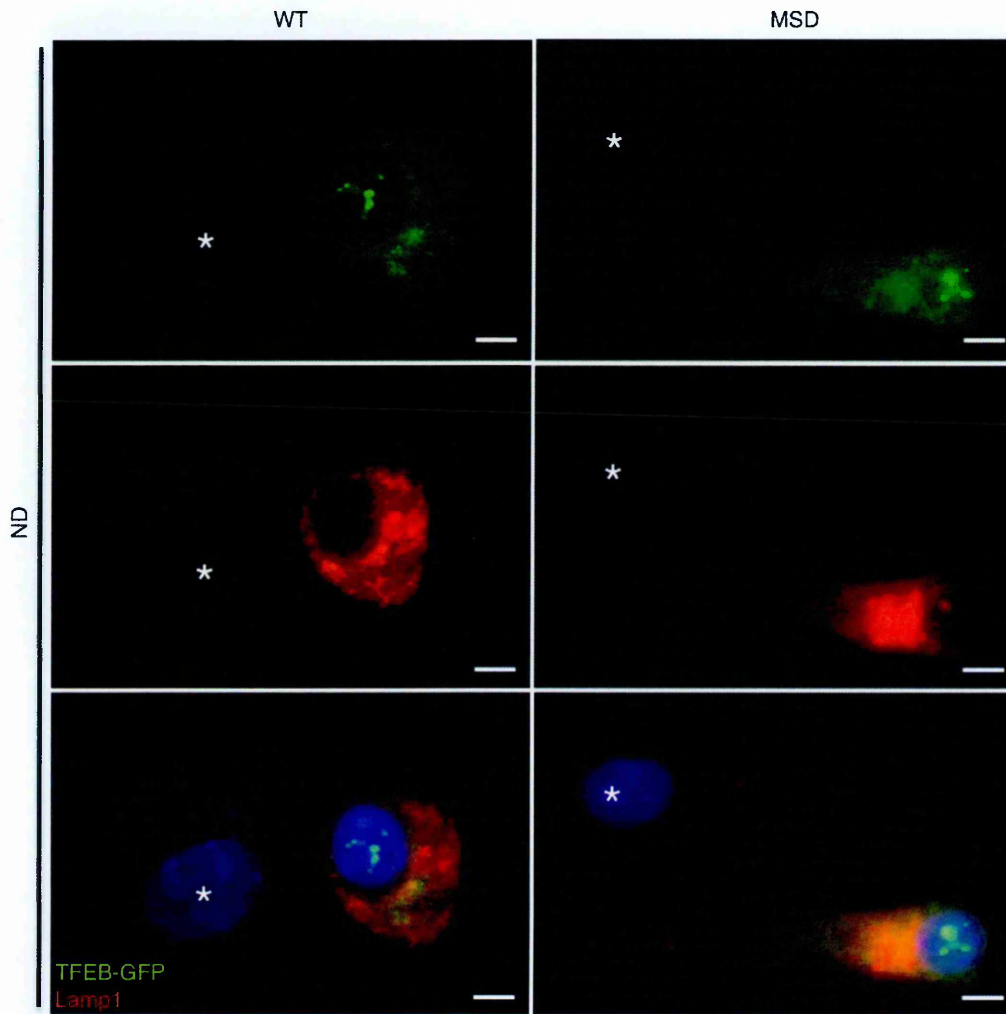
Figure 22. TFEB overexpression increases lysosomal compartment in NSCs

Epifluorescence microscopy image of immunofluorescence of LAMP1 in NSCs transfected with a bicystronic plasmid expressing TFEB-GFP. Cells expressing TFEB were localized by the expression of GFP; non-transfected cells are indicated by asterisks. Scale bars represent 10 μM.

TFEB promotes cellular clearance in MSD-NSCs

We tested whether TFEB-mediated increase of the lysosomal compartment could be exploited to induce cellular clearance in MSD-NSCs (Medina et al. 2011). Specifically, we evaluated the effect of TFEB overexpression on the clearance of GAGs in glia differentiated MSD-NSCs (Figure 23); we nucleofected cells with either a TFEB plasmid or with an empty plasmid and after 24 hours we induced glia differentiation for 48 hours; at the end of the differentiation program, we performed the alcian blue staining to reveal the amount of GAG accumulation within cells. Interestingly, TFEB overexpression resulted in a striking reduction of alcian blue stained GAGs in MSD glial cells (Figure 23-A), suggesting that TFEB is able to modulate cellular clearance also in cells affected by a lysosomal storage disorder.

This result was further confirmed by pulse-and-chase experiments using H^3-glucosamine to label GAGs (Figure 23-B). Wild-type and MSD-NSCs were nucleofected either with a TFEB plasmid or with an empty plasmid, and after 16 hours, cells were pulsed with H^3-glucosamine in differentiation medium and chased for 48 hours. Cell extracts obtained were quantified to determine the levels of labeled GAGs. The experiment showed a significant reduction of the levels of labeled GAGs in MSD glial cells overexpressing TFEB.

Finally, EM analysis revealed that TFEB-mediated clearance of GAGs in TFEB-overexpressing MSD cells was associated with both significant reduction of cellular vacuolization and recovery of normal intracellular morphology (Figure 23-C). Glia-differentiated MSD-NSCs were nucleofected with either TFEB or with an empty vector, fixed with glutaraldehyde and processed for standard electron

microscopy. The number of vacuoles per cell was significantly reduced in cells overexpressing TFEB compared to the empty vector, thus confirming the clearing potential of TFEB on pathologic vacuolization, typical of LSD cells and tissues.
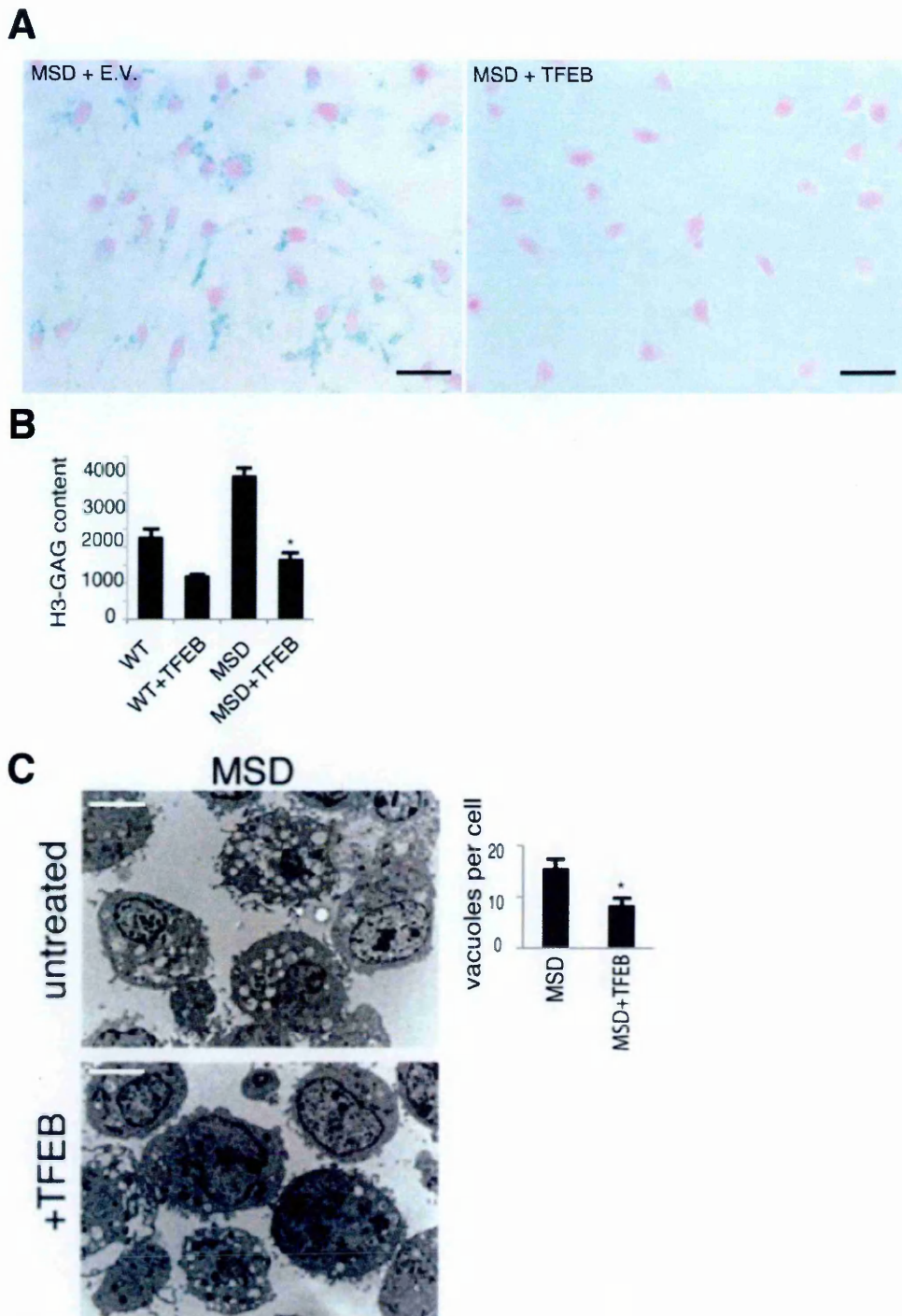
Figure 23. TFEB promotes cellular clearance in MSD-NSCs

(A) Alcian-blu staining of GAGs of MSD-derived glia cells nucleofected with either an empty vector or TFEB plasmid. (B) Pulse-and-chase incorporation of H^3-glucosamine of wild-type and MSD-derived glia cells nucleofected with either an empty vector or a TFEB plasmid. (C) Electron Microscopy of MSD-derived glia cells nucleofected with either an empty vector or TFEB plasmid. Scale bars represent 100 µM (A) and 10 µM (B). Data are mean of replicates (n=3) ± SEM; *p < 0.05 by Student's t-test (B and C)

TFEB Overexpression Induces Lysosomal Exocytosis

Since TFEB is not able to replace the missing *SUMF1* gene product in MSD NSCs, we postulated that the overexpression of TFEB may be able to induce the activation of lysosomal exocytosis, a secretory pathway that allows lysosomes to empty their content in the extracellular space. As we know, lysosomal exocytosis requires two sequential steps; in the first step lysosomes are recruited to the close proximity of the cell surface in a Ca^{2+}-independent manner (Jaiswal, Andrews, and Simon 2002), and in the second step the pool of predocked lysosomes fuse with the PM in a Ca^{2+}-dependent manner, thus emptying their content outside the cell (N W Andrews 2000; Jaiswal, Andrews, and Simon 2002; Tucker, Weber, and Chapman 2004).

Hence, a typical hallmark of lysosomal exocytosis is the translocation of lysosomal membrane markers to the PM (Reddy, Caler, and Andrews 2001; Rodríguez et al. 1997; Yogalingam et al. 2008). Therefore we detected LAMP1, a lysosomal membrane marker, using an antibody against its luminal portion, and in conditions of non-permeabilized wild-type NSCs, transfected with either a bicystronic plasmid expressing TFEB-GFP or an empty vector. Interestingly, TFEB overexpression resulted in an increased exposure of the luminal domain of LAMP1 on the PM, thus suggesting an increased translocation of lysosomes to the PM (Figure 24-A).

Consistently, also a quantitative analysis by flow cytometry (FACs) showed an increase of LAMP1 staining on the PM of TFEB-overexpressing wild-type NSCs (Figure 24-B).

88

Furthermore, a direct consequence of lysosomal exocytosis is the release of lysosomal enzymes into the cell culture medium (Rodríguez et al. 1997). Therefore we analyzed the presence of acid hydrolases in the culture medium of wild-type NSCs transfected with either a bicystronic plasmid expressing TFEB or an empty vector (Figure 24-C). Significantly higher levels of lysosomal hydrolases were detected in the medium of NSCs overexpressing TFEB compared with control cells. However, the increase of lysosomal enzymes in the medium was not associated with an increase in the levels of cytosolic lactate dehydrogenase (LDH) (Figure 24-D), thus excluding that the release of lysosomal enzymes was due to cell damage and that TFEB overexpression is cytotoxic. Together, these data indicate that TFEB induces lysosomal exocytosis (Medina et al. 2011).
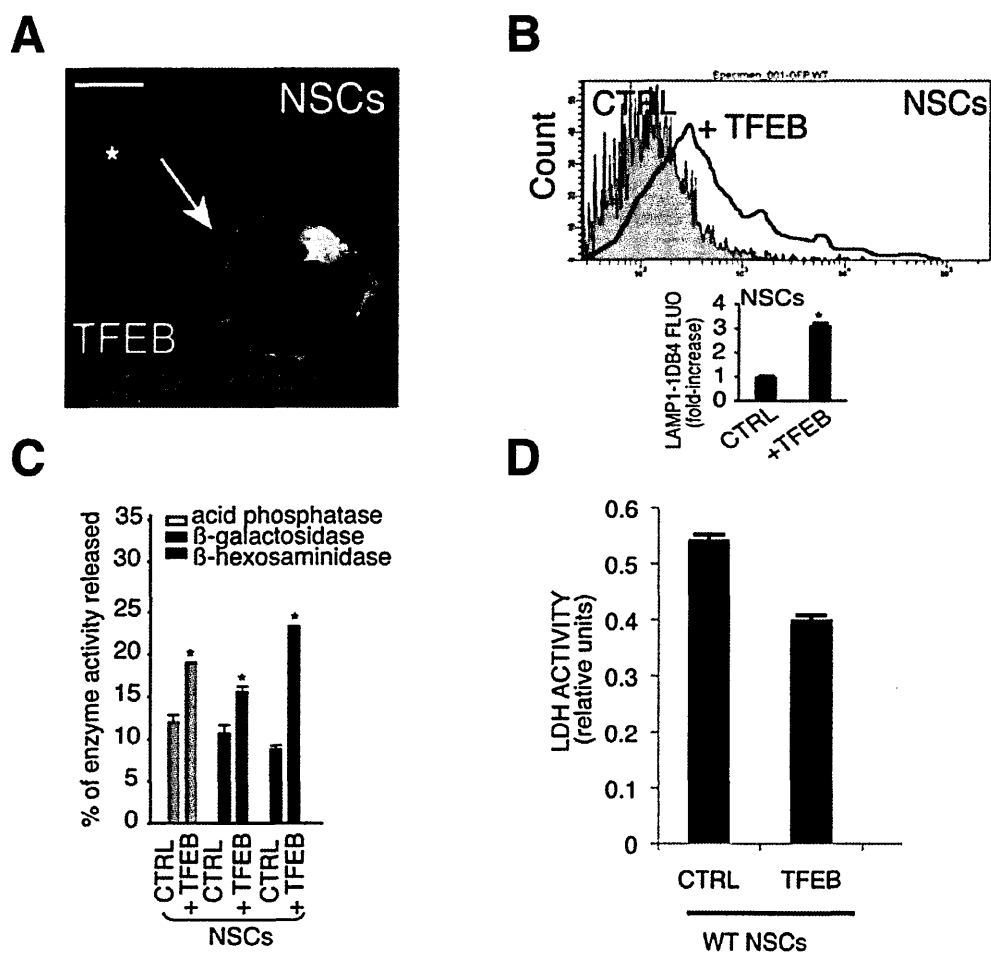
Figure 24. TFEB Overexpression Induces Lysosomal Exocytosis

(A) Confocal microscopy image of immunofluorescence detecting the luminal portion of LAMP1 (with the antibody LAMP1-1DB4) in non-permeabilized wild type NSCs transfected with either a bicystronic plasmid expressing TFEB-GFP or with an empty vector. TFEB-transfected cells were localized by the expression of GFP; non-transfected cells are indicated by asterisks. (B) Quantitative analysis by flow cytometry of LAMP1 levels on the PM in wild-type NSCs that express either a bicystronic TFEB-GFP plasmid or GFP. Bars represent the fold increase of LAMP1 fluorescence in TFEB-transfected versus GFP-transfected (CTRL) cells. (C) Activities of lysosomal enzymes acid phosphatase, b-galactosidase, and b-hexosaminidase in the culture medium of wild-type NSCs nucleofected with either an empty vector or with a TFEB-expression vector. The figure shows percentages of enzyme activities released compared with total activities. (D) LDH activity was determined following the manufacturer's manual (Abcam) in supernatants of NSCs transfected either with TFEB or with an empty vector. Data are mean of replicates (n=4) ± SEM (B, C and D). *p < 0.05 by Student's t-test (B and C)

TFEB Overexpression Enhances Lysosomal Predocking to the PM and Elevates Intracellular Ca^{2+}

Induction of lysosomal exocytosis involves the recruitment of lysosomes to the PM (Blott and Griffiths 2002). To clearly demonstrate that TFEB overexpression resulted in an increased motility of lysosomes we took advantage of a HeLa cell line stably expressing TFEB (HeLa CF7) using the immuno-EM approach (Figure 25-A). In HeLa control cells lysosomes were distributed randomly throughout the cells, whilst CF7 cells exhibited numerous lysosomes in the close proximity of the PM, indicating that TFEB overexpression significantly stimulates the recruitment of lysosomes to the PM, a step that is required for lysosomal exocytosis.

Several studies demonstrated that the elevation of Ca^{2+} concentrations is required for the fusion of lysosomes with the PM, but not for the previous step of the recruitment of lysosomes to the cell surface (N W Andrews 2000; Jaiswal, Andrews, and Simon 2002; Rodríguez et al. 1997). We also demonstrated that in HeLa control cells the overexpression of TFEB led to an elevation of intracellular Ca^{2+} levels (Figure 25-B), consistent with the role of TFEB in the induction of lysosomal exocytosis.

To assess whether the elevation of intracellular Ca^{2+} levels was responsible for the activation of lysosomal exocytosis and thus for the clearance of accumulated GAGs, we tested TFEB ability to reduce storage in presence of a Ca^{2+} inhibitor (Figure 25-C). Therefore, we nucleofected MSD-NSCs with a TFEB plasmid, we differentiated them to glia and at the same time we treated them with the Ca^{2+} chelator, BAPTA-AM, at increasing concentrations; then, after 48 hours,

alcian blue staining of GAGs was performed. We observed an inhibition of GAG clearance after the addition of BAPTA to TFEB overexpressing NSCs, confirming the involvement of Ca^{2+} in TFEB-mediated reduction of pathologic storage.

Together, these results indicate that in addition to promoting lysosomal recruitment to the PM, TFEB induces lysosomal exocytosis by enhancing Ca^{2+}-mediated fusion of lysosomes with the PM (Medina et al. 2011).
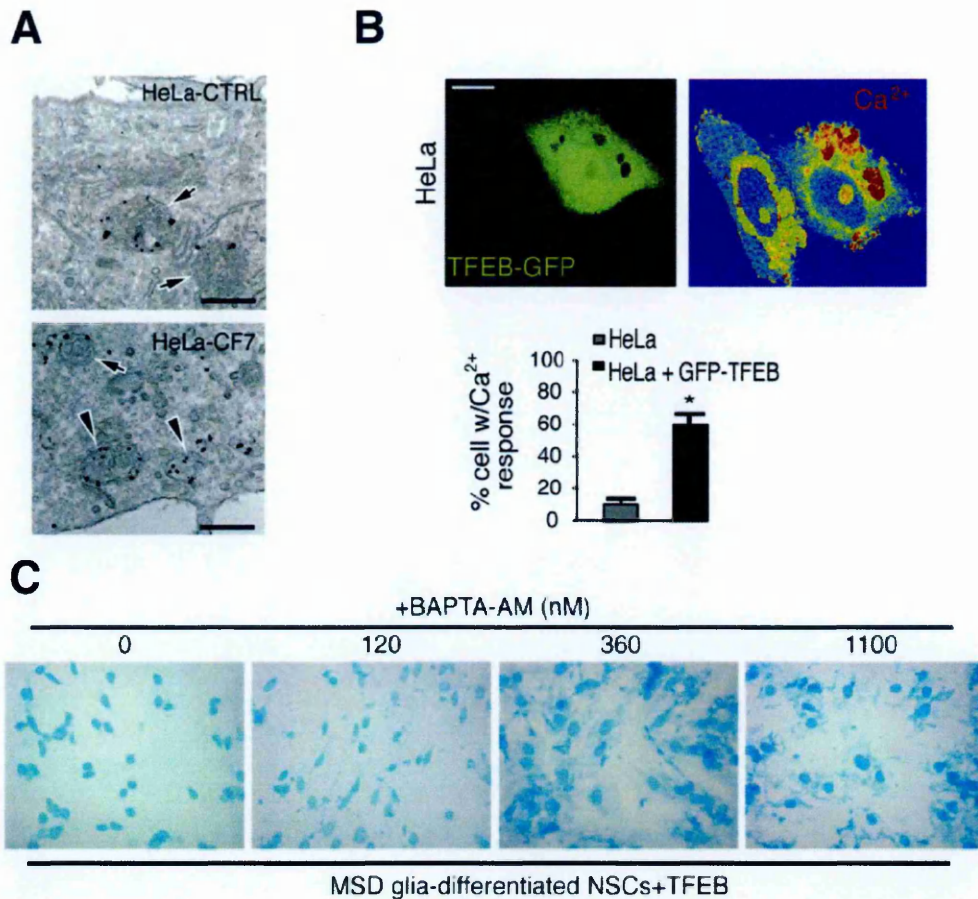
Figure 25. TFEB enhances PM proximity of lysosomes and Intracellular Ca^{2+} Release

(A) Immuno-Electron Microscopy in control and HeLa CF7. Cells were fixed and labeled with the antibodiy against LAMP1 and prepared for immuno-EM. LAMP1 detected at a substantial distance from the cell surface are indicated by arrows, while lysosomes that are close to the PM are indicated by arrowheads. The distance between lysosomes and PM was estimated in thin sections and 100 lysosomes were counted for each condition. Scale bars represent 350 nm. (B) Analysis of intracellular Ca^{2+} by confocal microscopy of HeLa cells transfected with a bicystronic TFEB-GFP construct. Data are displayed as the percentage of cells with Ca^{2+} response compared with the non-transfected cells. (C) Ca2+ involvement in TFEB-mediated GAG clearance in MSD-NSCs. Alcian blue staining of GAGs was performed on glia-differentiated MSD-NSCs nucleofected with a TFEB. The cells were treated with different concentrations of the Ca2+ chelator BAPTA-AM for 12 hours.

TFEB Releases Ca^{2+} from Acidic Stores through the Activation of Mucolipin-1

Although we demonstrated that TFEB overexpression strongly activates lysosomal exocytosis and eventually leads to the clearance of pathologic material, the mechanism of action was still left to clarify.

In a separate study, we demonstrated that among other genes belonging to the network, TFEB regulates the expression of the gene encoding MCOLN1 (Palmieri et al. 2011), a lysosomal non-selective cation channel that is mutated in MLIV, a severe type of LSD (Bargal et al. 2000; Bargal et al. 2002; Bassi et al. 2000). Previous studies reported that lysosomal exocytosis is reduced in MLIV cells (LaPlante et al. 2006) and increased in cells expressing gain-of-function MCOLN1 mutations (X. Dong et al. 2009). These observations made MCOLN1 an appealing candidate to mediate TFEB effects on lysosomal exocytosis, suggesting that MCOLN1, upon a proper cellular stimulation, mediates intralysosomal Ca^{2+} release to trigger lysosomal exocytosis.

Therefore, we performed a flow-cytometry Ca^{2+} flux assay in NSCs transfected with a control vector, a vector containing a scramble shRNA plus TFEB plasmid, and a vector containing a pool of four fluorescently tagged vectors carrying specific MCOLN1-shRNAs plus TFEB plasmid (Figure 26-A). Levels of Ca^{2+} were determined in resting condition and after stimulation with ionomycin (10 micromolar), an ionophore that raises the intracellular level of Ca^{2+}. Remarkably, TFEB-mediated increase of intracellular Ca^{2+} was blocked in NSCs overexpressing TFEB by transient silencing of MCOLN1 with shRNAs, suggesting a crucial role of

MCOLN1 in TFEB-mediated activation of lysosomal exocytosis. This observation was further confirmed also in HeLa cells stably depleted for MCOLN1 (HeLa shMCOLN1) (Figure 26-B) and in human MLIV fibroblasts (Figure 26-C). In addition, stable depletion of MCOLN1 in HeLa cells also impaired TFEB-mediated fusion of LAMP1 with the PM (Figure 26-D) and secretion of lysosomal enzymes (Figure 26-E).
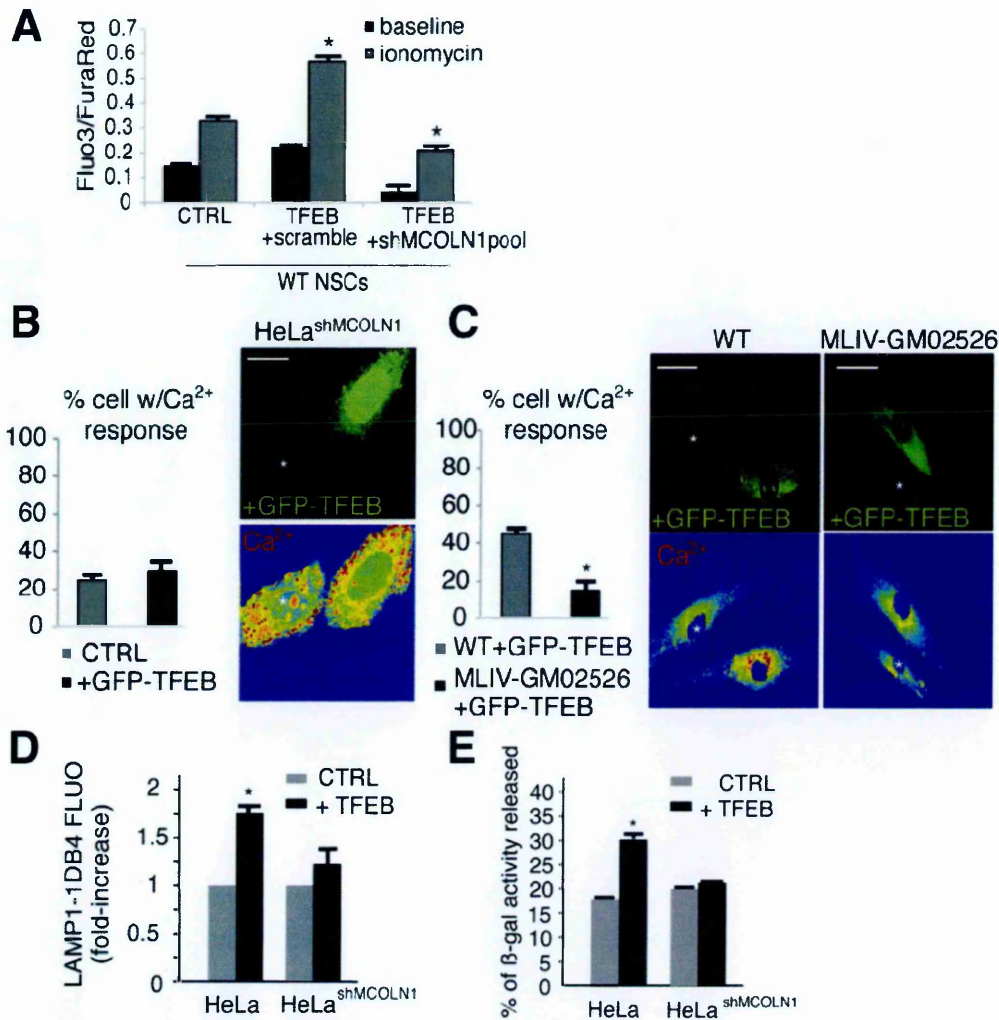
Figure 26. TFEB elevates intracellular Ca^{2+} levels through the activation of MCOLN1

(A) Flow-cytometry Ca^{2+} flux assay in NSCs transfected with a control vector, a vector containing a scramble shRNA plus TFEB plasmid, and a vector containing a specific shRNA against MCOLN1 plus TFEB plasmid. Ca^{2+} was determined in resting condition and after stimulation with ionomycin (10 µM). (B and C) Analyses of intracellular Ca^{2+} by confocal microscopy of (B) HeLa^{shMCOLN} and (C) human MLIV fibroblasts transfected with a bicystronic TFEB-GFP construct. Data are displayed as the percentage of cells with Ca2+ response compared with non-transfected cells. (D) Flow-cytometry analysis of LAMP1 on the PM of HeLa^{shMCOLN1} cells transfected with TFEB. (E) Secretion of lysosomal b-galactosidase in HeLa^{shMCOLN1} cells. Secretion efficiency was calculated as the % of enzymatic activity in the medium with respect to the total activity (medium and cellular pellet). Data represent mean of replicates (n=5) ± SEM; *p < 0.05 (A–E). Scale bars represent 10 mm (B) and 25 mm (C).

96

Furthermore, the lysosomal localization of MCOLN1 and its channel properties suggested that the elevation of intracellular Ca^{2+} levels induced by TFEB overexpression was due to the release of Ca^{2+} from lysosomal stores through MCOLN1. In fact, we treated cells with the inhibitor of the v-ATPase, Bafilomycin A1, which is responsible for the proton gradient driving lysosomal Ca^{2+} uptake (Christensen, Myers, and Swanson 2002), and therefore induces acidic Ca^{2+} release. The result clearly showed that both TFEB and MCOLN1 overexpressing HeLa cells were less sensitive to Bafilomycin A1-dependent depletion of lysosomal Ca^{2+} compared to HeLa control cells (Figure 27-A). This suggests that in a condition of induced lysosomal exocytosis, acidic stores of Ca^{2+} are actively being used to induce the fusion of lysosomes with PM and Bafilomycin A1 effect is less prominent if compared to control cells.

Moreover, we tested TFEB-mediated clearance of accumulated material in human MLIV fibroblast; cells were transfected with either TFEB-FLAG or with an empty vector and analyzed by confocal microscopy. TFEB overexpression did not reduce lipofuscin accumulation in human MLIV fibroblasts (Figure 27-B), thus confirming the importance of MCOLN1 function in TFEB-mediated clearance of lysosomal storage.

Together these data strongly suggest that MCOLN1-dependent release of Ca^{2+} from acidic Ca^{2+} stores plays a major role in TFEB-mediated lysosomal exocytosis (Medina et al. 2011).

At this point, we questioned whether MCOLN1 overexpression could mediate activation of lysosomal exocytosis, with no participation of TFEB. Thus, to assess whether MCOLN1 on its own was able to reduce GAG accumulation as well as

TFEB, we performed Alcian blu staining on glia-differentiated MSD-NSCs infected with either a lentiviral vector carrying TFEB or an adenoviral vector encoding MCOLN1. We observed reduced GAG accumulation in cells overexpressing MCOLN1, but to a lesser extent compared with TFEB, suggesting that Ca^{2+} elevation is required but not sufficient to mediate TFEB effects on lysosomal exocytosis. In fact, MCOLN1 overexpression only partially reduced GAG pathologic storage.
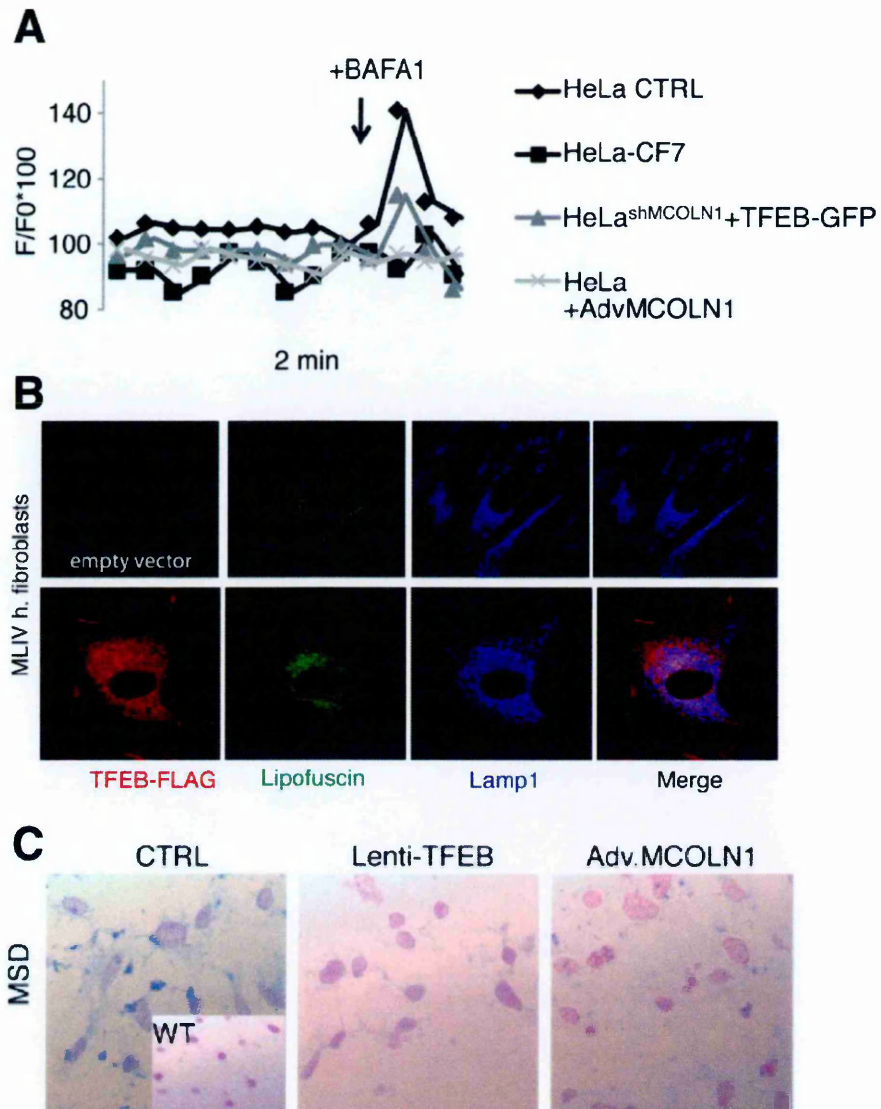
Figure 27. MCOLN1 plays a central role in TFEB-mediated activation of lysosomal exocytosis

(A) Ca2+ levels were measured by loading cells with the ratiometric fluorescent dye FuraRed. After 1.5 min of confocal time-lapse acquisition, cells were treated with 1uM Bafilomycin A1 to induce the release of Ca2+ from the acidic compartment. Data represent the F458/F488 ratio of each experimental group compared with their basal ratio before stimulation (F/F0*100). (B) Human MLIV disease fibroblasts were transfected with either TFEB-FLAG or with an empty vector and analyzed by confocal microscopy. (C) Alcian blue staining of GAGs in glia-differentiated MSD-NSCs infected with either a lentiviral vector carrying TFEB or an adenoviral vector encoding MCOLN1.

99

To further confirm that the clearance is effectively mediated by exocytosis, we measured the secretion efficiency of GAGs in the culture medium of MSD-NSCs overexpressing TFEB (Figure 28-A). Cells were nucleofected with either TFEB or an empty vector after pulse-chase incorporation of H^3-glucosamine and then radioactive GAGs were measured in the culture medium. When MSD-NSCs were overexpressing TFEB, we observed an increase in GAG secretion in the cell culture medium, thus confirming that clearance is mediated by exocytosis.

We then extended these studies to other types of LSDs associated with the storage of different types of lysosomal substrates, like Neuronal Ceroid Lipofuscinosls type 3 (CLN3 or Batten disease), and glycogenosis type II (or Pompe disease), characterized by the accumulation of lipofuscin and glycogen, respectively (Beratis, LaBadie, and Hirschhorn 1978; Persaud-Sawin et al. 2007). We found that TFEB overexpression strongly reduced lipofuscin autofluorescence in cells from a murine model of NCL3, and also in fibroblasts from a patient affected by glycogenosis type II, Pompe disease (Figure 28-B). These data indicate that induction of lysosomal exocytosis promotes cellular clearance in diseases due to accumulation of different types of lysosomal substrates. Notably, all diseases tested (i.e., MSD, Batten, and NCL) are due to deficiency of proteins whose activity is involved in crucial steps of different catabolic pathways. In addition, the cells analyzed were derived from patients and murine models carrying either null mutations or mutations that severely inactivate protein function. Therefore, it is unlikely that clearance of lysosomal substrates is due to either the enhancement of the activity of the defective enzymes, or to an overall induction of lysosomal catabolic processes, suggesting that clearance is mediated by exocytosis.
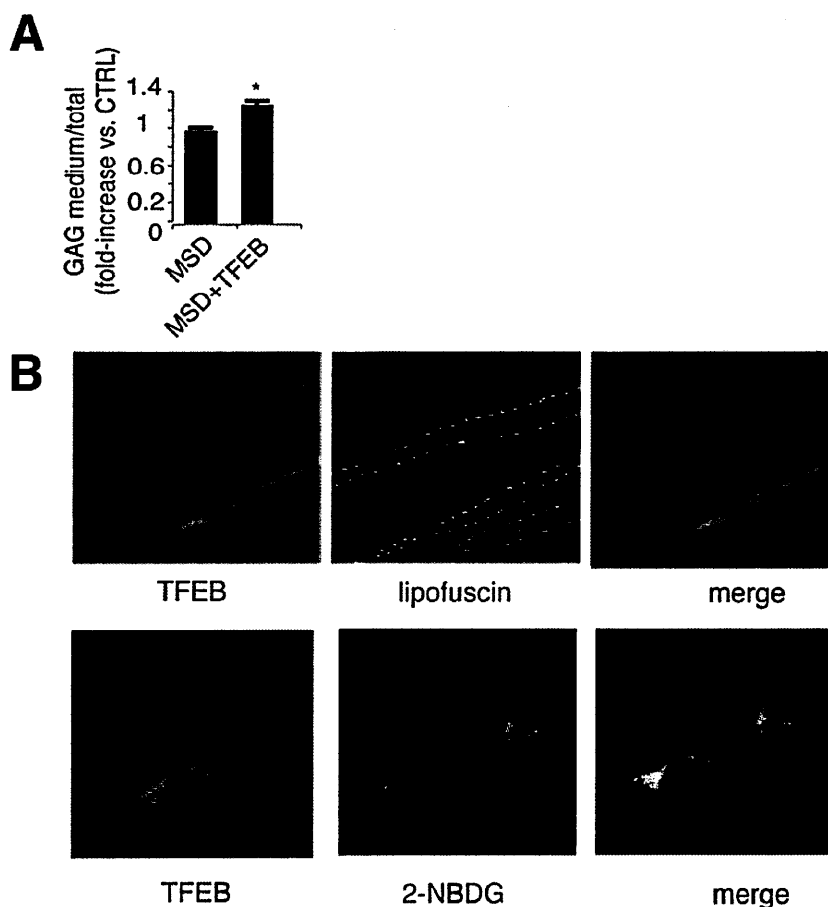
Figure 28. TFEB clearance is mediated by exocytosis and is tested in Batten and Pompe diseases' cellular model.

(A) Secretion efficiency of radioactive GAGs measured in the culture medium of MSD-NSCs nucleofected with either TFEB or an empty vector after pulse-chase incorporation of H^3-glucosamine. Data represent mean ± SEM; *$p < 0.05$ (B and C). (B) TFEB promotes clearance of lipofuscin in fibroblasts from a patient with Batten disease (upper panel) and of the fluorescent sugar 2-NBDG in human Pompe disease fibroblasts (lower panel). Cells were transfected with a vector carrying TFEB-Ruby (continuous red staining). After 24 hr, cells were examined by live imaging confocal analysis. Cells with increased TFEB (i.e., cells with red signal in the picture and outlined by dashed white lines in the middle panel) display highly reduced levels of lipofuscin or 2-NBDG (punctate green signal) and a normal cellular morphology compared with non-transfected cells (i.e., cells with intense green staining).

101

TFEB induces storage clearance in a mouse model of MSD

Finally, we tested whether *in vivo* overexpression of TFEB in the mouse model of MSD had similar effects on cellular clearance (Figure 29). To this end, we injected systemically into adult MSD mice an adeno-associated virus type 2/9 (AAV2/9) that carries TFEB-3xflag or GFP, both under control of the cytomegalovirus (CMV) promoter. One month after injection, several tissues were collected to monitor transduction efficiency and GAG storage; AAV-mediated TFEB delivery was detected using a specific anti-flag antibody and resulted in efficient TFEB transduction (Figure 29-A, left panel). In both liver and skeletal muscles we observed a significant reduction of GAG amount, as detected by alcian blue staining and GAG quantification (Figure 29-A, right panel, and B).

Subsequently, we investigated whether TFEB-mediated clearance of GAGs resulted in the reduction of the pathologic hallmarks of MSD, such as macrophage infiltration and apoptosis (Settembre et al. 2007) (Figure 30). We found a striking reduction of CD68-positive cells in the liver of AAV-TFEB injected MSD mice compared with MSD non-injected littermates (Figure 30-A). Most importantly, we also observed a significant reduction of apoptotic cells in liver of 4-month-old MSD mice injected with an AAV2/9-CMV-TFEB3xflag viral vector (Figure 30-B); we counted the number of TUNEL-positive cells and compared it with age matched MSD non-injected mice.

These results indicate that TFEB activation of lysosomal exocytosis reduces both primary accumulation of GAGs and secondary pathological processes associated with LSDs, such as inflammation and cell death (Medina et al. 2011).
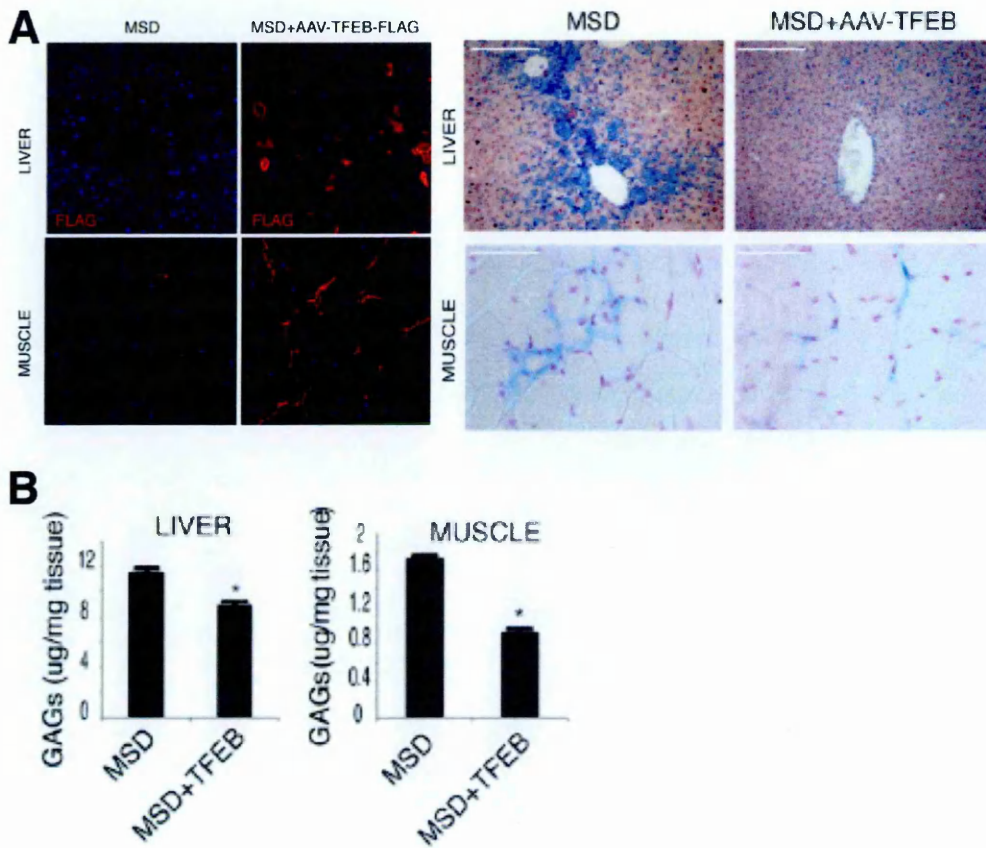
Figure 29. TFEB overexpression reduces GAG storage in a mouse model of MSD

(A) Representative immunofluorescence of liver and muscle sections infected with AAV-TFEB-FLAG (left panel) and alcian blue staining of GAG content in skeletal muscle and liver from mice injected systemically with either an AAV2/9-CMV-GFP or with an AAV2/9-CMV-TFEB3xflag viral vector (right panel). (B) Quantitative analysis of GAG content in liver (left panel) and skeletal muscle (right panel) of MSD mice injected with either an AAV2/9-CMV-GFP or with an AAV2/9-CMVTFEB3xflag viral vector. GAG content was displayed as mg of GAGs/mg of tissue extract. At least four mice per group were analyzed for each tissue examined (*p < 0.05). Data are mean of replicates (n=5) ± SEM; *p < 0.05.

A

MSD   MSD+AAV-TFEB

B

MSD   MSD+AAV-TFEB

TUNEL

LIVER

%TUNEL + CELLS

35
25
15
5
0

MSD   MSD+TFEB
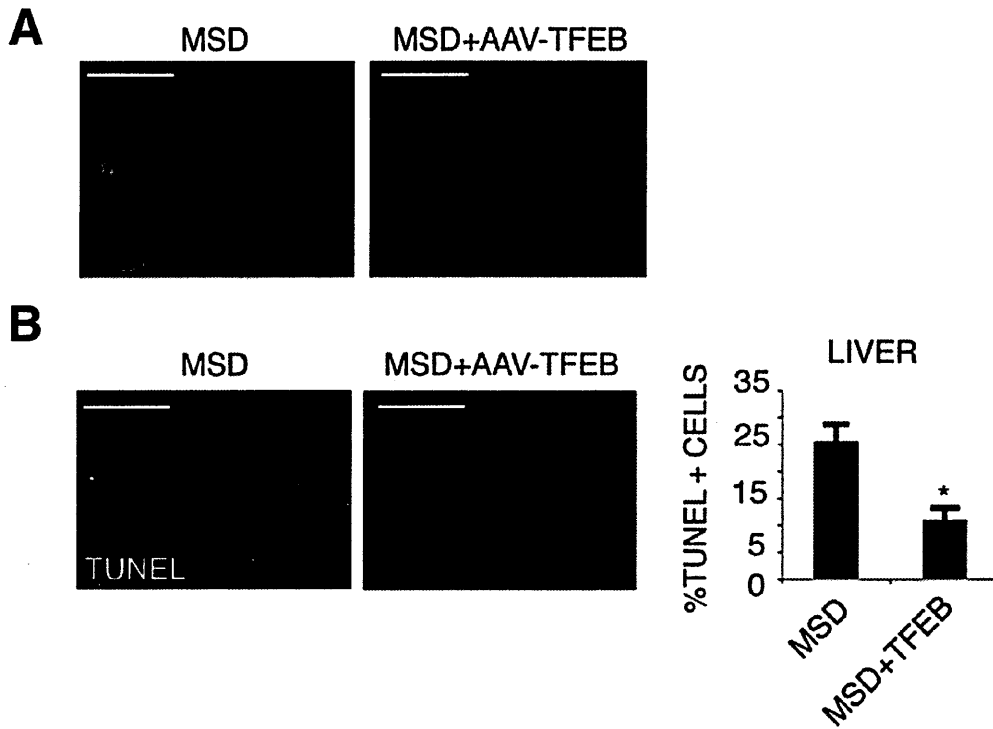
Figure 30. TFEB overexpression reduces tissue pathology in a mouse model of MSD

(A) TFEB reduces inflammation in the liver of MSD mice. Macrophages and macrophage-related inflammatory cells were detected in liver sections from mice injected systemically with either an AAV2/9-CMV-GFP or with an AAV2/9-CMV-TFEB3xflag viral vector by immunofluorescence analysis using an antibody against CD68. (B) Reduction of TUNEL-positive cells (arrows) in 4-month-old MSD mice injected with an AAV2/9-CMV-TFEB3xflag viral vector compared with age-matched MSD non-injected mice. At least four mice per group were analyzed for each tissue examined). Data are mean of replicates (n=5) ± SEM; *p < 0.05 (B and D). Scale bars represent 100 mm (A, C, and D).

DISCUSSION

NSCs recapitulate the main hallmarks of MSD pathology

Most of the LSDs present neurological involvement, but yet little is known about the mechanism of neurodegeneration. Intracellular accumulation of undegraded compounds are the primary effect observed in patients as well as in animal models, but whether they are the direct cause of cell death it is not clear; most likely the primary accumulation of undegraded substrates triggers secondary pathologic cascades, such as impaired autophagy, which in turn contributes together with global lysosomal dycfunction to neurodegeneration.

Post-mitotic neurons do not replicate in adult life and have a highly developed lysosomal network for membrane recycling: lysosomes are trafficked in axonal fibres using molecular motors to ensure that, as the cytoplasm extends to the far reaches and ramifications of the axon, distribution of the organelle is maintained (Tsukita and Ishikawa 1980). For this reason, the well-being of the lysosomal compartment has a particularly high relevance in post-mitotic neurons, and any perturbation of its proper function can cause neurological deficits, with or without cell death. In fact, in lysosomal diseases, electron microscopy of neurons, as well as other cell types, shows formation of vacuoles and lysosomes distended by recognizable cellular cargo (Walkley et al. 2010). Thus, it does not surprise that two-thirds of lysosomal diseases have potentially devastating consequences in the nervous system, and therefore future therapeutic research will require an

integrative understanding of the unitary steps in their neuronal pathogenesis.

Multiple Sulfatase Deficiency is a neurodegenerative lysosomal disease caused by the deficiency of a non-lysosomal protein; in fact, SUMF1 resides in the ER, where it is responsible for the post-translational activation of a whole family of enzymes, sulfatases. Patients suffering from MSD combine clinical symptoms of the different single sulfatase defects, with a severe impairment of neurological abilities. Sumf1 -/- mice recapitulate all the features of the disease observed in MSD patients, obviously including neurodegenerative aspects. The Sumf1 -/- mouse model not only allows a systematic study of the pathophysiology of MSD; importantly, it also provides a model to test therapeutic approaches to the treatment of MSD, similar to other mouse models of individual LSDs.

These observations prompted us to develop a reliable system to generate a neural cellular model from MSD mice, in order to investigate the signaling pathways involved in the neuronal pathology and eventually develop novel therapeutic strategies.

Furthermore, MSD mice are asymptomatic immediately after birth, but soon after they display congenital growth retardation and frequent mortality in the first weeks of life (Settembre et al. 2007). This observation suggested that early stages after birth are crucial for MSD pathology development, especially from the aspect of the neurological dysfunction, but that deeper investigation was needed.

Therefore, I isolated NSCs from neo-natal (P0) brain of wild-type and MSD mice, establishing a robust protocol that allows not only for the isolation, but also for the continuous *in vitro* expansion of NSCs, resembling and preserving all the morphological and molecular features of *in vivo* neuronal progenitors.

As a first observation, MSD-NSCs display similar properties of their wild-type counterpart, suggesting that, with SUMF1 lack of function, progenitor cells maintain their radial glia identity, although they seem to have a progressive reduction of self-renew.

It has been shown that Hematopoietic Stem Cells (HSCs) isolated from MSD mice are characterized by an altered extracellular signaling of FGF and Wnt pathways (Buono et al. 2010). Self-renewal and differentiation of HSCs are balanced by the concerted activities of FGF, Wnt, and Notch pathways; Sulf1 and Sulf2 are two non-lysosomal sulfatases, which are activated by SUMF1 post-translational modification and localize at the cell surface, where they mediate the remodeling of heparan sulfate proteoglycans (HSPGs). It has been demonstrated that Wnt controls tissue-specific cell fate decisions during embryogenesis, binding to the heparan sulfate moieties of HSPGs on the cell surface (Logan and Nusse 2004; Bejsovec 2005). Sulf enzymes remove the sulfate from heparan sulfate, and releases Wnt from HSPGs. This released Wnt associates with Frizzled (Fz) and LRP5/6 receptors, resulting in inactivation of a multiprotein destruction complex, which in turn leads to the translocation of β-catenin into the nucleus, where it activates several target genes (Hoppler and Kavanagh 2007). β-catenin translocation in the cell nucleus has been shown to enhance self-renewal and maintain totipotency of ESCs and multipotency of HSCs (Reya et al. 2003; Suh et al. 2007). Thus, the impairment in the activities of Sulf1 and Sulf2 in SUMF1 deficient cells may alter the action of important extracellular signals by modifying the sulfation state of the heparan sulfates contained in HSPGs. In fact, crystal structure studies have demonstrated that binding of FGF1 and FGF2 to the FGF

receptor is stabilized by sulfation of the heparan sulfates of the HSPGs (Pellegrini et al. 2000). It is also known that FGF signaling controls proliferation and subsequent lineage commitment of neural stem cells through a concerted action with β-catenin (Israsena et al. 2004).

Therefore, the self-renew impairment of MSD-NSCs observed in neurosphere assay may be ascribed to an alteration in the local concentration of important mitogenic factors bound to the HSPGs. Conversely, I did not observe any major block in the differentiation of MSD-NSCs, as glia and neurons expressed typical glia and neuronal markers, respectively, and they both did not show expression of the progenitor marker, as well as neurons outgrow neurites and were electrophysiologically active; nevertheless, more detailed and specific experiments are needed, in order to better quantify the extent of the differentiated cell population.

Isolated NSCs have the potency to generate the two main cell types of the CNS, glia and neurons, thus providing an *in vitro* model that can easily resemble all the main features of the developing brain. In fact, I did not observe defects in the differentiation of NSCs to the two main cell types of the brain, glia and neurons. However, the protocols I followed is one of the most accepted in order to visualize differentiation capacities in a grossly manner (Conti et al., 2005), but does not allow for a more specific analysis of diverse sub-populations actually present. In fact, NSCs can give rise also to oligodendrocytes, as well as different types of neurons; therefore, more detailed investigation on this aspect is needed, in order to evaluate whether the lack of SUMF1 can interfere with one or more of these cellular fate.

Furthermore, cellular differentiation is a progressive and committed process,

in which a single defect becomes usually amplified and triggers a cascade of subsequent intracellular malfunctions, eventually leading to a severe neurodegeneration. The more the cells proceed in the developmental progression, the more restricted they become in their differentiation potential, losing their self-renewal ability.

Altered autophagy is a hallmark of various LSDs, such as MSD and MPSIIIA (Settembre et al. 2008). As a degradative process, autophagy is responsible for constitutive protein turnover, a crucial function in neuronal cells and thus relevant to neurodegenerative diseases (Reggiori and Klionsky 2002). In agreement with *in vivo* data, we observed an increase of LC3-II levels in cellular extracts from differentiated MSD-NSCs, and accumulation of p62/SQSTM1 and poly-ubiquitinated proteins, both common components of protein aggregates in neurodegenerative diseases (Bjørkøy, Lamark, and Johansen 2006). We found that the accumulation of GAGs, poly-ubiquitinated proteins, and p62/SQSTM1 are likely to be associated with cell death. In fact, we observed that MSD-NSCs were more sensitive to apoptosis compared with wild-type cells, and in particular during the differentiation toward glial or neural cells. These findings correlate with the recent observation that defect in the recycling of dysfunctional mitochondria, through autophagy, could be involved in CNS neurodegeneration in MSD mouse model (de Pablo-Latorre et al. 2012).

Furthermore, I found a possible interesting correlation between impaired autophagy and aggresome formation and, although very preliminary, my data can suggest that, through this mechanism, the cell is protecting itself from toxic accumulation of proteins. Accumulation of misfolded proteins, indeed, is a

prominent pathological feature common to many neurodegenerative diseases, including Parkinson's disease (PD), Alzheimer's disease (AD), HD, Amyotrophic Lateral Sclerosis (ALS) and many others; protein quality control is particularly important to neuronal homeostasis and normal function because neurons are post-mitotic and unable to dilute cytotoxic misfolded proteins through cell division (Ross and Poirier 2004). Genetic mutations or environmental insults can induce many different proteins to misfold and aggregate, suggesting that a common pathological mechanism may link clinically distinct neurodegenerative diseases (Bucciantini et al. 2002; Kayed et al. 2003; Fändrich, Fletcher, and Dobson 2001; Glabe 2004). Interestingly, MSD and MPSIIIA cells present increased accumulation of overexpressed mutant forms of huntingtin and α-synuclein compared with wild-type counterparts, suggesting that protein aggregation may be activated in these diseases due to impaired autophagy (Settembre et al, 2008). In addition, recent findings demonstrate that certain regions of the brain of MPSIIIB mice show accumulation of hyperphosphorylated tau (Ptau) (Ohmi et al., 2009) and beta amyloid aggregations (Ohmi et al., 2011), both reminiscent of Alzheimer's disease, suggesting that LSDs are likely to produce toxic protein aggregates, as well as other more known neurodegenerative diseases. Therefore, in order to clarify the aspect of protein aggregation in MSD pathology, a future challange will be to detect endogenous toxic proteins, such as huntingtin and a-synuclein, and follow their accumulation in post-mitotic cells.

In cultured cell, when the production of misfolded proteins exceeds the capacity of the ubiquitin-proteasome degradation pathway, misfolded proteins are actively transported to the aggresome. Aggresome formation is recognized as a

cytoprotective response serving to sequester potentially toxic misfolded proteins and facilitate their clearance by autophagy (Fortun et al. 2003; Taylor et al. 2003; Iwata et al. 2005; Ravikumar, Duden, and Rubinsztein 2002). However, the deficit in autophagosome fusion with lysosome observed both *in vivo* and *in vitro* in MSD pathology can neutralize this cellular protective response, eventually worsening cellular viability.

TFEB-mediated activation of lysosomal exocytosis promotes the clearance of undegraded substrates

Establishing an *in vitro* system that was able to recapitulate all the main features of MSD prompted us to take advantage of the usefulness of this tool to explore possible therapeutic approaches, in the direction of reverting the pathologic phenotype. Towards this goal, we took advantage of the studies previously carried out in our laboratory on the bHLH transcription factor EB (TFEB) that was shown to coordinate lysosomal biogenesis, autophagy and also clearance in a neuronal model of HD (Sardiello et al. 2009; Settembre et al. 2011). Therefore we questioned whether TFEB-mediated increase of the lysosomal compartment could be exploited to induce the clearance of accumulated GAGs also in a neuronal cellular model of MSD.

Interestingly, TFEB overexpression resulted in a striking reduction of stored GAGs in MSD glial cells, as well as it induced recovery of normal intracellular morphology.

We also demonstrated that TFEB-mediated clearance of undegraded material

is actually due to the activation of one of his target genes, the cation-channel MCOLN1, which is then responsible for the promotion of lysosomal exocytosis; in fact, MCOLN1 triggers the intracellular Ca^{2+} elevation, required to induce lysosomes to move to the proximity of the PM; once there, they expel their content in the extracellular space, thus mitigating the intracellular burden (Figure 31).

Finally, we confirmed the importance of our result, showing that TFEB delivery rescues tissue pathology in a mouse model of MSD; one month after TFEB systemic injection, we observed a significant reduction of GAG storage in crucial organs, such as liver and muscles. Interestingly, also macrophage infiltration and apoptosis were strongly reduced, thus indicating that TFEB action ameliorates not only primary defects, such as the accumulation of undegraded material, but also those secondary defects, such as inflammation and cell death, that are the main pathologic features of MSD. Future studies overexpressing TFEB in the brain of MSD mice will allow us to further confirm the benefits of modulating lysosomal function in order to reduce pathologic lysosomal accumulation in the CNS.

Our data indicate that lysosomal exocytosis can be exploited to promote cellular clearance in lysosomal storage diseases, suggesting an alternative strategy to treat disorders due to intracellular storage, such as LSDs and other more common neurodegenerative diseases. Several approaches, such as gene delivery, pharmacological induction of TFEB, and target gene activation, could be exploited to promote cellular clearance in target tissues. These strategies will have to be tested by long-term *in vivo* studies in animal models to verify the therapeutic potential of this discovery.

As future investigation, it is particularly relevant that NSCs can be the perfect tool for drug screening, in order to find compounds able to induce cellular clearance, either in a TFEB-dependent or -independent way. As a second step, a very interesting goal will be to find genetic modulators of TFEB, whose expression can be modulated in order to achieve the clearance of undegraded substrates in LSDs. Toward this goal, I plan to use the very powerful and rapid genetic system of the fruit fly, *Drosophila Melanogaster*. Once generated all the fly models of LSDs, they can be used to validate *in vivo* drug targets that have been found to activate TFEB expression in a high-throughput cell screening. Furthermore, I can also conduct unbiased genetic screens on the whole fly genome to find genes that modify the phenotype of the diseases. This can enable us to uncover molecular pathways that are involved in the progression of LSDs. The screen may underlie interesting differences amongst them, but since several disorders share common features, it is very likely that the genetic screens will reveal common modifiers.
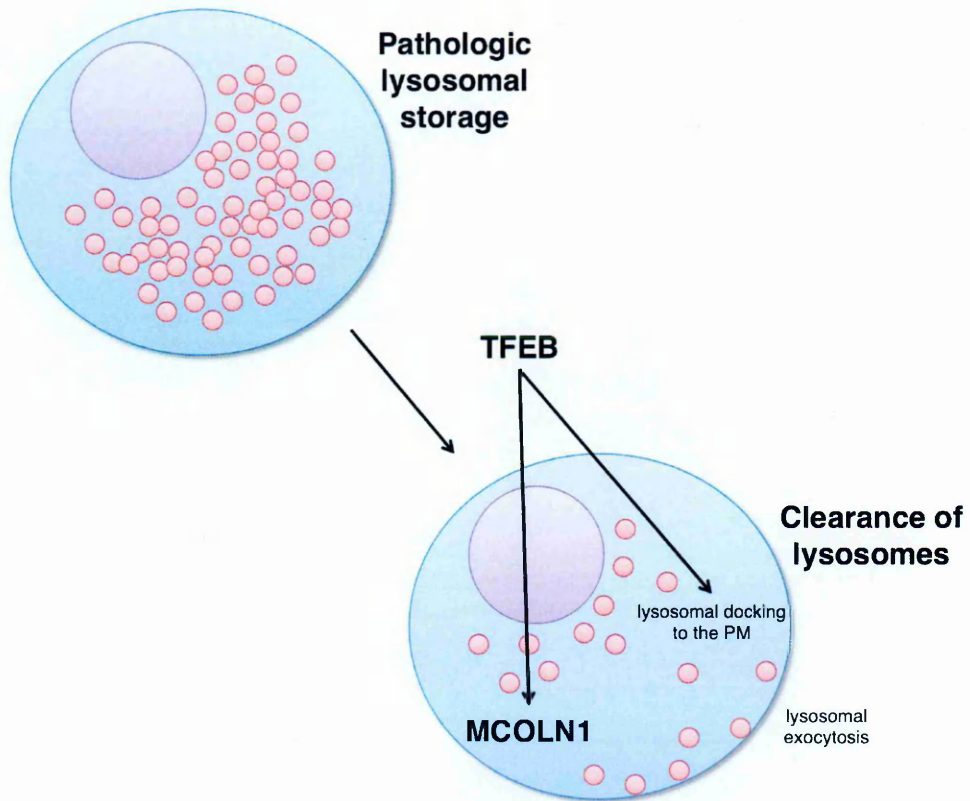
Figure 31. Schematic representation of TFEB mechanism of action.

TFEB overexpression activates the network of lysosomal genes, includind MCOLN1, which in turn promotes lysosomal exocytosis, reducing the storage of undegraded substrates.

MATERIALS AND METHODS

Isolation of NSCs and cell culture conditions

Neural tissue dissociation and positive selection of NSCs was achieved as described in results and following the manufacturer's manual (Miltenyi Biotec, Cologne, Germany).

Isolated neurospheres were propagated in culture at clonal density on uncoated plates (Nunclon, Waltham, Massachusetts, USA) in ESGRO medium (Chemicon, Temecula, California, USA) containing 20 ng/mL EGF and 10 ng/mL FGF2 (Peprotech, Rocky Hill, New Jersey, USA) and with penicillin/streptomycin (NSC expansion medium). Over 3–5 days, cells formed aggregates that, after harvesting and sedimentation to remove debris, subsequently attached to fresh plastic and outgrew NS cells.

For derivation from established neurospheres, NSC monolayer cultures was achieved by dissociating to single cells using Accutase (Sigma, St. Louis, Missouri, USA) and plating at 10^4 cells/ml on poly-ornithine (Sigma, St. Louis, Missouri, USA)/laminin (Invitrogen, Carlsbad, California, USA) coated culture plates in NSC expansion medium. For passaging established NSC lines, it was routinely used accutase (Sigma), cells were collected in phosphate-buffered saline (PBS) and split 1:3 to 1:5 every 2–3 days. The entire isolation protocol was carried out for three wild type and three Sumf1 -/- mice.

Flow cytometry analysis of nestin

NSCs were detached and dissociated with Accutase (Sigma, St. Louis, Missouri, USA), washed in PBS and triturated to a single cell suspension in PFN (15% PBS, 2% FCS, 0.1% sodium azide). Cells were distributed into 96-well U-bottomed microtiter plates (Nunc), fixed in 2% PFA (Sigma, St. Louis, Missouri, USA) for 20 minutes on ice, washed in PBS and incubated in PBS 0.5% saponin for 20 minutes on ice. After three washes in PBS 0.1% saponin, cells were incubated for 30 minutes on ice with a mouse anti-nestin primary antibody (Covance, Berkeley, California, USA) diluted 1:200 in PBS containing 0.1% saponin. After a further three washes, the cells were incubated for 30 minutes with a FITC-anti-mouse IgG diluted 1:200 in PBS 0.1% saponin. The cells were then washed in PBS 0.1% saponin and resuspended in PFN prior to Fluorescence-Activated Cell Sorting (FACS) analysis.

RT-PCR analysis

Total RNA was extracted from NSCs using RNeasy kit (Qiagen, Valencia, California, USA), and cDNA was generated using Superscript II (Invitrogen, Carlsbad, California, USA). Polymerase Chain Reaction (PCR) was performed for 30 cycles for all markers except B-actin (25 cycles). The primers used were as follows: β-actin (forward) 5'-GGCCCAGAGCAAGAGAGGTATCC-3' and (reverse) 5'-ACGCACGATTTCCCTCTCAGC-3'; oct4 (forward) 5'-GGCGTTCTCTTTGGAAAGGTGTTC-3' and (reverse) 5'-

116

CTCGAACCACATCCTTCTCT-3'; nanog (forward) 5'-

ATGAAGTGCAAGCGGTGGCAGAAA-3' and (reverse) 5'-

CCTGGTGGAGTCACAGAGTAGTTC-3'; pax6 (forward) 5'-

GCTTCATCCGAGTCTTCTCCGTTAG-3' and (reverse) 5'-

CCATCTTTGCTTGGGAAATCCG-3'; BLBP (forward) 5'-

GGGTAAGACCCGAGTTCCTC-3' and (reverse) 5'-ATCACCACTTTGCCACCTTC-

3'; sox2 (forward) 5'-GGCGGCAACCAGAAGAACAG-3' and (reverse) 5'-

GCTTGGCCTGCGTCGATGAAC-3'; olig2 (forward) 5'-

GGCGGTGGCTTCAAGTCATC-3' and (reverse) 5'-

TAGTTTCGCGCCAGCAGCAG-3'; mash1 (forward) 5'-

CTCGTCCTCTCCGGAACTGATG-3' and (reverse) 5'-

CGACAGGACGCCGCGCTGAAAG-3'; dlx2 (forward) 5'-

AACCACGCACCATCTACTCC-3' and (reverse) 5'-CCGCTTTTCCACATCTTCTT-

3'; nf-l (forward) 5'-GGATTCGCACAGCCTTCTC-3' and (reverse) 5'-

CCCTTCCTCTTCCAGCTTCT-3'.

Neurosphere assay

Cells were plated at 10 cells/µL in 24-well (0.5 mL/well) uncoated plates

(Nunclon) in NSC expansion medium. The total number of spheres that formed in

each well was counted after 7 days; the process was repeated similarly to generate

secondary and tertiary neurospheres, after 15 and 21 days, respectively. Only

colonies with a diameter > 100 µm were counted as spheres.

117

Cell Differentiation

For glia differentiation, NSCs were plated onto 24-well gelatine coated plates at 10^5 cells/well in ESGRO medium supplemented with 1% fetal bovine serum (FBS) (Hyclone, Logan, Utah, USA) and without any supplemented growth factor (glia differentiation medium); rapid differentiation of NSC to GFAP positive glia occurs within 2 days.

For neural differentiation, NSCs were plated onto 24-well poly-ornithine/laminin coated plates in ESGRO medium (Chemicon, Temecula, California, USA) 0.5% B27 (GIBCO, San Diego, California, USA) and with 10 ng/mL FGF2 (Peprotech, Rocky Hill, New Jersey, USA); a half volume of medium is replaced every 2-3 days to maintain conditioning of medium. After 7 days in these conditions, the media was switched to ESGRO medium (Chemicon, Temecula, California, USA) 0.5% B27 (GIBCO, San Diego, California, USA), without any growth factor. Half of the medium was exchanged every 2–3 days during the differentiation.

Immunofluorescence

To detect expression of intracellular markers, cells were plated on coated coverslips and fixed in PBS pH 7.4, 4% paraformaldehyde (PFA) (Sigma, St. Louis, Missouri, USA) for 20 minutes, quenched with 50 mM NH4Cl for 15 minutes. Cells were incubated per 20 minutes in FBS (Hyclone, Logan, Utah, USA) with 0.2% Triton X-100 to block nonspecific binding and permeabilize membranes, and

118

stained over-night with primary antibodies and for 2 hours with appropriate Alexa-594 and Alexa-488 conjugated secondary antibodies. Coverslips were mounted on glass slides in Vectashield with DAPI (Vector Laboratories, California, USA) to nuclear counterstaining and viewed on an epi-fluorescent or on a Zeiss LSM 510 META confocal microscope (LSM510; Carl Zeiss, Thornwood, New York, USA) equipped with a Plan-Neofluar 63x immersion objective (Carl Zeiss, Inc.). Mice tissues were collected after PBS perfusion and fixed with 4% PFA (Sigma, St. Louis, Missouri, USA) for 12 h at 4°C and then subjected to a sucrose gradient (from 10 to 30%) and incubated over night in 30% sucrose at 4°C, before OCT embedding. Immunofluorescence analyses were performed on 10-mm-thick serial cryosections. The specimens were incubated for 1 hour with blocking solution (PBS, 0.2% Tween-20) and 10% goat normal serum (Sigma) before incubation over-night with the specific primary antibody. After washing, sections were incubated for 40 min with secondary antibody. Stained sections were mounted with Vectashield with DAPI (Vector Laboratories, California, USA). Primary antibodies were used at the following dilutions: mouse anti-Nestin (1:400) (Covance, Berkeley, California, USA); mouse anti-GFAP (1:300) (Sigma, St. Louis, Missouri, USA); mouse anti-Tuj1 (1:200) (Covance, Berkeley, California, USA); rabbit anti-LC3 (1:200) (Novus Biological, Colorado, USA); mouse anti-CathD (1:500) (Sigma, St. Louis, Missouri, USA); rabbit anti-ubiquitin (1:100) (DakoCytomation, Carpinteria, California, USA); mouse anti-p62/SQSTM1 (1:200) (BD Biosciences, Franklin Lakes, New Jersey, USA); rabbit anti-HDAC6 (1:300) (Abcam, Cambridge, Massachusetts, USA); mouse anti-LAMP1 (1:500) (Sigma, St. Louis, Missouri, USA); rabbit anti-CD68 (1:250) (Serotech, Ontario, Canada) and mouse anti-flag

(1:200) (Sigma, St. Louis, Missouri, USA). Secondary antibodies were: goat anti-rabbit (1:3000) and donkey anti-mouse (1:5000) conjugated to Alexa Fluor 488 or 594 (Molecular Probes, Eugene, Oregon, USA).

Immunoblotting

Cells were lysed in cold lysis buffer (20 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1% TritonX-100) in the presence of protease inhibitors (Roche Diagnostics, Indianapolis, Indiana, USA) for 30 minutes on ice. Proteins were quantified by the Bradford method. 10-20 ug of protein samples were separated on SDS-PAGE acrylamide gel and transferred onto nitrocellulose membrane (Amersham Pharmacia Biotech, Uppsala, Sweden). Primary and HRP-conjugated secondary antibodies were diluted in 5% milk TBS-T. Bands were visualized using the ECL detection reagent (Pierce, Rockford, Illinois, USA) and normalized against actin. Antibodies were used as follows: mouse anti-GFAP (1:300) (Sigma, St. Louis, Missouri, USA); mouse anti-Tuj1 (1:200) (Covance, Berkeley, California, USA); rabbit anti-LC3 (1:200) (Novus Biological, Colorado, USA); rabbit anti-ubiquitin (1:100) (DakoCytomation, Carpinteria, California, USA); mouse anti-p62/SQSTM (1:200) (BD Biosciences, Franklin Lakes, New Jersey, USA); rabbit anti-HDAC6 (1:300) (Abcam, Cambridge, Massachusetts, USA); mouse anti-vimentin (1:300) (Santa Cruz Biotechnology, Santa Cruz, California, USA); and rabbit anti-Bactin (1:10000) (Sigma, St. Louis, Missouri, USA). HRP-conjugated secondary antibodies were: rabbit anti-mouse (1:3000) and mouse anti-rabbit (1:3000) (Amersham, Uppsala, Sweden).

Neurite Outgrowth Assay

Neurite assay was performed following the manufacturer's manual (Millipore). Differentiating neurons were placed in Millicell 12-well inserts containing neural differentiation medium at a concentration of 100,000 cells/100 μL (1 x 10^6 cells/mL). Plates were incubated at 37°C for 3 and 7 days. Following the neurite extension period, each insert was removed from the culture well, washed with PBS and fixed in cold methanol for 20 minutes at room temperature. Then the insert was stained for 15-30 minutes with Neurite Stain Solution, washed with PBS and then cell bodies were gently swabed from top of insert. Neurite Stain Extraction Buffer was added to the top of each swabbed inserts and incubate for 5 minutes at room temperature. Inserts were spinned and then read at an absorbance of 590 nm.

Electrophysiology and patch clamp recording

Recordings were made from NSC-derived neurons, differentiated between passages 20-25. Seals between electrodes and cells were established in a bath solution consisting of (in mmoles/l): 138 NaCl, 2 CaCl$_2$, 5.4 KCl, 1 MgCl$_2$, 10 glucose and 10 HEPES, pH 7.4 with NaOH; the pipette (intracellular) solution contained (in millimolar): 140 KCl, 2 MgCl$_2$, 10 EGTA, 10 HEPES, 5 Mg-ATP, pH 7.3–7.4 with KOH. The pCLAMP software (version 10.0.2) was used for data acquisition and analysis.

Ionic currents were recorded under voltage-clamp conditions using the patch-clamp whole-cell configuration at room temperature (20-24°C) with an Axopatch

200B patch-clamp amplifier (Axon Instruments, Burlingame, California, USA). For

Ionic currents recording the following protocol was applied: from an holding

potential of -90 mV, a short (50 ms) pulse to -100 mV followed by a series of

progressively increasing depolarizations (from -100 mV to +40 mV) of 50 ms

duration, before a final 50 ms step to -100 mV was applied. Data were filtered at 5

kHz and sampled at 50 kHz. Capacitance currents were compensated by analog

circuitry and subtracted on-line by using a p/-4 protocol from a subtracting holding

potential of -100 mV.

Electron Microscopy (EM) and immuno-gold analysis

Cells were washed with PBS and fixed at room temperature in 0.05%

glutaraldehyde (Polysciences, Warrington, Pennsylvania, USA) dissolved in 0.2 M

Hepes buffer (pH 7.4) for 30 minutes at room temperature. Then, cells were

scraped off the dish, pelletted by centrifugation and postfixed for 2 hours in 1%

OsO4 (Polysciences, Warrington, Pennsylvania, USA) in the same buffer. After en-

bloc staining with 1% uranyl acetate for 1 hour and ethanol dehydration, cells were

embedded in Epon-812 (Fluka, Ronkonkoma, New York, USA) and polymerized at

60°C for 72 hours. Thin sections were cut at the Leica EM UC6 and counterstained

with uranyl acetate and lead citrate. Images were acquired from thin sections using

a Philips Tecnai-12 electron microscope equipped with an ULTRA VIEW CCD

digital camera (Philips, Eindhoven, Netherlands).

For immuno-gold HeLa and CF7 cells were fixed with a mixture of 4%

paraformaldehyde and 0.05% glutaraldehyde, labeled with a monoclonal antibody

against LAMP1 according the gold-enhance protocol, embedded in Epon-812, and cut as described previously (Polishchuk et al. 2003). EM images were acquired from thin sections using a Philips Tecnai-12 electron microscope equipped with an ULTRA VIEW CCD digital camera (Philips, Eindhoven, Netherlands). Quantification of vacuolization was performed using the AnalySIS software (Soft Imaging Systems GmbH, Muenster, Germany). Evaluation of lysosome distance from the PM was done in EM images using the iTEM software (Soft Imaging Systems GmbH). Selection of cells for quantification was based on their suitability for stereologic analysis, i.e., only cells sectioned through their central region (detected on the basis of the presence of Golgi membranes) were analyzed.

Alcian Blue staining

Undifferentiated cells, glia and neurons were stained with 1% Alcian blue (Sigma, St. Louis, Missouri, USA) in hydrochloric acid. The counterstaining was performed for 2 minutes with Nuclear-Fast red (Sigma, St. Louis, Missouri, USA).

After the perfusion of the animals with PBS, the tissues were collected and fixed in methacarn solution (30% chloroform, 60% methanol and 10% acetic acid) for 24 hours at 4°C. The next day, the tissues were embedded in paraffin (Sigma, St. Louis, Missouri, USA) after their dehydration with a 70–100% ethanol gradient. Finally, the tissues were sectioned into 7 mm thick serial sections on a microtome. Sections of paraffin-embedded tissues were stained with 1% Alcian blue (Sigma, St. Louis, Missouri, USA) and counterstained with Nuclear-Fast red (Sigma, St. Louis, Missouri, USA).

GAG quantitative assay

The protein extracts were assayed with the dimethylmethylene blue-based spectrophotometry of glycosaminoglycans (de Jong et al. 1989). The samples were read at 520 nm and the GAG concentrations were determined using the heparan sulfate standard curve (Sigma, St. Louis, Missouri, USA). Tissue GAG amount was expressed as μg GAG/μg protein.

GAG pulse-and-chase analysis

Cells were grown in differentiation medium in presence of 7μCi/ml ^{3}H-glucosamine hydrochloride (37.75 Ci/mmol, Perkin Elmer, Massachusetts, USA) for 24 hours, washed extensively with PBS and then chased. At the end of the chasing time, cells were harvested, homogenized and subject to chromatography on Sephadex G-25 columns (GE Healthcare, Sweden) to eliminate unincorporated ^{3}H-glucosamine hydrochloride. The amounts of incorporated or secreted radioactivity was measured by liquid scintillation in a Beckman LS6500 counter (Beckman Instruments, Fullerton, CA, USA).

TUNEL assay

Cells apoptosis was detected by TUNEL assay following the manufacturer's manual (Roche, Basle, Switzerland). TUNEL-positive cells were quantitated microscopically.

TUNEL assay on tissue was performed by using the ApopTag Peroxidase In Situ Apoptosis Detection kit following the manufacturer's manual (Oncor, Gaithersburg, Maryland, USA).

Transfection and nucleofection

NSCs, HeLa cells and human fibroblasts were transfected using PolyFect Transfection Reagent (QIAGEN, Hilden, Germany), according to the manufacturer's protocols. HeLa CF7 stable cell lines was previously described (Sardiello et al. 2009). The HeLa^{shMCOLN1} cell line was generated by infection with MCOLN1 lentiviral shRNA. NSCs were transfected by using nucleofection (Amaxa, Lonza, Walkersville, Maryland, USA).

Staining for Surface LAMP1

Cells were grown on glass coverslips, incubated with anti-rat LAMP1 (1DB4; Santa Cruz Biotechnology, Santa Cruz, CA) diluted in PBS 1% BSA at 4°C for 30 minutes. Cells were washed in PBS and fixed in 4% PFA (Sigma, St. Louis, Missouri, USA) for 15 minutes at 4°C, washed in PBS, and incubated with Alexa-594 conjugated anti–rat secondary antibodies (Molecular Probes, Eugene, Oregon, USA) for 30 minutes at room temperature. Coverslips were mounted on glass slides with Vectashield (Vector Laboratories, California, USA). Finally, cells were analyzed on a confocal microscope (LSM510; Carl Zeiss, Thornwood, New York, USA) equipped with Plan-Neofluar 63x immersion objective.

125

Flow Cytometry analysis of surface LAMP1

Confluent cells transfected with TFEB-GFP constructs were trypsinized and washed twice with PBS before incubation with PBS for 5 min at 37°C. 10^6 cells for each assay were centrifuged and resuspended in PBS 1% BSA with anti-rat LAMP1-1DB4 at 4°C for 30 minutes. Cells were washed in PBS and fixed in 2% paraformaldehyde for 15 minutes. Cells were further incubated with Alexa-594 conjugated anti–rat secondary antibodies (Molecular Probes, Eugene, Oregon, USA) for 30 minutes at room temperature. Finally, cells were washed, resuspended in 0.5 ml PBS, and analyzed on a FACS Aria Flow Cytometer (Becton Dickinson & Co., Mountain View, California, USA). Forward angle scatter, right angle scatter, and fluorescence intensity were recorded from 50,000 cells whose forward angle scatter fell above a threshold used to distinguish intact GFP-positive cells from both non-transfected or damaged cells.

Enzymatic Activities

Enzyme activities were measured with the appropriate fluorimetric or colorimetric substrates. Specifically, acid phosphatase and ß-hexosaminidase activities were measured using the Acid-Phosphatase and ß-N-Acerylglucosaminidase assay kit (Sigma, St. Louis, Missouri, USA), repectively. ß-galactosidase was measured by a colorimetric assay using 4-methylumbelliferyl-β-D-galactopyranoside as substrate in 0,5M NaAc buffer, pH 5.0. Lactate de-hydrogenase (LDH) activity was measured using the LDH-Cytotoxicity Assay Kit

(Abcam, Cambridge, Massachusetts, USA). To measure enzyme activities in the medium, cells were incubated in medium containing 1% BSA (w/v). Protein concentrations were determined using the BCA-assay (Pierce, Rockford, Illinois, USA).

Calcium Measurements by Confocal Imaging

TFEB-GFP-transfected cells were treated with FuraRed for 30 minutes in a $Ca2+$ imagin buffer and analyzed according to the manufacturer's instructions (Invitrogen).

Flow Cytometric Calcium Flux Assay

Cell preparation and loading was performed following a protocol already described (Schepers et al. 2009) with some modifications. Briefly, cells were loaded with Fluo-3AM and FuraRed-AM (Molecular Probes, Eugene, Oregon, USA) for 30 minutes at 37°C. After a wash in PBS without $Ca2+$, the cells were resuspended in the appropriate buffer in accordance to the different conditions and kept at RT until analysis. $Ca2+$-entry is prevented by the use of a PBS buffer without $Ca2+$ and containing 1 mM sodium pyruvate, 25 mM HEPES and 5 mM EGTA, the latter to exclude remaining traces of $Ca2+$ contamination. In these Fluo3/FuraRed-loaded cells the release of $Ca2+$ from the intracellular stores can be studied. Samples were analysed using the FACS Aria Flow Cytometer (Becton

Dickinson & Co., Mountain View, California, USA). Following preparation, samples were aspirated during 30 seconds to determine the baseline fluorescence of the Fluo3 and FuraRed. Then, after the addition of ionomycin (5 µM), the acquisition was resumed with changes in Ca2+ concentration being recorded over a 150 seconds time period. Changes in the fluorescence (FL) intensity of the Fluo3 and FuraRed were measured on the FL-1 and FL-2 channels. The ratio of baseline Fluo3/FuraRed was plotted. Data are expressed as means ± standard deviation (SD). A p-value of less than 0.05 was considered significant.

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138

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151

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158

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