The role of ATP13A2/PARK9 protein in the lysosomal degradation pathway in neurodegeneration

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ABBREVIATIONS

3-AT, 3-amino-1,2,4-triazole

α –syn, α-synuclein

Aβ, β-amyloid peptide

AD, Alzheimer disease

ALS, amyotrophic lateral sclerosis

C. elegans, Caenorhabditis elegans

CMA, chaperone-mediated autophagy

CNS, central nervous system

Cub, C-terminal fragment of ubiquitin

DA, dopamine

DPI, day post-infection

EGFP, enhanced green fluorescence protein

EOPD, early-onset Parkinson disease

ER, endoplasmic reticulum

EV, empty vector

FTD, frontotemporal dementia

GFP, green fluorescence protein

GC, glucocerebrosidase

GO, gene ontology

HA, hemagglutinin

HD, Huntington disease

Htt, huntingtin protein

KD, knockdown

KRS, Kufor-Rakeb syndrome

LAMP-2A, lysosomal membrane associated protein

LDH, lactate dehydrogenase

LSDs, lysosomal storage disorders

MEFs, mouse embryonic fibroblasts

MF α, α-mating pheromone precursor

MTOC, microtubule-organizing center,

MVB, multivesicular body

MYTH, membrane yeast two-hybrid

NCLs, neuronal ceroid lipofuscinoses

NF, neurofilament

NPC, Neimann-Pick disease type C

Nub, N-terminal fragment of ubiquitin

PD, Parkinson disease

RNAi, RNA interference

ROS, reactive oxygen species

shRNA, short hairpin RNA

TF, transcription factor

Ub, ubiquitin

UBPs, ubiquitin specific proteases

UPS, ubiquitin-proteasome system

WT, wild type

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1. INTRODUCTION

Lysosomes are part of essential cell machinery responsible for degradation and recycling of bulky cell material to maintain cell homeostasis. Increasing evidence implicates lysosomal dysfunction in wide array of neurodegenerative disorders such as Parkinson (PD), Huntington (HD) and Alzheimer diseases (AD) (Nixon et al., 2008; Shacka et al., 2008; Jaeger and Wyss-Coray, 2009; Zhang et al., 2009). In addition, more than two-thirds of diseases characterized by the dysfunction of lysosomal pathways (i.e. lysosomal storage diseases) are exhibiting dysfunction in the central nervous system (CNS), emphasizing how neurons are particularly vulnerable to lysosomal impairment (Schultz et al., 2011).

Therefore, the study of lysosomal neurodegenerative disorders that are caused by mutations in lysosomal proteins presents an opportunity to elucidate specific molecular mechanisms and pathways that contribute to neurodegeneration.

1.1 Lysosomes

Discovery of the lysosome by Christian de Duve in 1955 started a new cell biology era and provided biological framework for understanding significance of lysosomes in pathogenesis of diseases (DE DUVE et al., 1955; de Duve, 2005). Lysosomes are membrane-bound organelles that play a major role in degradation of macromolecules through the adjusted action of more than 50 soluble hydrolytic enzymes (e.g. glycosidases, proteases, lipases, nucleases, phosphatases and sulfatases) and over 120 lysosomal membrane proteins (Braulke and Bonifacino, 2009). Lysosomes are acidic organelles, whose optimal pH (4.6-5.0) needed for maturation and activity of hydrolytic enzymes is maintained by V-type H^+-ATPase (Ruivo et al., 2009). Electron microscopy showed that lysosomes are of heterogeneous size and morphology and often have electron-dense cores and membrane whorls (membranous structures that look like multi-lamellar or arranged in spirals when observed in cross-section) (Luzio et al., 2007).

Lysosomes are dynamic organelles that interact and fuse with various cell compartments to perform their role in important cellular pathways such as autophagy, phagocytosis, endocytosis and exocytosis (Figure 1) (Luzio et al., 2007; Saftig and Klumperman, 2009). They are

responsible for degradation of extracellular material that enters the cell through endocytosis and phagocytosis as well as intracellular components through autophagy. During endocytosis, exogenous material, fluid, plasma membrane components and ligands are internalized in endosomes and via sequence of early and late endosomes delivered to the lysosomal lumen for degradation. This process is important for regulation and recycling of cell-surface molecules and ligand-receptor complexes (Martinez-Vicente and Cuervo, 2007). Lysosomes also participate in the cell defense against extracellular aggressors by playing a role in process of phagocytosis. During this process specialized cells engulf invading pathogens (e.g. bacteria, yeast or parasites) in a membrane-sealed compartments called phagosomes that fuse with lysosomes to degrade the undesired particles (Luzio et al., 2007; Martinez-Vicente and Cuervo, 2007). Further, autophagy-lysosomal pathway is responsible for degradation of intracellular proteins and entire organelles within the lysosomes. In addition, lysosomes participate in lysosomal exocytosis, a Ca^{2+}-regulated process, during which lysosomes dock to the cell surface and fuse with plasma membrane, releasing their content outside the cell. This process is important for secretion, cellular clearance, membrane repair, cell adhesion and migration (Figure 1) (Medina et al., 2011).

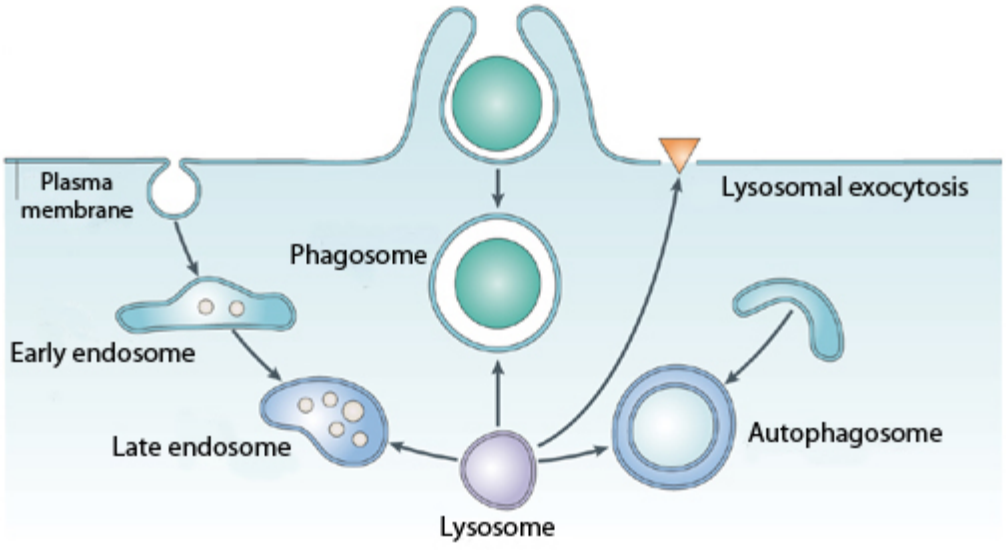


Figure 1. **Lysosomal fusion with different cellular organelles**
Lysosomes are fusing with endosomes, phagosomes and autophagosomes in order to perform their function as degradation organelle, and with plasma membrane during process of lysosomal exocytosis. Figure has been adapted and modified from Luzio et al., 2007 with permission from Elsevier press.

Since many pathways converge to the level of lysosomes, lysosomal dysfunction can disrupt many cellular processes. This can result in accumulation of undigested material and impairment of lysosomal pH, vesicle maturation and trafficking, synaptic release, neuronal remodeling, cell signaling, and calcium homeostasis. All these compromised processes can endanger cell viability (Schultz et al., 2011).

Particular interest in autophagy-lysosomal degradation pathway by neuroscientists has been sparked by recognition of lysosomal pathology and accumulation of protein aggregates in major neurodegenerative diseases (Nixon et al., 2008).

1.2 Clearance of misfolded and aggregated proteins

Cellular homeostasis and viability greatly rely on surveillance mechanisms that continuously control protein quality and participate in protein repair or its removal from the cell if there are any abnormalities (Martinez-Vicente and Cuervo, 2007; Wong and Cuervo, 2010). Major cellular pathways playing a role in quality control surveillance are intracellular proteolytic systems (ubiquitin-proteasome system and lysosomal degradation pathway) and molecular chaperones (Figure 2). Protein abnormalities that can occur relate to any type of protein modification that alters its three-dimensional folded, stable and functional structure, and results with misfolded or unfolded protein. These conformational alterations can be consequence of many cellular events, such as inappropriate protein folding during process of synthesis, errors in post-translational modifications or genetic mutations (Martinez-Vicente and Cuervo, 2007; Gao and Hu, 2008). Cellular stressors such as oxidative stress, endoplasmic reticulum stress or temperature perturbations can also cause protein unfolding or misfolding (Gao and Hu, 2008; Kirkin et al., 2009). **Molecular chaperones**, which mostly belong to a heat shock protein family (Hsp), are helping proper protein folding and refolding if abnormalities in protein structure occur. If the stable and functional structure of the protein cannot be accomplished proteins are targeted for degradation by **ubiquitin-proteasome system (UPS)** (Rubinsztein, 2006; Gao and Hu, 2008). Proteasomes are barrel-shaped multiprotein complexes that predominantly degrade short-lived nuclear and cytosolic proteins covalently modified with ubiquitin (Ub) molecules (Goldberg, 2003; Ciechanover, 2006; Rubinsztein, 2006). This process of conjugating ubiquitin molecules to a protein (i.e. ubiquitination) is achieved with highly orchestrated action of three major enzymes: E1 (ubiquitin-activating enzyme) that hydrolyses ATP, activates Ub and transfers it to a E2 (ubiquitin-conjugating enzyme) that binds E3 (ubiquitin ligase) which

connects to a protein and transfers Ub to a lysine residue of a target protein (Rubinsztein, 2006; Gao and Hu, 2008; Korolchuk et al., 2010). Ubiquitin molecule attached to the target protein presents a substrate for additional rounds of ubiquitination that occurs on his lysine (K) residues at positions 6, 11, 27, 31, 33, 48 or 63. Chain of four Ub molecules interconnected via K48 residues is thought to present a recognition signal for proteasome degradation. Proteasome, as a 26S proteolytic organelle, consists of a 20S central catalytic core and two 19S subunits that act as its lid. 19S subunits bind targeted proteins, remove Ub from it and control its entrance into the 20S catalytic domain that degrades proteins into oligopeptides, which are subsequently released into the cytoplasm and degraded into amino acids by soluble peptidases (Rubinsztein, 2006; Korolchuk et al., 2010).

In situations that promote acute cell damage, excessive production of misfolded proteins can occur and UPS may fail to remove them in short period of time. Accumulated misfolded proteins present a template for formation of more complex protein structures (Soto and Estrada, 2008). In the aqueous environment of cytoplasm, proteins are folded into native structure which is thermodynamically most favorable, with hydrophobic cores and which is stabilized through non-covalent interactions between amino acids' side chains (Berg et al., 2002). On the other hand, protein misfolding or unfolding expose hydrophobic residues in aqueous cytosolic environment, which further promotes formation of complex structures called **aggregates**. Aggregation is multistep process consisting of formation of intermediates like oligomers, protofibrils, fibrils, and finally of large aggregates (Kirkin et al., 2009; Yamamoto and Simonsen, 2011).

As previously mentioned, mutations can cause protein misfolding. Point mutations resulting in, for example exchange of hydrophobic amino acid with hydrophilic, can drastically disturb stable protein structure leading to its misfolding. Furthermore, some mutations are causing expansion of glutamine repeats, for example mutations in huntingtin protein. This long polyglutamine tracts are very adhesive and aggregate into β-sheet-rich oligomers, which further promote formation of protein aggregates (Perutz and Windle, 2001).

Aggregates present potential danger for a cell, since they can interfere with cellular trafficking and become a trap for another functional proteins (e.g. chaperones, transcription factors), promoting their aggregation (Gao and Hu, 2008). In addition, protein aggregates can cause oxidative stress that affects proteins, lipids and mitochondria (Behl et al., 1994; Hsu et al., 2000).

It has also been proposed that aggregates can form pores in cellular membranes, thereby disturbing ion homeostasis and cellular signaling cascades, which can consequently lead to cell death (Lashuel et al., 2002; Volles and Lansbury, 2002). Together, these potential threats of aggregates on cell viability emphasize the importance of their proper clearance.

Protein aggregates cannot be degraded with UPS, since substrates need to be monomeric, soluble and unfolded in order to pass through the narrow pore of the proteasome. Healthy cells overcome this problem through the **autophagy-lysosomal degradation pathway** as a system responsible for degradation of bulky cytoplasmic material including damaged organelles and misfolded and accumulated proteins (Rubinsztein, 2006; Martinez-Vicente and Cuervo, 2007).

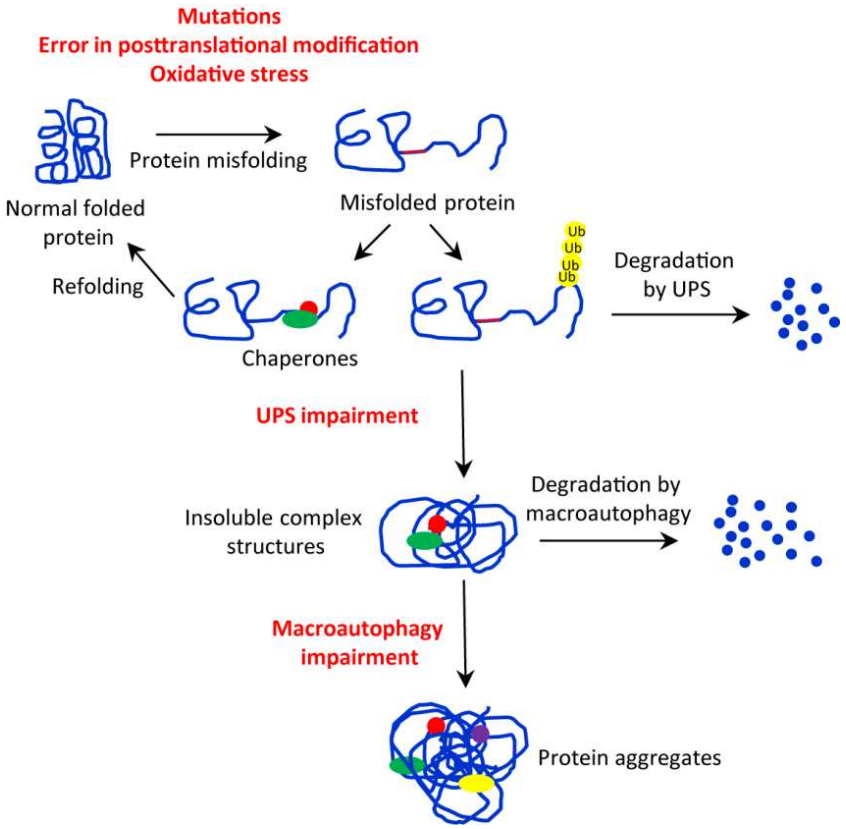


Figure 2. Quality control systems
Different cell stressors can cause protein misfolding (e.g. mutations, oxidative stress). Misfolded proteins are recognized by chaperones that help their refolding. In case those proteins cannot adopt their stable folded structure they become ubiquitinated and degraded by UPS. If this proteolytic system fails, proteins continue to form complex insoluble structures that can only be degraded by macroautophagy. In situations which cause impairment of macroautophagy, proteins start to aggregate. This illustration has been designed after a figure from Martinez-Vicente and Cuervo, 2007.

1.3 Autophagy and its molecular machinery

Autophagy - degradation of cytosolic components in lysosomes - can be divided in three subtypes based on the mechanism by which the degraded cargo is delivered to the lysosomes: 1) microautophagy, 2) chaperone-mediated autophagy (CMA) and 3) macroautophagy (Figure 3). In microautophagy, smaller cytosolic regions are engulfed directly into the lysosomal lumen by invagination of lysosomal limiting membrane. In CMA, proteins with lysosomal targeting motif KFERQ are selectively recognized by complex of chaperones that together with lysosomal receptors (e.g. LAMP-2A) help their translocation into lysosomal lumen to be degraded. During this process proteins need to be monomeric and unfolded that precludes the degradation of toxic multimeric protein complexes. That is when macroautophagy plays a crucial role as a pathway that involves degradation of complete bulk regions of the cytoplasm. In this process cytoplasm, protein complexes or entire organelles are engulfed by autophagosomes that fuse with lysosomes that provide enzymes required for degradation of cargo (Rubinsztein, 2006; Martinez-Vicente and Cuervo, 2007; Wong and Cuervo, 2010).

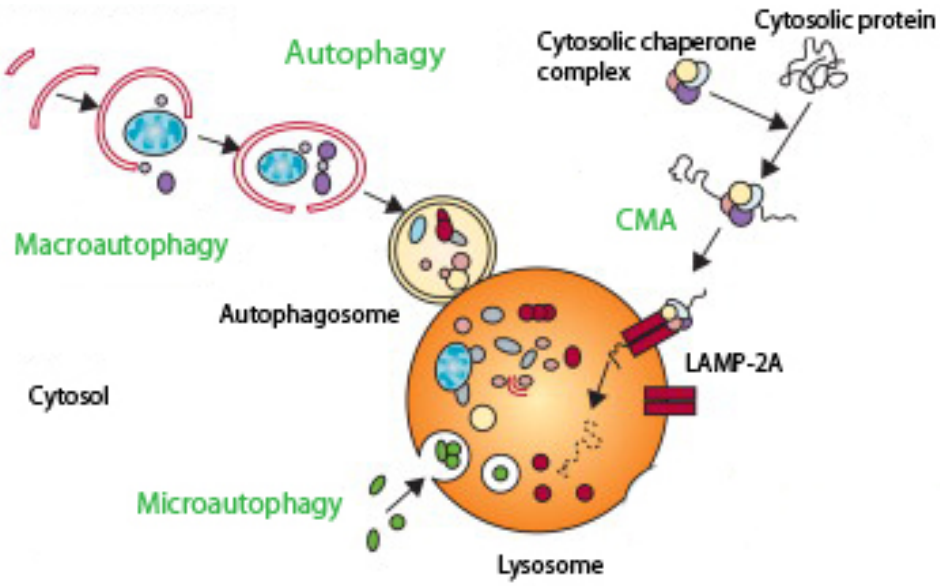


Figure 3. Autophagy system
The illustration represents three major autophagic pathways: macroautophagy, microautophagy and chaperone-mediated autophagy (CMA) involved in degradation of intracellular material. Figure has been adopted and modified from Martinez-Vicente and Cuervo, 2007, with permission from Nature Publishing Group.

Macroautophagy (hereafter referred to as autophagy) can be considered as a process consisted of autophagosome nucleation, elongation, maturation and fusion. Discovery of autophagy-related proteins (Atg) in yeasts that were found to be conserved throughout the evolution, greatly helped in elucidating molecular mechanisms of autophagy in mammalian cells (García-Arencibia et al., 2010; Mizushima et al., 2011). Early steps of autophagosome formation, nucleation and elongation, are driven by several protein complexes: 1) the Atg1/Unc-51-like kinase (ULK1) complex 2) the Vps34/class III phosphatidylinositol 3-kinase (PI3K) complex I, which forms phosphatidylinositol-3-phosphate (PI3P), 3) the transmembrane protein Atg9 and its associated cycling machinery; and 4) the two-ubiquitin-like systems Atg12-Atg5 and Atg8/MAP1-light chain (LC3) (Knaevelsrud and Simonsen, 2010; Yamamoto and Simonsen, 2011).

1.3.1 Initiation of autophagosome formation - nucleation

Autophagy is initiated by the formation of double–layered isolation membrane (called phagophore) at the phagophore-assembly site (PAS). The origin of this isolation membrane is still a debated topic, but recent studies proposed that ER, Golgi network and mitochondria membranes might be involved (Korolchuk et al., 2010; Mizushima et al., 2011; Yamamoto and Simonsen, 2011). Formation of phagophore depends on the recruitment and the activity of PI3K complex. Proteins Vps34, Vps15, Atg14 and Beclin-1 are major components of this complex that is responsible for generation of PI3P at PAS and allows recruitment of other Atg proteins (García-Arencibia et al., 2010; Yamamoto and Simonsen, 2011). Second ULK complex consisted of Atg1-ULK-Atg13-FIP200-Atg101 proteins is also important for autophagosome nucleation and recruitment of other Atg proteins involved in autophagosome synthesis, including Atg9 and its cycling machinery (Figure 4).

1.3.2 Elongation

Once nucleated, the phagophore elongates around the cargo and seals to form an double membrane layered autophagosome (Yamamoto and Simonsen, 2011). Elongation and expansion of phagophore membrane requires two-ubiquitin-like conjugation systems. First conjugation reaction includes conjugation of Atg12 to Atg5 by the action of Atg7 (E1-like) and Atg 10 (E2-

like) proteins (García-Arencibia et al., 2010). The Atg12-Atg5 conjugate interacts with membrane-bound Atg16L to form high molecular weight-complex. This association determines the site of the LC3 conjugation (a mammalian Atg8) to the lipid phosphatidylethanolamine (PE), that occurs as second conjugation reaction (Knaevelsrud and Simonsen, 2010). LC3 protein is cleaved by cysteine protease Atg4 to form cytosolic LC3-I, which is then conjugated to PE by the action of Atg7 and Atg3 (E2-like) proteins to generate membrane-bound LC3-II. The molecular mechanisms that are involved in final fusion of the phagophore to close the autophagosome are not well understood, although the role of LC3-II has been proposed (Nakatogawa et al., 2007). After the formation of autophagosome, Atg12-Atg5 conjugate is released from the vesicle, but LC3-II protein stays attached to the inner leaflet of the autophagosome, even after its fusion with the lysosomes, when it gets degraded together with the engulfed cargo (Figure 4). Therefore, LC3-II is generally used as a reliable autophagosomal marker, allowing to study both autophagosome initiation and degradation (Korolchuk et al., 2010; Mizushima et al., 2011; Klionsky et al., 2012).

1.3.3 Maturation and fusion

When autophagosome is formed it goes through the processes of maturation by a single or multiple fusion processes. In mammalian cells autophagosome might directly fuse with lysosome and form autolysosome or it can mature going through a stepwise process fusing with different endocytic compartments (early endosomes, multivesicular bodies and late endosomes) to first form amphisome before fusing with lysosome that degrades sequestered cargo (Knaevelsrud and Simonsen, 2010; Yamamoto and Simonsen, 2011) (Figure 4). The details about molecular machinery responsible for autophagosome maturation and fusion are still unclear but recent studies suggested few proteins and protein complexes that are involved in membrane tethering, docking and fusion, such as ESCRTs (endosomal sorting complex required for transport) complex, COPI (coat protein complex I), SNAREs (soluble N-ethylmalemide-sensitive factor activating protein receptors), Rab7, Rab11, UVRAG (ultraviolet radiation resistance-associated gene), and the class C vacuolar proteins sorting (Vps) proteins, HOPS (homotypic fusion and vacuole protein sorting) complex (Luzio et al., 2007; García-Arencibia et al., 2010; Yamamoto and Simonsen, 2011).

Protein complexes ESCRT and COPI are found to be involved in autophagosome maturation through a stepwise process (Knaevelsrud and Simonsen, 2010). COPI, found on early endosomes, is involved in sorting of endocytic cargo, whereas ESCRTs are responsible for 1) formation of multivesicular bodies (MVBs), 2) sorting of cargo determent for degradation and 3) fusion of MVBs with lysosomes (Luzio et al., 2007; Knaevelsrud and Simonsen, 2010). As previously described, Vps34 protein as a part of Vps34 /class III PI3K complex I is involved in nucleation of autophagophore, but it can also be engaged in Vps34/class III PI3K complex II. This complex, consisted of Vps35, p150 and Beclin-1 proteins, can also contain negative regulator Rubicon (Run domain protein as Beclin-1 interacting and cysteine-rich containing), and/or positive regulator UVRAG and both play a role in endosomal trafficking and autophagosome-lysosome fusion (Mizushima et al., 2011; Yamamoto and Simonsen, 2011).

Fusion between lysosomes and autophagosomes/amphisomes occurs in perinuclear region, near microtubule-organizing center (MTOC), so machinery responsible for a proper vesicular trafficking, such as microtubules and dynein, are also playing an important role in autophagosome maturation. In addition, LC3- and Rab7- binding protein named FYCO1 (FYVE and coiled-coil domain-containing protein 1) was identified and found to be involved in anterograde transport of autophagosomes via microtubule, thereby it plays a role in transport of autophagosomes to the fusion site (Knaevelsrud and Simonsen, 2010; Yamamoto and Simonsen, 2011).

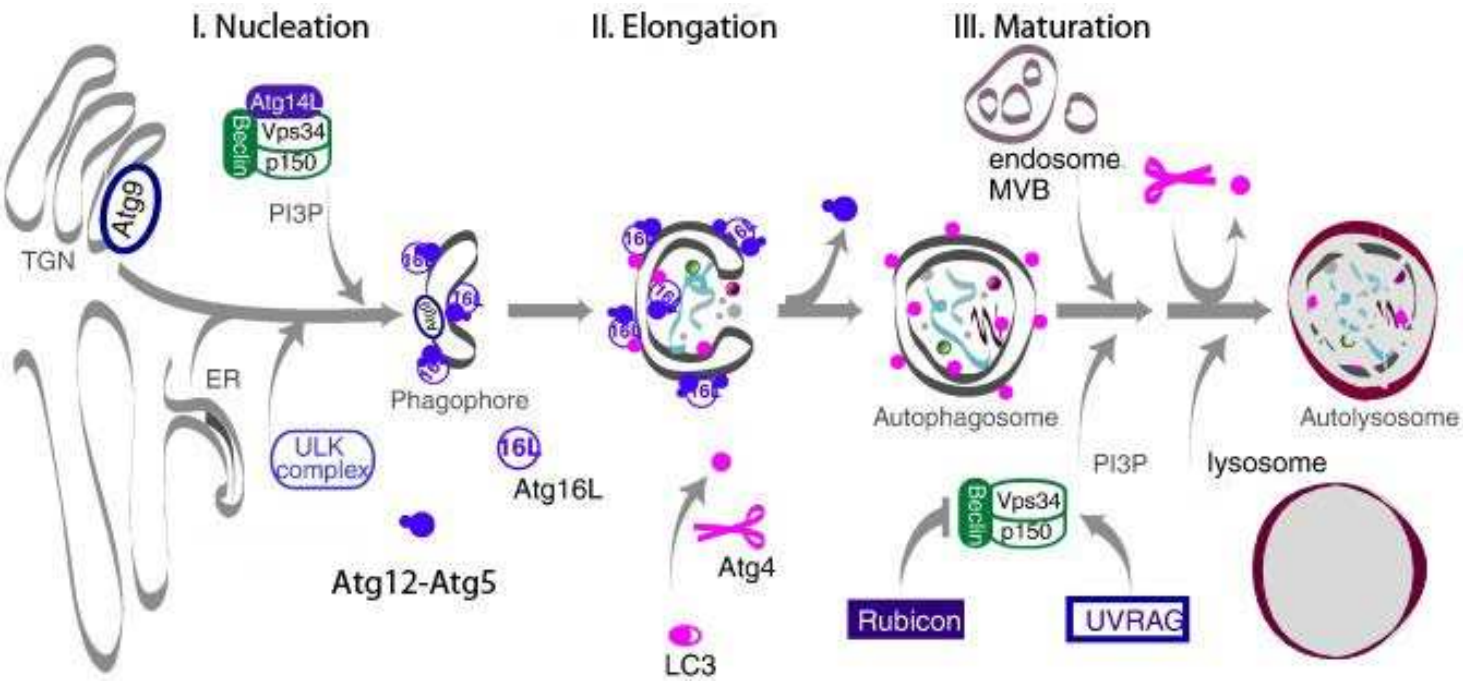


Figure 4. Schematic summary of macroautophagy main steps
Macroautophagy process can be divided into three main steps: I) nucleation, II) elongation and III) maturation. These steps require various protein complexes such as: a) Atg1/Unc-51-like kinase (ULK1) complex, b) the Vps34/class III phosphatidylinositol 3-kinase (PI3K) complex I, which forms phosphatidylinositol-3-phosphate (PI3P), c) the *trans*-Golgi network (TGN) protein Atg9 and its associated cycling machinery, and d) the two-ubiquitin-like systems Atg12-Atg5 and LC3. During maturation, the formed autophagosomes fuse with different endocytic compartments (early endosomes, multivesicular bodies (MVBs) and late endosomes) or they can fuse directly with lysosomes that degrade sequestered cytoplasmic cargo (see text for details). This figure has been adopted and modified from Yamamoto and Simonsen, 2011.

1.4 Autophagy signaling pathways

Autophagy plays an important role in cellular metabolism, degrading and recycling cellular components, providing a pool of macromolecules considered being cellular building blocks (i.e. amino acids, lipids, carbohydrates and nucleic acids). As a major housekeeping pathway, autophagy is under the control of many different signaling cascades (Jaeger and Wyss-Coray, 2009).

1.4.1 mTOR-dependent pathways

Mammalian target of rapamycin (mTOR) is considered as main negative regulator of the autophagy and is therefore, included in so called mTOR-dependent pathways (Figure 5) (García-Arencibia et al., 2010; Mizushima et al., 2011). mTOR is a central protein kinase that senses nutrient levels, energy status and growth factors signals. During starvation, autophagy is highly activated to support cell survival until the nutrients are replenished (Knaevelsrud and Simonsen, 2010; Mizushima et al., 2011). Under starvation or rapamycin treatment, deactivation of mTOR occurs and autophagy is promoted. Under nutrient-rich conditions, mTOR, as part of mTORC1 protein complex, inhibits autophagy by phosphorylating and thus compromising kinase activity of Atg13 and ULK1 proteins that are crucial for autophagosome nucleation (García-Arencibia et al., 2010; Mizushima et al., 2011). Activation of mTOR is regulated by nutrient sensitive MAPK/ERK1/2 complex (mitogen-activated protein kinase/extracellular signal-regulated kinase) that phosphorylates and inhibits TSC1/TSC2 complex (tuberous sclerosis complex 1/2) which further activates mTOR and thus inhibits autophagy (Ravikumar et al., 2010). In addition, ERK2 kinase and mTORC1 are also responsible for phosphorylation of transcription factor EB (TFEB) which regulates transcription of autophagosomal and lysosomal proteins, and is thus considered a master regulator of autophagosomal-lysosomal biogenesis. Phosphorylation of TFEB blocks its translocation to the nucleus causing the cytoplasmic localization of TFEB in the vicinity of lysosomes. During starvation, mTORC1 and ERK2 are inhibited to allow dephosphorylation of TFEB and its translocation to the nucleus where it can activate gene transcription and consequently induce autophagy (Settembre et al., 2011, 2012).

mTOR also acts as a cellular energy status sensor via AMP-activated kinase (AMPK). In response to elevated AMP to ATP ratio (presenting lower energy state), AMPK activates TSC2 resulting in mTOR inhibition and autophagy activation (García-Arencibia et al., 2010; Metcalf et al., 2012).

Further, mTOR can be regulated by insulin and growth factors that activate mTOR through PI3K/Akt signaling cascade. Activated Akt regulates TSC1/TSC2 complex activity and downstream mTOR and autophagy (García-Arencibia et al., 2010).

Autophagy can also be stimulated with oncogenic or genotoxic stress that is mediated by nuclear p53 and that via AMPK pathway stimulates autophagy. In contrast, p53 localized in cytosol inhibits autophagy (Jaeger and Wyss-Coray, 2009; García-Arencibia et al., 2010).

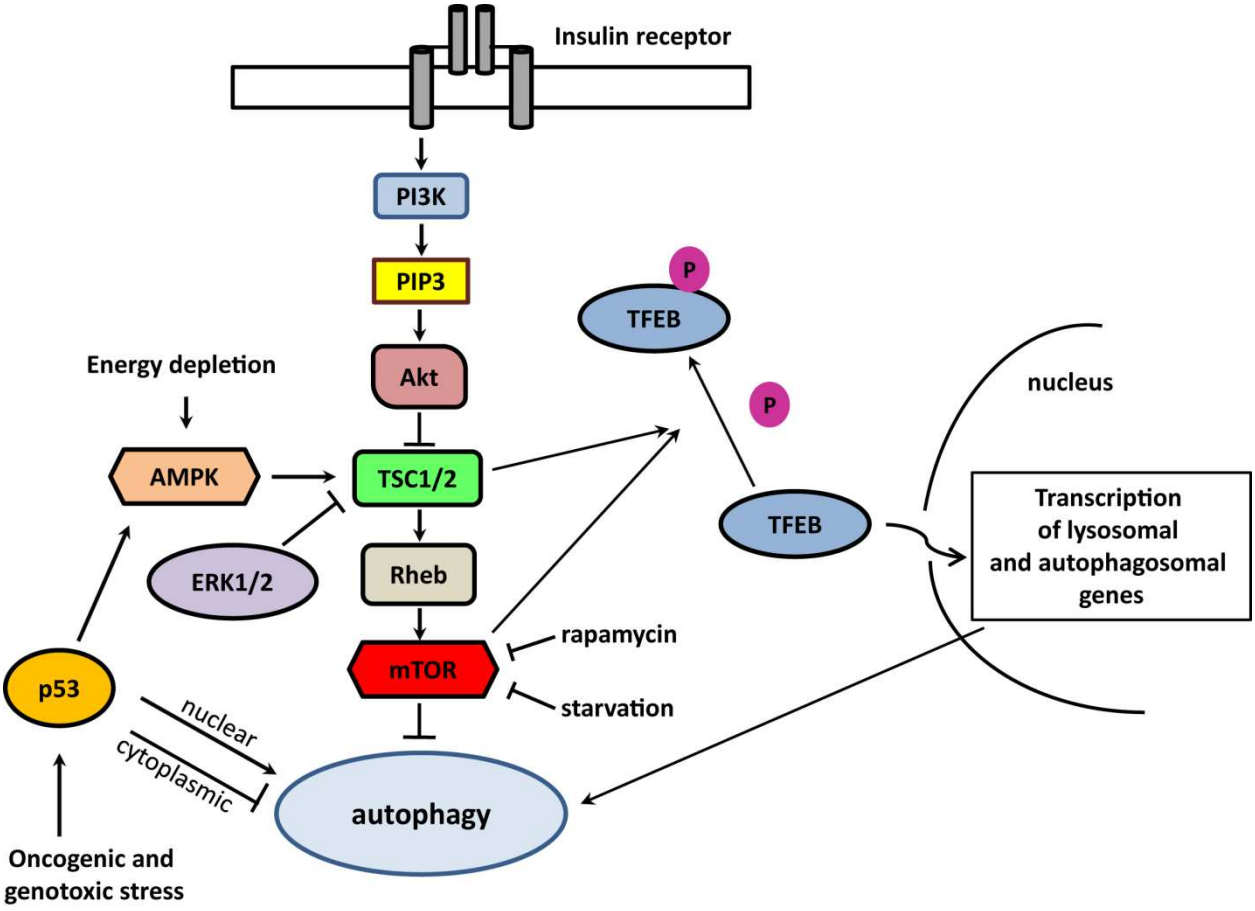


Figure 5. mTOR-dependent autophagy regulation
This illustration has been designed after a figure from García-Arencibia et al., 2010.

1.4.2 mTOR-independent pathways

Aside from mTOR-dependent autophagy regulation, there are pathways that do not go through mTOR, commonly named mTOR-independent pathways. Example is positive autophagy regulation achieved through Beclin-1. Beclin-1 activates autophagy through interaction with Vps35, AMBRA1, UVRAG and Bif-1 (García-Arencibia et al., 2010; Yamamoto and Simonsen,

2011; Metcalf et al., 2012). In addition, Beclin-1 can bind anti-apoptotic proteins Bcl-2 or Bcl-X_L that act as its negative regulators since they prevent Beclin-1 to interact with above mentioned components of complex responsible for autophagosome nucleation (Metcalf et al., 2012). Bcl-2 interaction with Beclin-1 is regulated by nutrient depletion and is considered to be mTOR-independent. In response to starvation Jun N-terminal kinase 1 (JNK1) phosphorylates Bcl-2 that results in its release from Beclin-1 and activation of autophagy (García-Arencibia et al., 2010; Metcalf et al., 2012).

Reactive oxidative species (ROS) can also modulate autophagy. The detailed mechanism still remains unknown, but it is proposed that ROS might affect Atg4 action on LC3. Atg4 in reduced form is responsible for the delipidation of LC3-II from the autophagosomal surface allowing its recycling. ROS might oxidize Atg4 protein that is inactive in oxidized stage and thus, allow LC3 lipidation to promote autophagosome formation (García-Arencibia et al., 2010). Autophagy is also negatively regulated by increased levels of inositol and inositol-1,4,5-triphosphate (IP3) through the action off cAMP, release of calcium from the ER, and activation of calpains (García-Arencibia et al., 2010; Metcalf et al., 2012).

Autophagy can be stimulated by the presence of intracellular protein aggregates and damaged organelles. Even though the precise underlying mechanisms remain unknown, activated autophagy was found crucial for removal of toxic accumulated cell components (Ravikumar et al., 2002; Jaeger and Wyss-Coray, 2009). In addition, inhibition of the autophagy exacerbated cellular accumulation of aggregate-prone proteins and cytotoxicity lending further support for the autophagy role in clearance of protein aggregates (Martinez-Vicente and Cuervo, 2007). Recently it was found that degradation of aggregates via autophagy can also be mTOR-independent since it was found that clearance of aggregation-prone proteins proceeds in cells treated with insulin, the known mTOR activator thus, autophagy inhibitor (Yamamoto et al., 2006; Knaevelsrud and Simonsen, 2010).

1.5 Selective autophagy

Autophagy has long been considered a non-selective process, randomly sequestering and degrading cytoplasmic material during starvation. However, recent studies suggested that autophagy also has an important role in quality control process, removing damaged organelles and cell substrates targeted for autophagy degradation. It has become more clear that autophagy

can be selective process recognizing and degrading specific organelles, proteins and pathogens (Knaevelsrud and Simonsen, 2010; Yamamoto and Simonsen, 2011; Metcalf et al., 2012). This was supported by the discovery of proteins functioning as autophagy receptors, recruiting the autophagy machinery to its substrates. Some of the proteins that are considered to be autophagy receptors or to facilitate degradation of specific cargo via autophagy are p62 (SQSTM/A170), NBR1 (neighbor of BRCA1 gene 1), Alfy (autophagy FTVE protein), HDAC6 (histone deacetylase 6), NIX (BCL2/adenovirus E1B 19kDa interacting protein 3-like, BNIP3), NDP52 (nuclear dot protein 52 kDa), Optineurin and Atg30 (Kirkin et al., 2009; Knaevelsrud and Simonsen, 2010; Wong and Cuervo, 2010; Novak and Dikic, 2011).

1.5.1 Aggrephagy

Selective autophagy clearance of protein aggregates named aggrephagy has emerged as more evident process after the discovery of the proteins that help recognition of aggregates, and that recruit autophagy machinery to enhance their degradation. It addition, proteins degraded by UPS need to be soluble and unfolded in order to pass through the narrow catalytic core of the proteasome, which precludes the clearance of bulky protein aggregates, thus suggesting the existence of specific process responsible for their clearance.

Autophagy receptors p62 and NBR1 are specific for clustering and degradation of ubiquitinated protein aggregates. They bind to Ub but also to autophagosome-specific LC3 protein to recruit Ub-tagged cargo into autophagosome. Both of these proteins are degraded within the lysosomes, and thus often are used to monitor autophagy activity (Kirkin et al., 2009; Yamamoto and Simonsen, 2011). Ubiquitin binding domains of p62 and NBR1 showed preferential binding to K63-ubiquitin chain over K48, suggesting that K63 ubiquitination might present a specific tag for cargo degradation via autophagy. p62 was also found to interact with E3 ubiquitin ligase TRAF6, which preforms K63 polyubiquitination and it is likely that this interaction contributes to additional ubiquitination of protein aggregates enhancing their degradation via autophagy (Moscat and Diaz-Meco, 2009). In addition to ubiquitination, other post-translational modifications such as acetylation and phosphorylation have shown to enhance the clearance of aggregated proteins via autophagy (Jeong et al., 2009; Thompson et al., 2009).

Recently, large protein Alfy (autophagy FTVE protein) was identified to be necessary for selective clearance of protein inclusions. Alfy binds to p62 and Atg5 proteins (Clausen et al., 2010; Filimonenko et al., 2010) and is recruited to protein aggregates thus, providing the platform for interaction between autophagy machinery components (Atg5-Atg12-Atg16 complex and LC3) and Ub- and p62- positive protein inclusions (Filimonenko et al., 2010).

It was discovered that aggregated proteins tend to concentrate in complex ubiquitinated proteinous structures called aggresomes at the perinuclear microtubule organizing center (MTOC). This cell region is enriched with autophagosomes and lysosomes, thus suggesting that the formation of aggresomes might facilitate autophagy clearance of accumulated ubiquitinated proteins (Kopito, 2000). HDAC6 is microtubule associated deacetylase that provides a link between aggresomes and autophagy. HDAC6 participates in transport of cargo through interaction with dynein motors. It was discovered that HDAC6 mediates formation of aggresomes and transports them to the close proximity of autophagic machinery and thus promotes their clearance in the autophagy dependent manner (Kawaguchi et al., 2003; Iwata et al., 2005; Pandey et al., 2007). It was shown that HDAC6 preferentially binds K63 Ub-chains in vivo, further supporting the role of specific ubiquitination as a signal for autophagy degradation (Olzmann et al., 2007). In addition, it has recently been found that HDAC6 is recruited to ubiquitinated protein aggregates, where it promotes F-actin remodeling, which facilitates fusion of autophagosomes and lysosomes. This process was not starvation induced, lending a support to the existence of different autophagy mechanisms other than nutrient-regulated one (Lee et al., 2010b).

1.5.2 Mitophagy

Aside from selective clearance of accumulated and aggregated Ub-positive proteins, mounting evidence suggests that autophagy might also be responsible for specific degradation of mitochondria. Proper removal of aged and impaired mitochondria via autophagy (process called mitophagy) is important process in maintaining cellular homeostasis since accumulated dysfunctional mitochondria are the primary source of highly damaging reactive oxygen species (ROS). In addition, extensive release of cytochrome c from damaged mitochondria could enhance programmed cell death (Batlevi and La Spada, 2011). Link between mitochondria and

autophagy machinery was provided by identification of NIX protein that acts a receptor for mitochondrial degradation via autophagy. NIX is mitochondrial outer membrane protein that recruits and interacts with autophagic machinery components LC3 and GABARAP (Atg8 homologs) to mediate clearance of damaged mitochondria (Novak et al., 2010). In addition, ubiquitin ligase Parkin and serin/threonine kinase PINK1 were found to mediate the clearance of dysfunctional mitochondria via selective autophagy (Geisler et al., 2010b). PINK1 accumulates selectively on impaired mitochondria and recruits Parkin that subsequently ubiquitinates mitochondrial proteins and attracts p62 that recruits autophagic machinery (Narendra et al., 2008; Geisler et al., 2010a). One of the newly identified Parkin mitochondrial substrates is VDAC1 (voltage-dependent anion channel 1). Polyubiquitination of this protein might attract p62 and thus generate the link between mitochondria and newly forming autophagosome (Geisler et al., 2010a). Deficiency of these proteins result in disrupted clearance of damaged mitochondria (Geisler et al., 2010b).

In summary, the vast of the fine-tuned and complex mechanisms that cells use to regulate quality control system emphasize how processes of protein and organelle clearance are important for cellular survival. Unfortunately, this complex regulatory system can get disrupted on many levels but synchronized interplay between autophagy and UPS allows cells to rapidly adjust to changes of concentrations of altered and accumulated components. Detrimental for the cells are conditions that cause primary failure of quality control system that results in cellular imbalance that cannot be repaired and leads to irreversible cellular damage and death (Martinez-Vicente and Cuervo, 2007).

Accumulation of toxic protein species or protein aggregates and dysfunctional mitochondria can have destructive effect on neurons since they cannot reduce the concentration of undigested toxic cytosolic contents by cell division. Non-degraded cellular material can further act as a trap for other cellular components promoting their aggregation, while accumulated mitochondria present a source of toxic ROS (Wong and Cuervo, 2010).

Persistence of toxic cellular material leads to a progressive loss of neurons that can eventually give a rise to a symptoms (Martinez-Vicente and Cuervo, 2007). Accumulation of misfolded and aggregated proteins presents the basis of several neurodegenerative disorders, including Parkinson (PD), Huntington (HD) and Alzheimer diseases (AD) (Nixon et al., 2008; Jaeger and Wyss-Coray, 2009). Even the exact nature of cytotoxic species (soluble monomers, oligomers or

larger aggregates) is still subject of debate, mounting evidence suggests that aggregation-prone proteins mediate toxicity via gain-of-function mechanisms associated with their propensity to aggregate (Rubinsztein, 2006; García-Arencibia et al., 2010). In addition, clearance of these cellular aggregates was found beneficial and it attenuated cellular dysfunction and toxicity (Yamamoto and Simonsen, 2011). Therefore, both activity and dysfunction of autophagy, as a pathway responsible for removal of protein aggregates, have become a major topic in research of neurodegenerative disorders (Wong and Cuervo, 2010).

1.6 Neurodegeneration and protein aggregates

Neurodegeneration is a process of gradually progressive disintegration of neuronal system that eventually leads to wide spectrum of neurodegenerative disorders. Depending on which part of neuronal system is affected variety of the clinical manifestations can occur. Classifying neurodegenerative disorders based on the clinical features is considered the most helpful in practice to clinicians (Ropper and Samuels, 2009). However, development of molecular biology techniques and discovery of genes causative of neurodegenerative disorders have greatly improved the knowledge of molecular pathogenesis of this disorders and provided a platform for development of effective therapies. Therefore, another classification of neurodegenerative disorders can be made based on the cellular and molecular characteristics/abnormalities that are present in certain disorders. Several neurodegenerative disorders were recognized to share accumulation of misfolded and aggregation-prone proteins as their pathological hallmark. These disorders are known as proteinopathies and even though they have different clinical characteristics, the mechanisms of neurodegeneration seem to be overlapping (Rubinsztein, 2006). The most common proteinopathies are PD, HD and AD. PD is a progressive neurodegenerative disorder with accumulation of α-synuclein (α-syn) protein in intracellular inclusions named Lewy bodies. AD brains are characterized by pathological accumulation of β-amyloid peptide (Aβ) and hyperphosphorylated microtubule-associated protein Tau in plaques and tangles, respectively. In pathological conditions of HD, mutated huntingtin protein (Htt) with the expansion of polyglutamine (polyQ) on its N terminus accumulates and forms aggregates (Zhang et al., 2009). In addition, frontotemporal dementia (FTD) and amyotrophic lateral sclerosis (ALS) are another neurodegenerative disorders pathologically characterized with ubiquitinated-protein aggregates (Talbot and Ansorge, 2006).

1.6.1 Parkinson and Huntington disease

PD and HD are also known as movement disorders of basal ganglia. Basal ganglia and cerebellum are considered an extrapyramidal pathway that modulates corticospinal (pyramidal pathway) and cortical-brain stem-spinal motor systems, and therefore are involved in controlling and planning of movements and posture maintenance (Kandel et al., 2000; Barrett et al., 2012). The basal ganglia consist of four major subcortical nuclei 1) the striatum (the caudate nuclei and the putamen), 2) globus pallidus (divided into external and internal segments, GPe and GPi), 3) substantia nigra (divided into pars compacta and pars reticulata, SNc and SNr) and 4) subthalamic nucleus (STN). Basal ganglia receive main inputs from cerebral cortex that are excitatory (glutaminergic) signals and which terminate in the striatum that is receptive part of basal ganglia. The output nuclei of basal ganglia are GPi and SNr and they send their signals back to motor cortex via the thalamus. Output projections of GPi and SNr are inhibitory (GABAergic) while thalamic ones are excitatory (glutaminergic). Apart from the external connections, the basal ganglia have they internal pathways. Striatal inhibitory neurons (GABAergic) project to both GPi and GPe (striatopallidal circuit). Another circuit called nigrostriatal circuit consists of dopaminergic projections of SNc (pigmented portion) to striatum and striatal GABAergic projections to SNr (non-pigmented portion). Neurons of the subthalamic nucleus (STN) are the only excitatory (glutaminergic) projections of the basal ganglia and they influence both GPi and GPe, while GPe sends its inhibitory signals to STN (Figure 6) (Kandel et al., 2000; Barrett et al., 2012).

All these afferent and efferent projections in basal ganglia are interconnected and can be summarized in 2 main pathways, direct and indirect pathways that regulate cortical voluntary movements. In the *direct* pathway striatum receives glutaminergic inputs from the sensorimotor cortex and dopaminergic inputs from the SNc. This results in further inhibition of GPi and SNr that in turn disinhibit thalamus. As a consequence, thalamocortical excitatory pathway is enhanced and cortically initiated movements are facilitated. In the *indirect* pathway striatal neurons have an inhibitory effect on the GPe that in turn disinhibits the subthalamic nucleus and activates subthalamic pathway that results in the activation of GPi and SNr (Figure 6). The final effect is thalamic inhibition that reduces thalamocortical signals and inhibits voluntary movement (Ropper and Samuels, 2009).

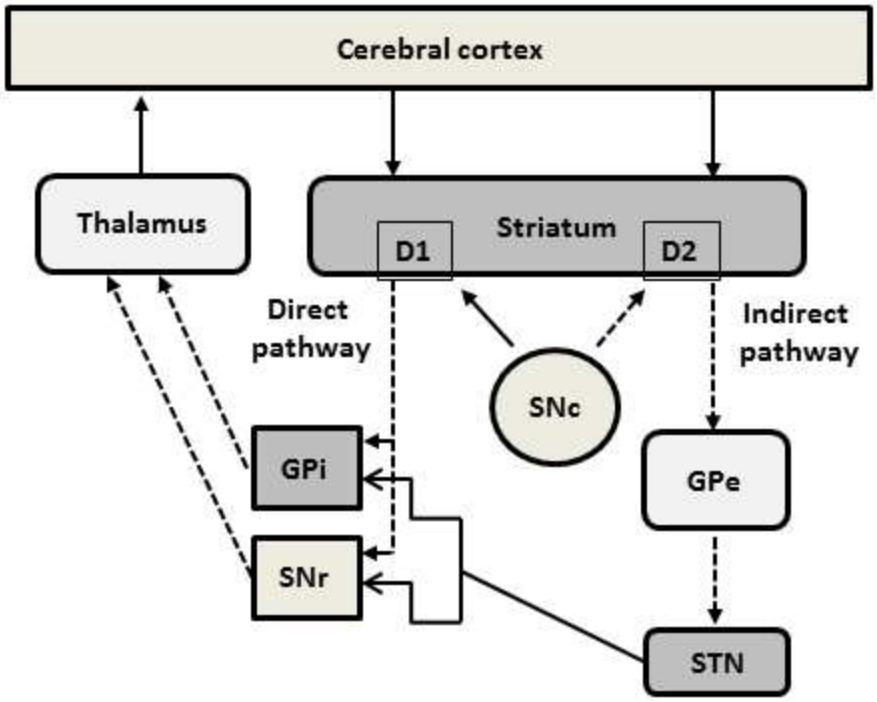


Figure 6. Simplified diagrammatic representation of basal ganglia circuits
Solid lines indicate excitatory pathways and dashed lines inhibitory pathways. SNc, substantia nigra pars compacta; SNr, substantia nigra pars reticulata; GPi, globus pallidus internal, GPe, globus pallidus external; STN, subthalamic nucleus, D1 and D2, dopaminergic receptors.

If one of the parts of basal ganglia becomes damaged and balance between the excitatory and inhibitory pathways with in the basal ganglia becomes disrupted, characteristic motor dysfunctions occur. Dysfunction in basal ganglia can lead to two general types of movement disorders: hypokinetic and hyperkinetic disorders (Kandel et al., 2000; Barrett et al., 2012). Hypokinetic disorders are characterized by: a) impaired initiation and execution of a movement, b) decreased spontaneous movement (akinesia), c) reduced amplitude and speed of voluntary movements (bradykinesia), d) muscular rigidity and tremor in rest. Hyperkinetic disorders are characterized by excessive motor activity and are represented with symptoms such as involuntary movements (dyskinesia: chorea, athetosis, ballismus, dystonia) and decreased muscle tone (hypotonia). Parkinson disease is the well-known example of hyperkinetic disorders that is characterized with progressive loss of dopaminergic neurons of substantia nigra pars compacta (SNc). This loss of dopaminergic input on striatum leads to increased activity of indirect pathway and decreased activity of direct pathway because of the excitatory and inhibitory effect of dopamine on the striatum through the D1 and D2 dopaminergic receptors, respectively (Figure 6) (Kandel et al., 2000). The final result is increased inhibition of the thalamocortical pathway

and reduced excitation of the cortical motor system, which lead to hypotonic symptoms. Huntington disease is an example of hyperkinetic disorders that is characterized by the initial atrophy of the striatum (the caudate nuclei and the putamen). Striatal neurons that are involved in indirect pathway are preferentially lost and therefore; inhibition of GPe is decreased that causes its excessive discharge and inhibition of subthalamic nucleus. On the other hand, direct pathway initially still stays active and since it cannot be abolished through the indirect pathway via subthalamic nucleus, inhibitory activity of GPi and SNr is diminished and excitatory function of thalamus on motor cortex is enhanced. A consequence of this way unbalanced excitatory and inhibitory pathways is leading to hyperkinetic, choreiform symptoms (random movements of limb and facial structures) in early stage of HD (Kandel et al., 2000).

Parkinson disease (PD) is progressive neurodegenerative disorder that was first identified to be caused by a deficiency of a specific neurotransmitter. The characteristic clinical features of PD are bradykinesia, resting tremor, postural instability and rigidity. In addition to the motor hypokinetic symptoms, patients develop non-motor symptoms such as cognitive impairment (dementia), sleep problems and neuropsychiatric symptoms. Symptoms start to appear when 60-80% of the nigrostriatal dopaminergic neurons degenerate (Crosiers et al., 2011). Beneficial response to a precursor of dopamine levodopa (L-dopa) or to dopamine agonists is another characteristic of PD and therefore, these drugs are used as a therapy for suppressing PD symptoms. Unfortunately, patients develop resistance to a L-dopa after few years of treatment (Ropper and Samuels, 2009).

Aside from loss of pigmented neurons in the substantia nigra a second pathologic hallmark of PD are eosinophilic cytoplasmic inclusions named Lewy bodies in the surviving neurons (Dickson et al., 2009). The major component of these inclusions is α-syn; therefore PD is classified as a proteinopathy, more precisely synucleinopathy.
Inclusions are seen in majority of sporadic PD than commonly effects older population over age of 65. A small percentage (5-10%) of PD is genetically inherited (Crosiers et al., 2011).
Discovery of several genes responsible for rare monogenic PD (Table 1) offered a unique opportunity to elucidate the function of these genes and uncover pathogenic mechanisms responsible for neurodegeneration in genetic as well as sporadic PD.

These genes/proteins and their cellular functions suggested that processes such as inadequate protein clearance and accumulation of toxic protein, impaired vesicular trafficking, dysfunctional mitochondria and oxidative stress might present underlying molecular mechanisms of PD neurodegeneration (Moore et al., 2005).

Table 1. Parkinson disease-associated genes and their proteins' functions

Gene	Function	Ref.
α-synuclein (PARK1or SNCA)	- Aggregation prone protein, major component of Lewy bodies, associated with membranes and vesicular structures - Involved in vesicular trafficking and recycling at the synapses and ER-Golgi trafficking - Overexpressed and/or mutant α-syn impairs UPS and CMA, cellular trafficking, reduces mitochondrial function and increases oxidative stress	(Kahle et al., 2000; Ko et al., 2000; Tanaka et al., 2001; Snyder et al., 2003; Cuervo et al., 2004; Henchcliffe and Beal, 2008; Gitler et al., 2009a; Auluck et al., 2010)
Parkin (PARK2)	- E3 ubiquitin ligase, interacts with 19s proteosomal subunit and mediates transfer of poly-Ub substrates to proteasome - Involved in mitophagy and maintaining mitochondrial integrity, interacts with PINK1 to promote mitochondrial fission - Mutations in Parkin cause mitochondrial dysfunction and oxidative stress - Overexpression of Parkin suppresses α-syn mediated inhibition of UPS	(Petrucelli et al., 2002; Darios et al., 2003; Greene et al., 2003; Sakata et al., 2003; Palacino et al., 2004; Pesah et al., 2004; Narendra et al., 2008; Poole et al., 2008; Yu et al., 2011)
PINK1 (PARK6)	- Serine-threonine kinase, localized to mitochondrial membrane, together with Parkin involved in mitochondrial fission and selective clearance of damaged mitochondria. - Mutations are causing mitochondrial dysfunction and increased oxidative stress.	(Clark et al., 2006; Poole et al., 2008; Yang et al., 2008; Geisler et al., 2010b; Yu et al., 2011)
DJ-1 (PARK7)	- Chaperone, oxidative stress sensor, protects against oxidative stress, during oxidative stress localizes to mitochondria to protect cell against mitochondria-dependent cell death. Interacts with α-syn and prevents its accumulation - Loss of DJ-1 function increases cellular sensitivity to oxidative stress and inhibits UPS	(Yokota et al., 2003; Canet-Avilés et al., 2004; Martinat et al., 2004; Shendelman et al., 2004; Park et al., 2005; Zhang et al., 2005)
LRKK2 (PARK8)	- Leucine-rich repeat kinase 2, implicated in many cellular processes: vesicular trafficking and endocytosis, protein synthesis, autophagy-lysosomal pathway - LRRK2 gain-of-function mutations seam to alter these processes. In LRRK2 PD models autophagy process is altered resulting in accumulation of autophagosomes, ubiquitinated proteins and α-synuclein-positive inclusions	(Smith et al., 2005; Cookson, 2010; Manzoni, 2012)
UCH-L1 (PARK5)	- Ubiquitin ligase and deubiquitinating enzyme, responsible for the maintaining pool of ubiquitin monomers necessary for proper UPS degradation - Mutations promote α-syn accumulations in cultured cells presumably affecting its UPS degradation	(Liu et al., 2002; Osaka et al., 2003; Moore et al., 2005)

Huntington disease (HD) clinically presents with chorea (involuntary arrhythmic movements of rapid, jerky type), behavioral and psychiatric disturbance (e.g. personality changes, depression, psychosis, paranoia) and cognitive deterioration (e.g. dementia). Disease commonly starts after third to fifth decade of patient's life, and after 10-15 years of symptoms most patients deteriorate to vegetative state (Kandel et al., 2000; Ropper and Samuels, 2009). Although the striatal neurons are preferentially affected, as disease progresses, degeneration of other parts of the brain was observed, such as cortex, thalamus and subthalamic nucleus (Krainc, 2010).

HD is hereditary autosomal dominant neurodegenerative disorder caused by mutations in huntingtin gene (*HTT*) localized to chromosome 4 (Gusella and MacDonald, 1993; Locke et al., 1993). This gene normally contains 11 to 34 consecutive repetitions of the CAG triplet that cods for glutamine amino acid. Mutations cause the expansion of the CAG triplet and the individuals with more than 42 CAG repeats will most invariably develop symptoms of Huntington disease (Ropper and Samuels, 2009). The number of the repetitions of CAG correlates to the disease onset and the severity. *HTT* gene codes for huntingtin protein (Htt) normally localized in the cytoplasm. Major characteristic of mutant Htt is that it is prone to aggregate and it forms intracellular inclusions. Accumulation of Htt interrupts diverse cellular pathways that can affect neuronal viability, such as gene transcription, energy metabolism, axonal transport, synaptic transmission, and vesicle release (Roze et al., 2008; Krainc, 2010). Although the neurotoxicity of the aggregated vs. non-aggregated Htt in disease pathogenesis is still topic of the debate, the existence of intracellular aggregates of mutant Htt protein in brains of HD patient suggest that mutant Htt is not adequately cleared from the neurons and therefore selective degradation of Htt might be efficient therapy in HD.

1.6.2 Alzheimer disease

Alzheimer disease (AD) is the most common neurodegenerative disease characterized with defuse cerebral atrophy (i.e. shrinkage in size and weight of the brain), with particularly affected area of hippocampus and medial parts of the temporal lobes. This pathologic brain changes give a rise to a progressive dementia that presents the most frequent mental illness occurring in majority of patients after their sixties. The incidence rate of clinically diagnosed AD is 125 new cases yearly per 100.000 of individuals older than 60 years of age. This incidence increases with the age of population and it seems that women get more affected than men. Major symptom of AD is gradual development of forgetfulness, starting with forgetting words, names and small

day-to-day happenings, language deficit, which further progresses into severe memory loss and cognitive impairment (e.g. motor incapacity, apraxia), all of which interferes with patients' everyday life (Kandel et al., 2000; Ropper and Samuels, 2009). Patients show personality and mood changes, and frequently they develop anxieties, phobias and sometimes even hallucinations. In advanced stages of the disease, difficulty in locomotion and parkinsonian symptoms can be perceived in patients. Ultimately, patients lose the ability to stand and walk, ending up wheelchair-bounded (Ropper and Samuels, 2009).

Aside from significant atrophy of hippocampus, AD disease brains are characterized with extracellular neuritic plaques and intraneuronal neurofibrillary tangles. These inclusions contribute to the pathogenesis of the disease and catalogue AD as one of the proteinopathies like HD and PD (Irvine et al., 2008; Bekris et al., 2010). The main component of plagues is aggregated amyloid β protein (Aβ). Aβ derives from amyloid-precursor proteins (APP) in the processes of sequential cleavage by α, β and γ secretase. This APP processing can result in Aβ40 and Aβ42 protein form (Shoji et al., 1992). Aβ40 consists of 40 amino acids and it is more abundant form of the protein, than longer Aβ42, that is associated with AD and is prone to oligomerize and form toxic amyloid aggregates (Iwatsubo et al., 1994).

Tangles are formed of hyperphosphorylated and aggregated protein tau. Tau (microtubule associated protein) is cytoskeletal protein involved in microtubule assembly and is therefore responsible for maintaining neuronal structure and synaptic plasticity (Wood et al., 1986). Studies have proposed that neurofibrillary tangles are disturbing neuronal cytoskeleton and are impairing trafficking of proteins and vesicles, which presumable lead to impaired axonal trafficking and neuronal viability (Ropper and Samuels, 2009). Presence of neurofibrillary tangles closely reflects disease severity (Braak and Braak, 1991).

Sporadic form of AD is the most prevalent, but a genetic studies have also identified rare mutations in several genes that are causing familial early-onset AD. Those genes are *APP*, presenilin 1 (*PSEN1*) and 2 (*PSEN2*). Multiplication of *APP* locus results in increased production of Aβ including the toxic Aβ42 form. Presenilins are part of catalytic core of γ secretase and mutations of this genes are associated with overproduction of toxic Aβ42 (Bertram and Tanzi, 2004; Bekris et al., 2010). In addition, the presence of the isoform ε4 of the apolipoprotein E (*APOE*) gene has been identified as a strongest genetic risk factor for the late onset AD. It was proposed that APOE interacting with APP and tau promotes their aggregation and neuronal toxicity (Huang, 2006). Identification of genes causing AD has led to a number of animal models

that have significantly improved understanding of the biology of AD pathogenesis (Bekris et al., 2010).

Aside from AD the second most common progressive dementia is **frontotemporal dementia (FTD)** characterized with neurodegeneration of the frontal and temporal lobes of the brain. This dementia is also an example of proteinopathies, more precisely tauopathies, since it is associated with inclusions consisted of accumulated and hyperphosphorylated tau protein. Mutations in tau gene were identified as rare cause of FTD (Froelich-Fabre et al., 2004). In addition to Tau positive inclusions, ubiquitin-immunoreactive inclusions have been also recognizes as a pathologic characteristic of FTD (Talbot and Ansorge, 2006; Filimonenko et al., 2007). Interestingly, several findings proposed a hypothesis of common molecular pathogenesis between FTD and another neurodegenerative disease called **amyotrophic lateral sclerosis (ALS).** ALS is a complex neuronal disease defined as motor system disorder characterized with degeneration of upper and lower motor neurons (Talbot and Ansorge, 2006). ALS clinically manifests by muscular weakness, atrophy (due to the loss of lower neurons), and corticospinal tract signs in varying combinations (eg. fasciculations, hyperreflexia, paralysis in the extremities). As disease progresses patients end in vegetative state and death occurs within 3-6 years from the onset when respiratory muscle can no longer perform their function (Talbot and Ansorge, 2006). Approximately 5 to 10% cases of ALS present with FTD (Parkinson et al., 2006). Moreover, cytoplasmic intraneuronal ubiquitinated proteinaceous inclusions were found to be a pathological hallmark in both ALS and FTD. Additionally, genetic analysis identified mutations in CHMP2B gene (unit of the ESCRT) as a cause of both ALS and FTD. Together these finding suggest that ubiquitin-containing protein aggregates and disturbance of endosomal-lysosomal pathway might lead to neuronal loss in both of these clinically different disorders (Parkinson et al., 2006; Filimonenko et al., 2007).

In conclusion, here described neurodegenerative disorders show diverse clinical features but they share common pathological changes that are accumulation of protein aggregates, which are potential threat to neuronal viability. This emphasizes how it is important to study protein aggregates and their clearance in order to deepen knowledge of underlying molecular mechanisms of neurodegeneration. In addition, extensive studies suggested that autophagy-lysosomal pathway is implicated in the pathogenesis of these diseases (Nixon et al., 2008; Shacka et al., 2008; Jaeger and Wyss-Coray, 2009; Zhang et al., 2009). Interestingly, disturbed

autophagy-lysosomal pathway presents the bases of the pathogenesis of lysosomal storage disorders (LSDs). The fact that more than two-thirds of these inborn metabolism disorders have devastating consequence on the nervous system suggests that proper lysosomal function is crucial for neuronal viability and that lysosomal dysfunction might play a unitary role in neuropathogenesis of these disorders (Cox and Cachón-González, 2012).

1.7 Lysosomal storage disorders with neurodegeneration

LSDs belong to a diverse class of metabolic genetic disorders characterized by the storage of non-degraded lysosomal material. They are caused by dysfunction of lysosomal enzymes, lysosomal membrane proteins and proteins responsible for their modification and trafficking. LSDs were classified based on the biochemistry of the stored substrates, which resulted in groups such as mucopolysaccharidoses, sphingolipidoses, mucolipidoses, glycoproteinoses and so forth (Walkley et al., 2010). There are more than 50 genetically distinct LSDs and they exhibit different clinical and pathological features. In LSDs autophagy-lysosomal dysfunction is almost universal but involvement of other complex pathogenic cascades was also recognized such as, altered calcium homeostasis and lipid trafficking, as well as oxidative stress, ER stress, chronic inflammation and autoimmune response (Vitner et al., 2010; Cox and Cachón-González, 2012). These pathogenic pathways lead to a progressive dysfunction of many organ systems (e.g. skeletal and cardio muscles, spleen, liver, bone marrow), but most notably the brain (Walkley et al., 2010; Cox and Cachón-González, 2012). Diverse brain pathology was observed. Aside from neurons that are highly vulnerable and show abnormalities in perikarya, dendrites and axons, leading to gray matter degeneration, some LSDs also exhibit abnormalities in oligodendroglia and myelination of axons, leading to white matter lesions (Walkley et al., 2010). For example Krabbe's disease (caused by deficiency of β-galactosylceramidase) features demyelination with loss of oligodendroglia and proliferation of astroglia in the brain and spinal cord; widespread axonal degeneration and degeneration of peripheral nerves; Tay-Sachs disease (also known as GM2 gangliosidosis), caused by deficiency of β-hexosaminidase A is characterized with increased brain weight due to gliosis, and neuroaxonal atrophy (Cox and Cachón-González, 2012).

In addition to intraneuronal macromolecular storage that appears universally in brain in most LSDs, other cellular changes occur such as axonal swelling (also named neuroaxonal dystrophy) (Walkley et al., 2010; Cox and Cachón-González, 2012). These axonal alterations result from

formation of focal enlargements (call spheroids) that are predominantly consisted of accumulated organelle-type structures. Axonal spheroids are found in numerous areas of the CNS (cerebral cortex, cerebellum and basal ganglia) but it appears that they are predominantly affecting GABAergic neuronal populations (particularly Purkinje cells), giving a rise of various neurological symptoms from ataxia, tremor, features of cerebellar dysfunction to seizures (Walkley et al., 1991, 2010). Spheroids might compromise axonal trafficking and propagation of action potentials, neuronal activity and connectivity, which consequently leads to neurodegeneration. The incidence and the distribution of axonal spheroids correlate closely with onset and severity of the disease in animal models of LSDs, suggesting that they play an important role in neuronal dysfunction and death (Walkley et al., 2010). Interestingly, neuroaxonal dystrophy was also observed in pathogenesis of common neurodegenerative diseases such as AD and HD, further implicating lysosomal dysfunction as a common mechanisms leading to neurodegeneration (Yagishita, 1978; Chevalier-Larsen and Holzbaur, 2006; Nixon et al., 2008).

1.8 Lysosomes in neurodegeneration

1.8.1 Autophagy-lysosomal pathway in common neurodegenerative disorders

Autophagy has attracted neuroscientists since it is the pathway that is crucial for clearance of aggregated proteins that present pathological hallmark of several neurodegenerative disorders, including PD, HD and AD (Nixon et al., 2008; Jaeger and Wyss-Coray, 2009).

Since the presence of the protein aggregates is connected with cellular toxicity it was postulated that removing the aggregates via autophagy might be beneficial. Indeed, pharmacological or genetic activation of autophagy-lysosomal pathway in cell or animal models of these disorders facilitates removal of aggregated forms and decreases the toxic, while blockage of this proteolytic pathway worsens the pathological manifestation (Martinez-Vicente and Cuervo, 2007). For example, induction of autophagy resulted in clearance of Htt aggregates in cell culture, fly and mouse models of HD, whereas inhibition had an opposite effect (Ravikumar et al., 2002, 2004; Qin et al., 2003; Jaeger and Wyss-Coray, 2009). Similar findings were confirmed for α-syn in cell cultures (Webb et al., 2003; Williams et al., 2006; Sarkar et al., 2007; Vogiatzi et al., 2008a; Mazzulli et al., 2011) and for tau in a fly model (Berger et al., 2006;

Khurana et al., 2010). These data provide support for the participation of autophagy-lysosomal pathway in the degradation of altered proteins and their toxic multimeric complexes that eventually promote neuronal death (Martinez-Vicente and Cuervo, 2007).

Furthermore, pathological accumulation of autophagic-lysosome compartments and abnormality of the endosomal-lysosomal pathway were recognized in patients and in different models of these clinically different neurodegenerative diseases, providing further evidence of an important role that lysosomal pathway plays in neurodegeneration (Table 2) (Martinez-Vicente and Cuervo, 2007; Nixon et al., 2008; Jaeger and Wyss-Coray, 2009; Zhang et al., 2009). For example, in Alzheimer disease, degradative capacity of lysosomal system decreases as disease progresses, resulting in poor clearance of intracellular material and accumulation of autophagosomes. Recently it was proposed that accumulated autophagosomes contain proteases needed for production of Aβ that contributes to amyloid deposition (Yu et al., 2005; Martinez-Vicente and Cuervo, 2007). In addition, mutant presenilin-1 that causes familial AD was found to enhance lysosomal system pathology, amyloid formation and neurodegeneration (Cataldo et al., 2004). Another proof of autophagy involvement in accumulation of amyloid β protein (Aβ) and neurodegeneration in AD was provided by strongly reduced levels of Beclin-1 in brains of AD patients. Decreased levels of Beclin-1 that is important for autophagosome formation could impair autophagy degradation system. Indeed, AD mouse models with decreased Beclin-1 levels exhibited reduced neuronal autophagy, disrupted lysosomes and increased accumulation of Aβ, while increasing Beclin-1 expression rescued the phenotype (Pickford et al., 2008). Studies of autophagy in PD showed that CMA and UPS can be blocked by mutant soluble and aggregated wild type α-syn respectively, that results in impaired degradation of other substrates of these proteolythic systems (Tanaka et al., 2001; Snyder et al., 2003; Cuervo et al., 2004). Inhibition of these systems can be compensated with autophagy but overwhelming this system with substrates of other pathways may result in its failure, accumulation of non-degraded products and neuronal toxicity (Wong and Cuervo, 2010).

Table 2. Neurodegenerative disorders associated with lysosomal system dysfunction

Disease	Endo/lysosomal phenotype	Ref.
Parkinson disease	Impaired autophagy/mitophagy, accumulation of autophagosome-like structures	(Anglade et al., 1997; Cuervo et al., 2004; Pan et al., 2008; Vogiatzi et al., 2008b; Geisler et al., 2010b)
Alzheimer disease	Impairment of autophagy degradative steps, accumulation of autophagosomes, lysosomes, endosomal-lysosomal abnormalities	(Cataldo et al., 1995; Nixon et al., 2000, 2000, 2005; Ditaranto et al., 2001; Lee et al., 2010a)
Huntington disease	Impaired sorting/degradative steps of autophagy, accumulation of autophagosome-like structures	(Kegel et al., 2000; Ravikumar et al., 2002, 2004; Qin et al., 2003; Qin and Gu, 2004; Sarkar et al., 2007; Jaeger and Wyss-Coray, 2009)
Amyotrophic lateral sclerosis	Impaired early endosome, sorting/degradation of autophagosomes, accumulation of ubiquitinated proteins	(Filimonenko et al., 2007; Morimoto et al., 2007; Johnson et al., 2010)
Frontotemporal dementia	Impaired endosome maturation, accumulation of enlarged endosomes, autophagosomes and ubiquitinated proteins	(Lee et al., 2007)

Recent studies further implicated endosomal-lysosomal pathway in neurodegenerative diseases finding that mutations in CHMP2B, unit of the endosomal sorting complexes required for transport (ESCRT), are responsible for frontotemporal dementia (FTD) and also for amyotrophic lateral sclerosis (ALS). Role of ESCRT complex in process of endocytosis (i.e. fusion of endosomes with MVBs and lysosomes) has been well described, but recent findings indicated that it is also involved in autophagy pathway. Depletion of ESCRT subunit or overexpression of mutant CHMP2B impairs autophagic degradation resulting in accumulation of autophagosomes and protein aggregates (containing ubiquitinated proteins, p62 and Alfy), and subsequent cell death (Filimonenko et al., 2007; Lee et al., 2007; Lee and Gao, 2008). Furthermore, mutations in VCP (valosin-containing protein), an AAA+ ATPase, were found to cause FTD and ALS. These disease-causing mutations effect maturation of ubiquitin-containing autophagosomes, their fusion with lysosomes that result in formation of ubiquitinated protein aggregates within the cells (Ju et al., 2009; Custer et al., 2010; Johnson et al., 2010; Tresse et al., 2010).

Together these findings suggest that regardless which delivery pathway to lysosomes is impaired (i.e. autophagy or endocytosis), collapse of overall lysosomal system can occur and result in deficient protein turnover and cell dysfunction and death.

Most neurodegenerative diseases associated with protein aggregates occur late in life, for example after 60 years of age. Interestingly, it was observed that autophagy activity reduces with ageing suggesting that progressive gradual reduction of this degradation pathway could at least partially contribute to the neuropathogenic process and late onset of these disorders particularly the idiopathic one (Ward, 2002; Cuervo et al., 2005; Terman et al., 2007).

Apart from being neuroprotective through degradation of toxic protein species, autophagy plays important role in selective degradation of aged and dysfunctional mitochondria (mitophagy) that present a primary source of oxidative damage (Batlevi and La Spada, 2011). Accumulated damaged mitochondria and oxidative stress were recognized in the pathogenesis of neurodegenerative disorders such as PD and HD (Martinez-Vicente et al., 2010; Oliveira, 2010; Batlevi and La Spada, 2011). Dysfunction of PINK1and Parkin proteins related to inherited PD was associated with disrupted mitophagy, accumulation of damaged mitochondria and neuronal loss, which presumably contributes to disease pathogenesis (Clark et al., 2006; Narendra et al., 2008; Geisler et al., 2010b). Recent studies showed that HD pathogenesis is associated with inefficient autophagy substrates recognition leading to accumulation of dysfunctional mitochondria (Martinez-Vicente et al., 2010). Together these finding further emphasized proper function of autophagy-lysosomal pathway in clearance of damaged mitochondria and in neuronal homeostasis and viability.

Additional support for the active role of autophagy-lysosomal pathway in neuronal survival comes from knockout mice lacking autophagy-related genes (*Atg*). Knockouts of *Atg5* and *Atg7* genes lead to early neonatal mortality that made studies of the adult CNS impossible. Conditional neuronal *Atg5* and *Atg7* knockout mice were created to circumvent this limitation, and these models developed intraneuronal ubiquitinated-aggregates and neurodegeneration. These results suggest that clearance of cytosolic proteins though basal autophagy is crucial for preventing abnormal accumulation of proteins that can subsequently disrupt neuronal homeostasis and lead to neurodegeneration (Hara et al., 2006; Komatsu et al., 2006).

1.8.2 Lysosomal pathology in lysosomal storage disorders with neurodegeneration

Disturbed autophagy-lysosomal pathway has been observed in various lysosomal storage disorders (LSDs) with neurological deficits, giving another proof of lysosomal involvement in neurodegeneration (Table 3). (Nixon et al., 2008; Ruivo et al., 2009; Schultz et al., 2011; Cox and Cachón-González, 2012)

Example is Danon disease that is caused by mutations in LAMP-2, a lysosomal membrane protein responsible for lysosomal maturation and integrity. In this disorder, lysosomal fusion with autophagosomes, mediated by dynein, is impaired and causes accumulation of autophagosomes in many tissues, suggesting that defective autophagy plays a role in the development of this fatal disease (Nishino, 2006; Saftig et al., 2008). Furthermore, in several lysosomal disorders such as multiple sulphatase deficiency (deficiency of the sulfatase modifying factor 1) and mucopolysaccharidosis type IIIA (sulfamidase deficiency) activation of autophagy and its functional arrest has been reported. This was presented by accumulation of LC3 positive vesicles, but with their decreased co-localization with LAMP1 lysosomal marker, suggesting that the fusion between autophagosomes and lysosomes is impaired. As a consequence, ubiquitinated proteins, normally destined for degradation with in the lysosomes, accumulated in the embryonic cells of the mouse models of these diseases (Settembre et al., 2008a). The similar cellular abnormalities were seen in brains from mouse model of Neimann-Pick disease (NPC) caused by mutations in endosomal/lysosomal membrane proteins NPC1 or NPC2 and characterized with excessive accumulation of cholesterol and glycolipids (Walkley et al., 2010). For example, Npc1(-/-) mice showed accumulation of LC3-labeled autophagosomes, increased levels of LC3-II protein and accumulation of undigested ubiquitinated proteins. These data suggest that accumulation of cholesterol alters autophagy-lysosomal function leading to neurodegeneration in NPC disorder (Liao et al., 2007).

As previously mentioned, axonal swellings that present a pathologic feature of majority of LSDs are consisted of accumulated membranous tubular structures and various organelles, like autophagosomes, mitochondria and multivesicular bodies (Walkley et al., 2010; Cox and Cachón-González, 2012). Although it is not precisely clear why and how spheroids are made, it was proposed that inadequate autophagy-lysosomal degradation and accumulation of undigested material can: 1) disrupt retrograde neuronal trafficking, resulting in accumulation of organelles

in axons and 2) cause accumulation of diverse organelles (e.g. autophagosomes and mitochondria). This was supported by the studies showing that mice with impaired retrograde transport exhibited similar axonal pathology and mice lacking autophagy related gene *Atg7,* necessary for process of autophagy, showed spheroid accumulations just without the autophagosomes (Liu et al., 2003, 2007; Komatsu et al., 2007). Subsequently these cellular changes can additionally impair axonal trafficking and firing, closing a vicious circle that leads to neuronal dysfunction and death. The axonal swelling was also identified in AD and HD, suggesting a common mechanism of neurodegeneration with accent on lysosomal dysfunction (Nixon et al., 2000, 2008). Additionally, recent studies have suggested neuropathological similarities between lysosomal storage disorders called GM2 gangliosidoses and age related AD. GM2 gangliosidoses are group of LSDs with severe neurodegeneration caused by accumulation of GM2 ganglioside and related sphingolipids due to the mutations in either hexamidase A (*HEXA*) gene causing Tay–Sachs disease or in hexamidase B (*HEXB*) causing Sandhoff disease (Keilani et al., 2012). Altered gangliosides metabolism was observed in connection with aging and AD (Yanagisawa, 2007; Ariga et al., 2008; Yamamoto et al., 2008) while lysosomal lipid accumulation and altered lipid composition of lysosomal membranes was found to impair autophagy-lysosomal degradation pathway (Boland et al., 2008; Tamboli et al., 2011). Therefore it was proposed that altered ganglioside metabolism might contribute to AD pathogenesis by promoting autophagy dysfunction and Aβ aggregation (Tamboli et al., 2011; Yanagisawa, 2011). Indeed, studies in mouse models of Sandhoff disease showed that accumulation of gangliosides disrupts autophagy-lysosomal degradation, leading to intraneuronal accumulation of autophagosomes and Aβ like, α-synuclein-like, and tau-like immunoreactive materials (Keilani et al., 2012). Together these results provide a potential common pathway between LSDs and AD that connects accumulation of gangliosides, autophagy-lysosomal dysfunction and intraneuronal accumulation of proteins associated with AD.

In summary, accumulation of undigested substrates within lysosomes, due to deficiency of specific lysosomal enzyme, disrupts lysosomal degradation capacity, fusion of lysosomes and autophagosomes (i.e. normal autophagic turnover), that consequently results in accumulation of protein aggregates and autophagosomes and mitochondria (Settembre et al., 2008a, 2008b). Together, these findings suggest that neurodegeneration in LSDs may share some mechanisms with late-onset neurodegenerative disorders (e.g. PD, AD, HD) in which accumulation of protein aggregates and lysosomal dysfunction are prominent features (Settembre et al., 2008b; Zhang et

al., 2009). Findings that showed that pathology of endosomal-lysosomal pathway is present in both LSDs with CNS deficits and in major neurological diseases (Table 2 and 3), provide further support of the pivotal role that lysosomes play in neurodegenerative processes (Nixon et al., 2008; Jaeger and Wyss-Coray, 2009; Zhang et al., 2009).

It is interesting to note that parkinsonism has been reported in patients with Gaucher disease suggesting a possible pathogenic link between the two disorders (Neudorfer et al., 1996; Tayebi et al., 2003; Sidransky, 2005). Gaucher disease is caused by mutations in *GBA1, a* gene for lysosomal enzyme β-glucocerebrosidase (GC) that results in accumulation of its lipid substrates (Cox and Cachón-González, 2012) . Importantly, mutations in *GBA1* were identified as the most common genetic risk factor for PD. Previously was shown that depletion of GC also results in decreased lysosomal degradation capacity and accumulation of α-syn, providing a mechanistic link between these neurodegenerative disorders (Mazzulli et al., 2011). Furthermore, parkinsonism has been observed in and neuronal ceroid lipofuscinoses (NCLs) diseased families (Nijssen et al., 2002; Burneo et al., 2003), and brains of NCL patients with deficiency in lysosomal protease Cathepsin D showed accumulation of α-syn (Cullen et al., 2009). NCLs are a heterogeneous group of lysosomal storage diseases that present with the lysosomal accumulation of autofluorescent lipopigments.

Together, this evidence provides a clinical link between LSDs and Parkinsonian disorders and suggests that they share pathobiological features centering on lysosomal dysfunction.

Lysosomal dysfunction has been further implicated in neurodegeneration by identification of mutations in novel lysosomal protein ATP13A2/PARK9 that cause a rare form of autosomal-recessive parkinsonism, Kufor-Rakeb syndrome (KRS), and NCL.

To this end, studies of neurodegenerative disorders caused by mutations in lysosomal proteins offer an opportunity to elucidate mechanisms that lead to neurodegeneration and potentially identify specific therapeutic targets. We focused our studies on the role of ATP13A2/PARK9 protein since it is a novel lysosomal protein associated with neurodegenerative disorders.

Table 3. Lysosomal storage disorders with neurodegeneration

Disease	Endo/lysosomal phenotype	Ref.
Gaucher disease	Impaired lysosomal degradation, accumulation of lysosome like structures	(Sun et al., 2010; Kinghorn, 2011; Mazzulli et al., 2011; Westbroek et al., 2011)
Neuronal ceroid lipofuscinosis	Impaired lysosomal degradation, accumulation of autophagosome/lysosome like structures	(Koike et al., 2003, 2005; Cao et al., 2006)
Niemann-Pick disease type C	Endosome-lysosomal system dysfunction, disrupted autophagy, accumulation of autophagosomes	(Jin et al., 2004; Ko et al., 2005; Liao et al., 2007)
Mucopolysaccharidosis type IIIA and Multiple Sulfate Deficiency	Increased number of autophagosomes, reduced autophagy, impaired organelle turnover	(Settembre et al., 2008a, 2008b)
Danon disease	Impaired fusion of lysosomes with autophagosomes, accumulation of enlarged lysosomes and autophagosomes	(Nishino, 2006; Malicdan and Nishino, 2012)
GM2 gangliosidoses (Tay-Sachs disease and Sandhoff disease)	Impaired autophagosomal-lysosomal turnover, autophagy, accumulation of autophagosomes and aggregation prone proteins	(Tamboli et al., 2011; Keilani et al., 2012)

1.9 ATP13A2/PARK9

Ramirez and colleagues (Ramirez et al., 2006) were the first to link ATP13A2 and neurodegeneration, finding that mutations in *ATP13A2* gene, encoding for lysosomal membrane protein, are causing hereditary early-onset parkinsonisam with pyramidal degeneration and dementia (additional multysystemic features), named Kufor-Rakeb syndrome (KRS) (Figure 7) (Najim al-Din et al., 1994; Williams et al., 2005). KRS is characterizes with typical features seen in PD such as rigidity, bradykinesia, postural instability, mask-like face, initial good response to levodopa, and with the early onset of the condition between age of 11-16. Interestingly, tremor in patients extremities has not been observed yet, only very mild tremor in lower lip and chin during the talk (Hampshire et al., 2001; Di Fonzo et al., 2007; Schneider et al., 2010). Importantly, in addition to parkinsonian signs, KRS shows features characteristic for

corticospinal tract degeneration (i.e. pyramidal tract), such as spasticity, supranuclear gaze paresis, extensor plantar response (Babinski sign), and cognitive dysfunction (variable presence and degrees of dementia, hallucinations, behavior disorders) (Hampshire et al., 2001; Santoro et al., 2011). The disease appears to be rapidly progressive with increasing parkinsonism, pyramidal signs, cognitive deterioration and secondary non-responsiveness to levodopa, and the patients mostly end up wheelchair-bound due to severe dystonia (Williams et al., 2005; Schneider et al., 2010). The pathology of KRS remains largely unexplored, although MRI brain scans of KRS patients have shown progressive, diffuse brain atrophy (with initial atrophy of globus pallidus and pyramids), and DaTSCAN (brain scaning with injection of radioactive contrast agent, ioflupane iodine-123, that binds to dopamine transporters) and SPECT (single photon emission computed tomography) have shown significant nigrostriatal dopaminergic defects (Hampshire et al., 2001; Williams et al., 2005; Schneider et al., 2010; Santoro et al., 2011).

Since the initial report, nine disease-causing mutations (homozygous or compound heterozygous) have been described in four families (Ramirez et al., 2006; Crosiers et al., 2011; Eiberg et al., 2011; Park et al., 2011) and three isolated KRS patients (Di Fonzo et al., 2007; Ning et al., 2008; Paisán-Ruiz et al., 2010). Another nine mutations were identified in single heterozygous state in patients with early-onset Parkinson disease (EOPD) (Di Fonzo et al., 2007; Lin et al., 2008; Djarmati et al., 2009), but their role in the disease causation remains unclear. One possibility is that these heterozygous mutations might act as risk factor for development of EOPD.

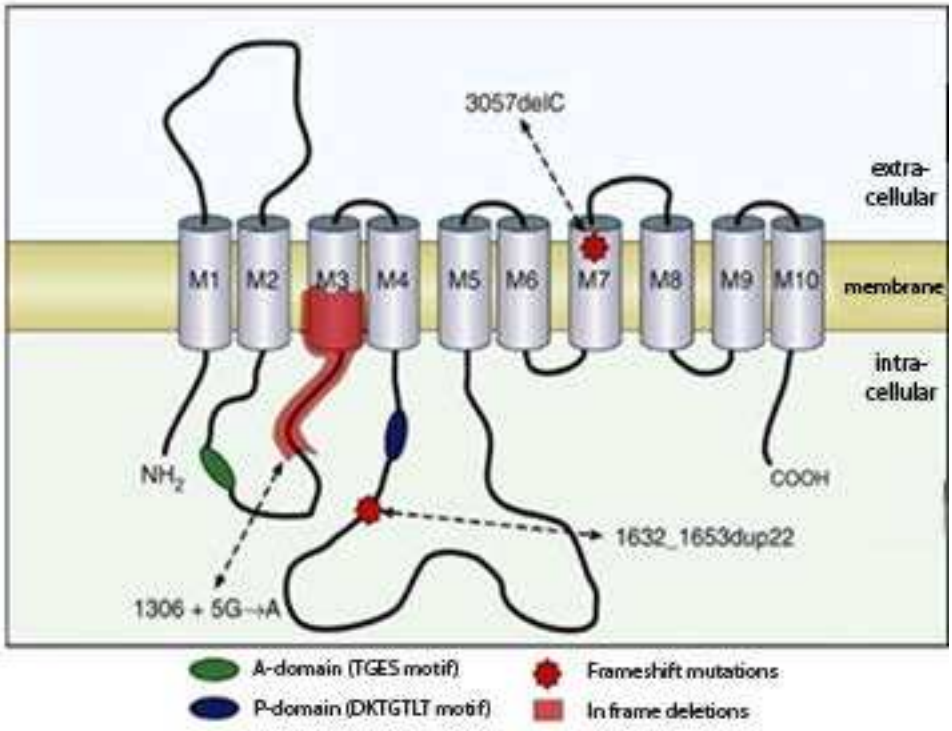


Figure 7. Predicted topology of ATP13A2 protein with mutations that were first to be discovered as a cause of Kufor-Rakeb syndrome
Figure has been adopted from Ramirez et al., 2006 with permission from Nature Publishing Group.

To this end, little is known about the physiological role of ATP13A2 as well as the mechanisms though which mutations in ATP13A2 are mediating neurodegenerative processes.

ATP13A2 is large lysosomal membrane protein with 1180 amino acid and 10-transmembrane domains, which belongs to 5 P-type ATPase family (Figure 7) (Schultheis et al., 2004). P-type ATPases transfer ions across the membrane using the energy derived from ATP hydrolysis (Kühlbrandt, 2004). Substrate specificity of ATP13A2 is still unknown, but recent studies suggest that this protein is involved in maintaining intracellular homeostasis of several cations including manganese and calcium (Gitler et al., 2009a; Schmidt et al., 2009; Tan et al., 2011; Chesi et al., 2012; Ramonet et al., 2012).

Discovered mutations in ATP13A2 are diverse: some are causing protein truncation and others substitution of amino acid in conserved protein domains. Regardless of the severities of mutation, mutant ATP13A2 proteins are unstable, retained in endoplasmic reticulum (ER) and subsequently degraded by UPS (Ramirez et al., 2006; Park et al., 2011; Ugolino et al., 2011; Ramonet et al., 2012). Recent evidence showed that mutant ATP13A2 proteins are causing ER

stress and are increasing cell sensitivity to ER stress-induced cell death. (Park et al., 2011; Ugolino et al., 2011). These results suggest that ER stress might play a role in the development of KRS. Another important aspect of these findings is that premature removal of the ATP13A2 mutant presumably leads to a loss of normal ATP13A2 function in lysosomes.

Recent findings showed that overexpression of ATP13A2 suppresses α-syn-mediated toxicity in yeast and *Caenorhabditis elegans* (*C. elegans*), whereas ATP13A2 loss-of-function enhances α-syn accumulation in body wall muscle cells in a *C. elegans* model of PD (Hamamichi et al., 2008; Gitler et al., 2009a). The evidence that suggest that autophagy-lysosomal pathway mediates clearance of accumulated and aggregated α-syn (Cuervo et al., 2004; Vogiatzi et al., 2008a; Mazzulli et al., 2011) give an idea of possible involvement of ATP13A2 in lysosomal function and α-syn clearance. In addition, recent studies showed that loss of ATP13A2 function caused accumulation of fragmented mitochondria, suggesting disrupted mitochondrial clearance via lysosomes and lending further support of ATP13A2 involvement in lysosomal function (Grünewald et al., 2012; Gusdon et al., 2012; Ramonet et al., 2012). Interestingly, mutations in *ATP13A2* were found in NCL patient and in a dog model for NCL, suggesting that mutations in ATP13A2 might be the cause of another neurodegenerative disease linked to lysosomal dysfunction (Farias et al., 2011; Wöhlke et al., 2011; Bras et al., 2012). In summary, these recent data suggest possible involvement of ATP13A2 in lysosomal degradation pathway and in α-syn misfolding and toxicity. However, the role of ATP13A2 protein in lysosomal function, as well as the mechanistic link between ATP13A2 and α-syn, has not been explained.

We believe that studying the role of the lysosomal ATP13A2 protein may not only lead to a novel insights into lysosomal function in the rare form of Parkinson-like disease, KRS, but may also help understand lysosomal degradation pathway in other neurodegenerative disorders, and provide a novel targets for development of neuroprotective therapies.

2. AIMS OF RESEARCH AND HYPOTHESES

While increasing evidence implicates lysosomal dysfunction in the pathogenesis of neurodegenerative disorders, the molecular underpinnings of the role of lysosomes in neurodegeneration remain largely unknown. Studies of lysosomal proteins linked to neurodegenerative disorders present an opportunity to directly examine the role of lysosomes in disease pathogenesis and to uncover specific molecular mechanism that contribute to neurodegeneration. Therefore, we focused our research on lysosomal ATP13A2 protein that, when mutated, causes Kufor-Rakeb syndrome (KRS) characterized by early-onset Parkinsonism, pyramidal degeneration and dementia.

Hypothesis 1. Since ATP13A2 is lysosomal protein we hypothesized that it plays a role in lysosomal degradation pathway and that lack of ATP13A2 function caused by loss-of-function mutations leads to disruption of lysosomal function.

Aim 1. Investigate the role of ATP13A2 protein in lysosomal degradation pathway.

Using primary mouse cortical neurons with silenced ATP13A2 and fibroblasts from KRS patient we tested lysosomal function using well established methods for studying lysosomal degradation capacity: long-lived protein degradation, degradation of epidermal growth factor receptor (EGFR) and clearance of autophagosomes. We also examined the lysosomal number and size that represent lysosomal staining profile and that can be altered if lysosomal pathway is compromised.

Hypothesis 2. Recent studies implicated ATP13A2 in α-syn misfolding and toxicity in yeast and *C. elegans* (Gitler et al., 2009a), but the underlying mechanism has not been established. Since inadequate clearance of α-syn via lysosomes results in accumulation of its toxic species (Mazzulli et al., 2011), we hypothesized that inhibition of lysosomal degradation capacity caused by loss-of-ATP13A2 function is responsible for accumulation of α-syn and neurodegeneration.

Aim 2. Examine the mechanism that connects ATP13A2 and α-syn, and their common involvement in neurodegeneration.

We examined α-syn levels in primary cortical neurons with silenced ATP13A2 that should be elevated if α-syn clearance is disrupted. To test if ATP13A2 loss-of-function results in neurotoxicity we applied well established methods for evaluating neuronal toxicity:

neurofilament staining, release of lactate dehydrogenase in cell medium and nuclear condensation. To test if ATP13A2 loss-of-function toxicity is α-syn mediated we silenced endogenous α-syn and examined neuronal cell death.

Hypothesis 3. Since ATP13A2 is a protein of unknown function, we hypothesized that identifying its interacting proteins will help us elucidate its normal biological role and cellular processes that ATP13A2 is involved in. In addition, the fact that loss of ATP13A2 function leads to α-syn accumulation and neurotoxicity in *C. elegans* suggests their functional connection and involvement in common pathways. Therefore we hypothesized that our newly identified ATP13A2 interactors might also be involved in α-syn functional network and modify aggregation and neurotoxicity in *C. elegans.*

Aim 3. Identify interacting partners of ATP13A2 and their role in α-syn-mediated neurodegeneration.

We applied membrane yeast two-hybrid, method specifically developed to discover interactors of full-length membrane proteins. To strengthen the functional link between α-syn and ATP13A2 we tested the ability of our newly identified ATP13A2 interactors to modify α-syn aggregation and neurotoxicity in *C. elegans.*

3. MATERIALS AND METHODS

3.1 Fibroblasts and primary cortical neurons

Primary human dermal fibroblasts carrying mutation 1550C>T in ATP13A2 (L6025) and matching healthy control (WT1) were a gift from Christine Klein (University of Luebeck, Luebeck, Germany). Another sex-matched control (WT2) was purchased from American Type Culture Collection, CRL-2522. Fibroblasts were cultured in DMEM medium (Invitrogen) supplemented with 15% Fetal Bovine Serum (FBS), L-glutamine, sodium pyruvate and penicillin/streptomycin.

Mouse embryonic primary cortical neurons were prepared from E17 embryos of C56BL/6 mouse as previously described (Jeong et al., 2009).

Neurons were infected at a multiplicity of infection (MOI) of 1 at day in vitro (DIV) 7, and harvested 7 seven days post-infection (DPI) for immunostaining and western blots. For leupeptin treatment, cells were treated with 50μM of leupeptin (EMD chemicals) 4 days prior to harvesting. Fibroblasts were transfected using Amaxa Basic Nucleofector kit (Lonza, VPI-1002) following manufacturer's protocol.

3.2 Plasmids

Lentiviral plasmids expressing short hairpin RNA (shRNA) targeting ATP13A2 and scrambled (non-targeting) sequence control, cloned in pLKO.1-puro vector backbone, were purchased from Sigma-Aldrich. shRNA plasmids for silencing of α-syn was obtained from Open Biosystems. Lentivirus was generated following published protocol (Tiscornia et al., 2006). Virus titers were determined using HIV-1 p24 Antigen ELISA kit (Zeptometrix).

3.3 Quantitative PCR

Total RNA was isolated from primary neurons day post infection 7 (DPI 7) using Trizol reagent (Invitrogen) and then treated with DNase (RNAase Free DNase Set, Qiagen). Reverse transcription was performed using SuperScript II First-Strand Synthesis SuperMix (Invitrogen) followed by quantitative PCR using SYBR GreenER SuperMix (Invitrogen) on the iCycler (Bio-Rad). Primers used in this study were following:

ATP13A2-Forward: AACTCCACAGGCAGGAGAG, Reverse: ATTGGGGCC GTAAATGGTCT;

18S-Forward: GTAACCCGTTGAACCCCATT, Reverse: CCATCCAATCGGTAGTAGCG

GAPDH-Forward: ATGACATCAAGAAGGTGGTG; Reverse: CATACCAGGAAA TAGAGCTTG. Relative mRNA abundance was calculated by the ΔΔCT method.

3.4 Western blot analysis

All western blots were performed and quantified using Odyssey Infrared Imaging System (Li-Cor) and Odessey software V2.1. Primary cortical neurons were lysed on ice in 1% Triton X-100 buffer, fibroblasts were lysed in RIPA buffer, and resolved on 12 or 8% Tris-Glycine gels. Antibodies that were used for detection were: anti-α-syn 202 (mAb, Covance, MMS-529R, 1:1000), anti LC3B (pAb, Cell Signaling, #2775, 1:500), anti-EGFR (pAb, Millipore, # 06-847, 1:1000), anti-Tau (pAb, Dako, #A0024, 1:1000)

3.5 Immunofluorescence analysis

Cells that were growing on poly-D-Lysine coated cover slips were washed in PBS and fixed in 4% paraformaldehyde (PFA) for 15 min at room temperature (RT). After brief wash with PBS cell were permeabilized in blocking buffer (1X PBS, 4% goat serum, 0.1% BSA, 0.1% TritonX-100) for 1h at RT. Subsequently cell were incubated with primary antibody at 4 ^{0}C. For immunostaining we used following antibodies: anti LC3, pAb, Cell Signaling, #2775, 1:50; anti LAMP1, rat mAb, Developmental Studies Hybridoma Bank, University of Iowa, # ID4B, 1:50; anti LAMP1, pAb, Santa Cruze Biotechnology, #sc-20011, 1:50). The day after, cover slips were washed 3 times with PBS and incubated for an hour with secondary antibody on RT.

For LysoTracker staining fibroblasts were incubated with LysoTracker Red DND-99 (Invitrogen, Molecular probes, 1:1000 dilution) for 30 min on 37 ^{0}C, washed 3 times with PBS and fixed as described previously. Neurons were treated with 200 nM LysoTracker Green DND-26 (Invitrogen, Molecular probes) for 5 min on 37 ^{0}C and immediately after imaged by confocal microscope.

Activity of lysosomal protease Cathepsin B was detected within live cells using Magic Red Cathepsin detection kit following suggested protocol of manufacturer (Immunochemistry Technologies, 937). Confocal microscopy was performed with Lieca TCS SL using 63x 1.4 numerical aperture objective. Live cell imaging was performed using Zeiss LSM 510 META microscope with 25x objective. Quantitative analysis of fluorescence intensities ware performed using ImageJ (Fiji) software.

3.6 Monitoring autophagosome maturation

Generation of the mouse embryonic fibroblast cell line stably expressing mCherry-EGFP-LC3 was previously described (Tresse et al., 2010). RNAi knockdown were performed by transfection of ON-TARGET plus-Smartpool siRNA (Dharmacon, 050430 for ATP13A2 and 057592 for VCP) with Lipofectamine RNAi Max (Invitrogen, 13778-075). The cells were imaged and red and yellow puncta quantified with a Marianas 2 confocal microscope using a 63x objective. All analyses were performed with Slidebook 5.0 software.

3.7 Lysosomal degradation studies

Primary cortical neurons on DPI 7 were treated with murine epidermal growth factor (EGF) (Preprotech, #31509, 50ng/ml) to stimulate epidermal growth factor receptor (EGFR) endocytosis. Subsequently cells were harvested at the indicated time points (Figure 12B, C). Fibroblasts were treated with 150ng/ml of human EGF (Preprotech, #AF-100-15). To follow degradation of EGFR, protein lysates were subjected to sodium dodecyl sulfate- polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotted with anti-EGFR antibody (pAb, Millipore, # 06-847, 1:1000).

Measurement of the degradation of long lived proteins in fibroblasts was performed as previously described (Kaushik and Cuervo, 2009). In order to preferentially label long lived proteins, cells were incubated in complete DMEM with L-^{3}H-leucine (final 5 μCi/ml, PerkinElmer) for 48h. To remove unincorporated radioactive leucine cells were extensively washed in DMEM containing 10-fold excess of unlabeled (cold) leucine (2.8 mM final). Then, cells were incubated in cold medium for a one hour to remove amino acids coming from degradation of short-live proteins. After washes cells were incubated in complete cold media and at 0, 8 hr and 24 hr time points, 60 μl of media was removed and precipitated with

trichloroacetic acid (10%, final) and BSA (0.5mg/ml, final) for 16h on 4 ^{0}C. Samples were centrifuged at 20 000g for 15min, 4^{0}C. Supernatants were collected; precipitated protein pellets and cells were lysed in Na deoxycolate, 0.1M NaOH; and radioactivity was measured by scintillation counting. Percentage of proteolysis was calculated as described (Kaushik and Cuervo, 2009).

3.8 Neuronal toxicity

Cell death of neurons treated with lentivirus carrying ATP13A2 shRNA and scrambled shRNA was evaluated by LDH-Cytotoxicity assay Kit II (BioVison). Cortical neurons were seeded at 40 000/well in 96-well plate. Media from cultured neurons were collected on days post-infection 5, 7, 9 and 12 and activity of lactate dehydrogenase (LDH) enzyme was measured following manufacturer's suggested protocol with minor changes. Standard curve was generated using known concentrations of β-NAD-reduced disodium (Sigma, N6660), based on which we calculated units of LDH enzyme in harvested samples.

Neurofilament staining was performed with in-cell western blot as previously described (Mazzulli et al., 2011). Cortical neurons were seeded in 96-well plate and fixed in 4% paraformaldehyde (PFA) at DPI 5-12 followed by staining against neurofilament (anti-neurofilament, mAb, Developmental Studies Hybridoma Bank, University of Iowa, # 2H3, 1:1000). To analyze percentage of condensed nuclei, cortical neurons DPI9 were stained with DAPI and visualized with fluorescence microscope.

3.9 Statistical analysis

Statistical analyses were calculated by two-tailed Student's t test and Mann-Whitney rank sum test using SigmaPlot 11.0 software. Values are expressed as mean ± standard error of the mean (SEM) (In Figures 9-16).

3.10 Membrane yeast two-hybrid screen

3.10.1 Design and validation of the bait construct

The cDNA of full-length wild type ATP13A2 (bait) was cloned into the vector pTMBV-MFα-Cub-TF by *in vivo* recombination in yeast strain THY.AP4 (MATa leu2-3,112 ura3-52 trp1-289lexA::HIS3 lexA::ADE2 lexA::lacZ) [detailed protocols in (Iyer et al., 2005; Snider et al., 2010). Using primers:
Forward:CCGAACCAGTGGCTGCAGGGCCGCCTCGGCCAAAGGATGAGCGCAGACAG CAGCCCT;
Reverse:CGAACCAGTGGCTGCAGGGCCGCCTCGGCCAAAGGATGAGCGCA GACAGCAGCCCT, human ATP13A2 was fused C terminally to Cub-TF.

Signal sequence of yeast α-mating pheromone precursor (MFα) was added to the vector to facilitate insertion of human ATP13A2-Cub-TF into a yeast membrane. After the pTMBV-MFα-ATP13A2-Cub-TF sequence had been confirmed, expression, correct insertion of the fused protein into the membrane and non-self-activation of reporter genes were verified with "NubG/I" test [detailed protocols in (Snider et al., 2010)]. In this assay, we used two non-interacting yeast proteins (preys); Ost1 (a component of the oligosaccharyl transferase complex in the endoplasmic reticulum membrane) and Fur4 (an uracil permease localized to the plasma membrane). Those proteins were fused to either NubI (Ost1-NubI and Fur4-NubI) or NubG (Ost1-NubG and Fur4-NubG). NubI constructs were positive controls while NubG constructs were negative controls. Yeast cells co-expressing control preys and ATP13A2-Cub-TF were spotted on selective medium lacking Trp, Leu, Ade and His (drop-out medium) to test the interaction with control fusion proteins. This assay was also used to select the optimal concentration of 3-amino-1,2,4-triazole (3-AT), a competitive inhibitor of HIS3 gene, to suppress background growth. MFα-ATP13A2-Cub-TF construct did not show any background growth on selective media SD-Trp-Leu-Ade-His, but to increase the stringency of the HIS3 selection we supplemented media with 10 mM 3-AT in following screen for interactors.

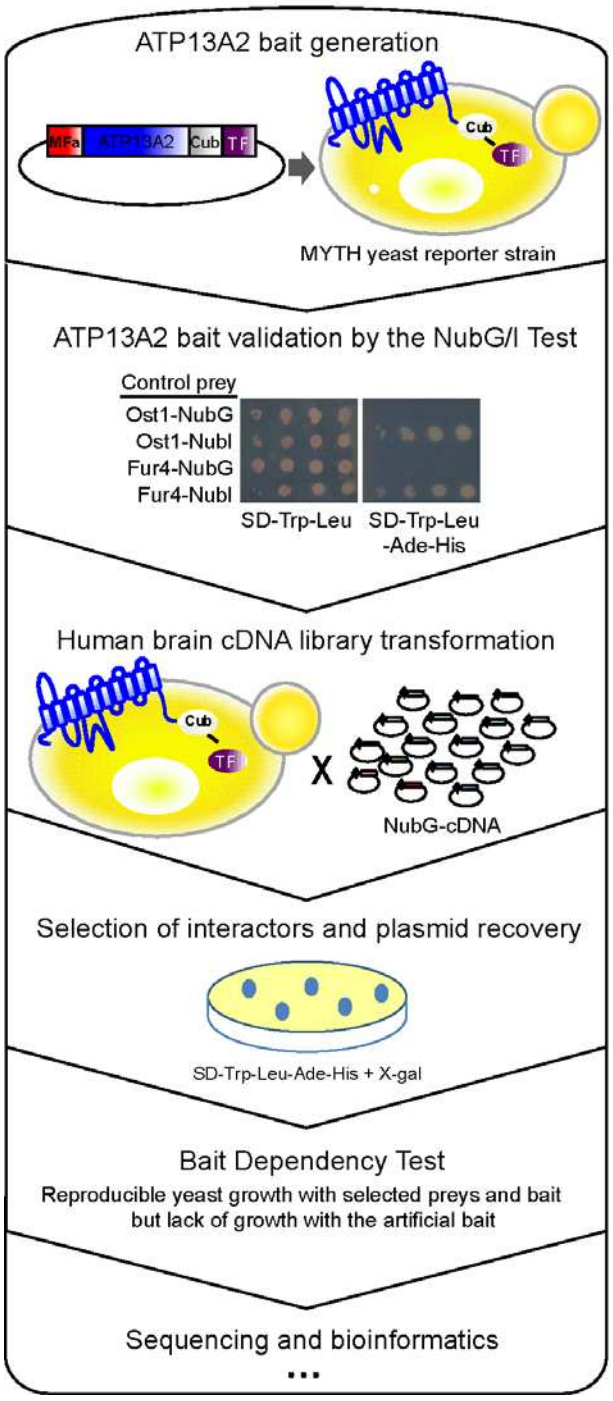


Figure 8. Flowchart of MYTH system showing general steps to identify interactors of membrane proteins

The ATP13A2 bait was generated and tested for expression and self-activation of reporter genes by the "NubG/I test" which uses negative and positive control preys; human brain cDNA library was introduced in the ATP13A2-Cub-TF expressing yeast strain and putative interactors are selected on the special selective interaction media. A "bait dependency test" which employs an unrelated bait protein, is used to identify spurious preys and to omit them from the final list of interactors. Prey plasmids were extracted and identified by sequencing.

3.10.2 Screen for ATP13A2 interactors and bait dependency test

Yeast strain THY.AP4 expressing pTMBV-MFα-ATP13A2-Cub-TF construct was transformed with human brain cDNA library (DualSystems Biotech Inc, Zurich, Switzerland) (preys) fused to the 3' end of NubG moiety (NubG-prey orientation) by the lithium acetate protocol (Gietz and Schiestl, 2007). The library has an average insert size of ~1.5kb and complexity of approximately $2x10^6$ independent clones (DualSystems Biotech Inc, Zurich, Switzerland). The total number of transformants in this screen was of around 4 $x10^6$. 480 colonies were selected on SD-Leu-Trp-Ade-His + X-gal plates supplemented with 10 mM 3-AT. Plasmids from these yeast colonies were isolated by the zymolase method (Iyer et al., 2005) and amplified in Escherichia coli XL10 gold with a standard protocol (Inoue et al., 1990). To assay the specificity of the interaction, 480 selected prey plasmids were transformed back into THY.AP4 yeast strains expressing ATP13A2-Cub-TF (bait) and artificial bait MFα-CD4-Cub-TF (MFα signal sequence, the single-pass transmembrane domain of human T-cell surface glycoprotein CD4 and a Cub-TF tag). Since MFα-CD4-Cub-TF contains minimal extraneous sequence in addition to the tag, it does not interact with other proteins. 43 preys that activated ADE2/HIS3/*lac*Z reporter genes in yeast bearing ATP13A2-Cub-TF but didn't grow when co-expressed with artificial bait CD4-Cub-TF, were sequenced and further analyzed.

3.11 Co-immunoprecipitation

To reconfirm interaction between ATP13A2 and its putative interacting proteins, HEK293FT cells were co-transfected with plasmids expressing ATP13A2-V5, FLAG-HDAC6, FLAG-NIX, EGFP-SYT11, EGFP-GAK, EGFP-AAK1, HA-YIF1A and EGFP-HSPA8 (constructs were generously provided by Christian Kubisch, Tso-Pang Yao, Ivana Novak, Stefan Pulst, Lois Green, Sean Conner and Hiro Nakamura, respectively; HSPA8 construct was purchased from Addgen Inc). Cells were lysed by incubation for 30 min at 4 °C on a rocking platform in lysis buffer [50 mM Hepes, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 10% glycerol, 1% Triton X-100, 25 mM NaF, 10 uM $ZnCl_2$ (pH 7.5)] with protease inhibitor cocktail (Complete Mini, Roche Applied Science). To remove the insoluble fraction, collected cell lysates were centrifuged at 20000g for 20min. Supernatants were pre-cleared with protein A-Sepharose beads (Santa Cruz Biotechnology) for 1h, incubated with V5 antibody (Invitrogen) overnight at 4 °C

and with protein A-Sepharose beads for 2h. Proteins were detected on SDS-PAGE using antibodies to FLAG, GFP, HA and V5 tag.

3.12 Bioinformatic analysis

Physical protein interactions were obtained from two sources. First, the Interologous Interaction Database (I2D, version 1.71) (Brown and Jurisica, 2005) was queried with Entrez Gene IDs for the bait ATP13A2 and 43 hits to extract a network of interacting proteins involving the screen hits. Second, tab-delimited interaction files were downloaded from BioGRID (v3.1) (Stark et al., 2006) and parsed to identify proteins known to interact with the screen hits. The network of MYTH hits plus known and predicted interaction were visualized using the NAViGaTOR graph visualization software (v2.2.1) (Brown et al., 2009). Edges, which represent physical protein-protein interactions, were colored according to the interaction source. Node color represents the functional category each gene was manually assigned to, based on its Gene Ontology annotations. Enrichment for Gene Ontology terms was performed using the DAVID functional annotation tool (v6.7; http://david.abcc.ncifcrf.gov/). GO_FAT categories comprising a more informative set of GO terms were used for enrichment of the GO Biological Process, Molecular Function, and Cellular Compartment trees.

3.13 *C. elegans* strains

C. elegans (nematodes) were maintained following standard protocols (Brenner, 1974). Strain UA50 (baInl3, [P_{unc-54}::α-syn::gfp, P_{unc-54}::tor-2, rol-6 (su1006)]) was generated as described (Cao et al., 2005). Strain TU3401 (uIs69, [pCFJ90 (P_{myo-2}::mCherry), $P_{unc-119}$::sid-1]; sid-1(pk3321)), a gift from Martin Chalfie (Calixto et al., 2010), was crossed to UA44 (baIn11, [P_{dat-1}::α-syn, P_{dat-1}::gfp]) to generate UA197 (uIs69, [pCFJ90 (P_{myo-2}::mCherry), $P_{unc-119}$::sid-1]; sid-1(pk3321); baIn11, [P_{dat-1}::α-syn, P_{dat-1}::gfp]). Strain UA228 (uIs69, [pCFJ90 (P_{myo-2}::mCherry)]; vtIs7, [P_{dat-1}::gfp]) was generated by crossing TU3401 with BY250 (vtIs7, [P_{dat-1}::gfp]) (a gift from Randy Blakely, Vanderbilt University).

3.14 *C. elegans* experiments

For α-syn misfolding studies in body wall muscles of *C. elegans*, RNAi silencing was performed as described (Hamamichi et al., 2008) by feeding UA50 worms with RNAi clones (Geneservice,

Cambridge, UK) targeting the worm orthologs of positive candidates from ATP13A2 MYTH screen with the following modification. Worms were grown on RNAi bacteria for an additional generation and then analyzed for α-syn misfolding at young adult stage (day 4; Figure 21). Analysis was performed in duplicate, and candidates were scored as positive if RNAi significantly enhanced misfolding (80% of worms exhibited increased size and quantity of α-syn aggregates). Likewise, for neurodegeneration assay in dopamine neurons, RNAi was conducted by feeding UA197 and UA228 worms with RNAi clones targeting the worm orthologs of ATP13A2 and the positive candidates from the α-syn misfolding screen with the following modification. UA197 worms were analyzed for α-syn-induced dopamine neurodegeneration on Day 6 (Figure 22 A-I) and UA228 worms were analyzed for dopamine neuron degeneration on Day 7 (Figure 23 A-C). A total of 90 animals for each gene were analyzed (3 trials of 30 animals/trial). Worms were considered wild-type when all four CEP and both ADE neurons were intact and had no visible signs of degeneration. If a worm displayed at least one degenerative change (dendrite or axon loss, cell body loss), the animal was scored as exhibiting degenerating neurons (Cao et al., 2005; Hamamichi et al., 2008). For statistical analysis, quantitative data were displayed as arithmetic means ± SD in triplicate, and the One-way ANOVA followed by the Dunnett's multiple comparison test ($p < 0.05$) was used to examine significance (GraphPad Prism Software, version 5.0). All images were taken by fluorescence microscopy (Hamamichi et al., 2008) on the same day of analysis.

4. RESULTS

4.1 ATP13A2 involvement in lysosomal function and a-syn accumulation

4.1.1 Loss-of-ATP13A2 function causes accumulation and enlargement of vesicles positive for lysosomal markers.

To study the effect of loss-of-function of ATP13A2 on lysosomal pathway, we first examined KRS patient fibroblasts harboring mutation 1550C>T that presumably leads to retention of the mutant protein in the endoplasmic reticulum (Ramirez et al., 2006; Park et al., 2011; Tan et al., 2011; Ugolino et al., 2011). Lysosomal profile was examined by lysosomal markers LAMP1 (lysosome-associated membrane protein-1), a well-characterized lysosomal transmembrane protein (Eskelinen, 2006; Lee et al., 2010a) and LysoTracker, a pH sensitive fluorescent dye that specifically labels acidic lysosomes. Immunofluorescence analysis revealed a significant increase in LAMP1 immunostaining in patient fibroblasts (L6025) compared to fibroblasts from healthy individuals (Figure 9A). In addition, we found a significant increase in the number and size of LysoTracker positive vesicles in patient fibroblasts (Figure 9B). While in control fibroblasts the average number of LysoTracker positive puncta was 33 per cell with average diameter of 0.5μm, both parameters were approximately doubled in patient fibroblasts (Figure 9B graph), suggesting that lysosomes accumulate and increase in size in the presence of ATP13A2 mutation.

We next examined the activity of Cathepsin B, a lysosomal cysteine protease that has been previously implicated in degradation of aggregation-prone proteins (Mueller-Steiner et al., 2006; Liang et al., 2011). To measure Cathepsin B activity, fibroblasts were treated with fluorogenic substrate MR-(RR) 2 that emits red fluorescence after enzymatic cleavage allowing visualization of intracellular activity and location of Cathepsin B in live cells. As shown in Figure 9C, the fluorescence signal of MR-(RR) 2 is weaker and more diffuse in patient fibroblast compared to controls. Quantification revealed that fibroblasts carrying mutated ATP13A2 exhibited 25% lower intensity of post-cleavage MR-(RR) 2 compared to wild type fibroblasts (Figure 9C). Together, these results suggest that despite the observed increase in lysosomal number, the activity of lysosomal Cathepsin B is decreased in the presence of ATP13A2 mutation.

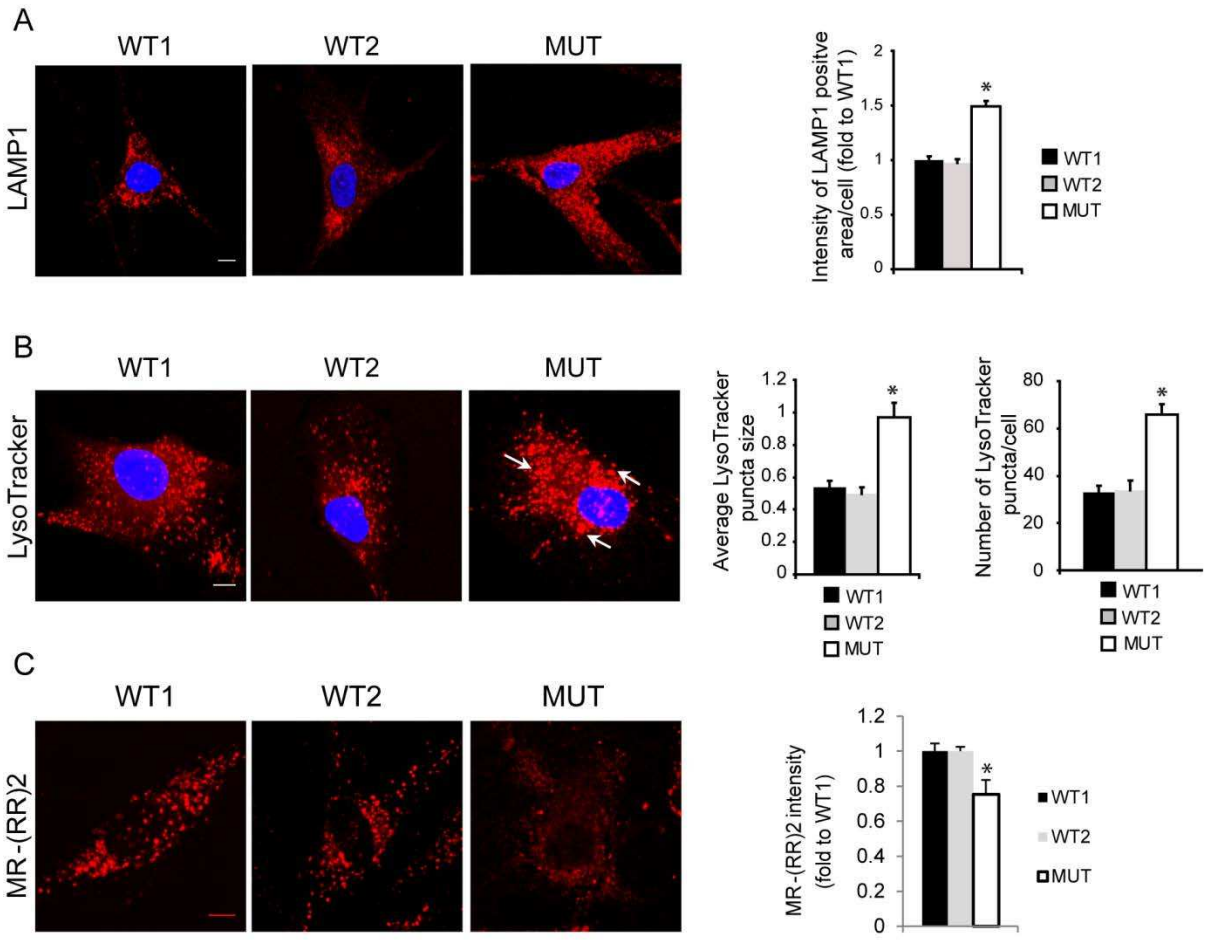


Figure 9. Accumulation of vesicles positive for lysosomal markers in ATP13A2 mutant fibroblasts
A) Representative images of LAMP1 immunostaining of two wild type fibroblast lines (WT) and fibroblasts carrying ATP13A2 mutation (MUT) (left panel), quantification of intensity of cell area stained by LAMP1 (right panel) (n=80, *p<0.001). **B)** Imaging of LysoTracker labeled fibroblasts showed increase in number and size of LysoTracker positive vesicles (n=80, *p<0.001), arrows indicate enlarged LysoTracker positive vesicles. **C)** Fibroblasts treated with fluorescent Cathepsin B substrate MR-(RR)2 (left panel), quantification of fluorescent signal is shown on the right (n=100, *p<0.001). In all graphs error bars represent standard error of the mean (SEM). Scale bars=10 μm.

In order to examine ATP13A2 function in primary cortical neurons, we developed lentiviral transduction to knockdown ATP13A2. Using this approach, about 90% reduction of ATP13A2 expression was achieved 7 days post-infection (DPI 7) compared with scrambled (scrb) shRNA control (Figure 10C). Importantly, silencing of ATP13A2 in neurons resulted in significant increase in the number and size of LAMP1 positive puncta when compared to scrambled shRNA (Figure 10A). As additional control, we used leupeptin, a well-established inhibitor of lysosomal proteases. Treating primary neurons with leupeptin also resulted in increased staining intensity

and the size of LAMP1 positive puncta as seen in the presence of ATP13A2 knockdown (Figure 10A). Moreover, live cell imaging revealed a significant increase in the number of LysoTracker positive puncta in ATP13A2 KD neurons compared to scrambled shRNA control neurons (Figure 10B). A similar pattern of LysoTracker staining was observed in neurons treated with leupeptin (Figure 10B).

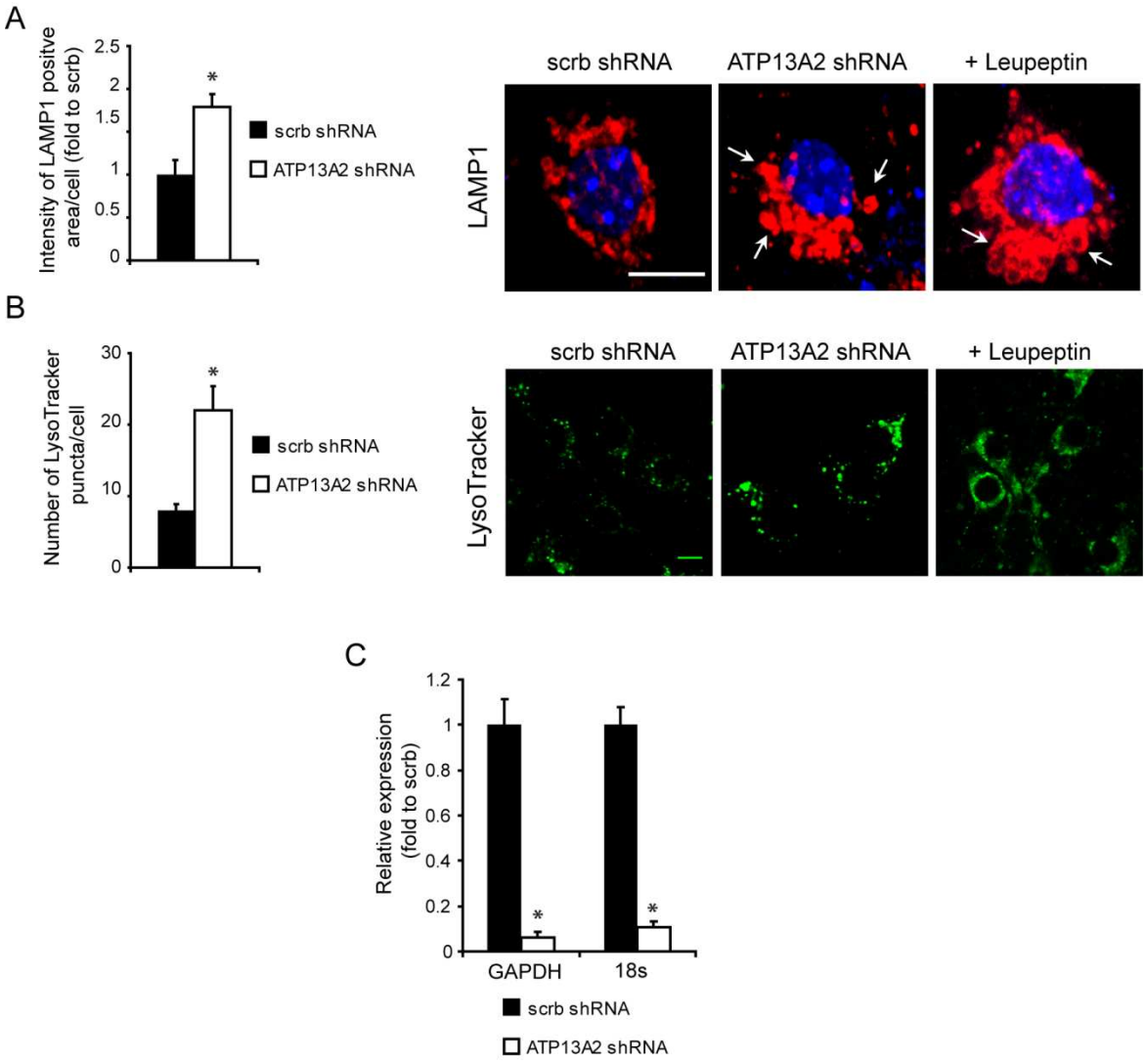


Figure 10. LAMP1 and LysoTracker staining in primary cortical neurons
A) Representative images of LAMP1 immunostaining of primary cortical neurons, graph on the left represents fold change of LAMP1 positive area in ATP13A2 KD neurons compared to scrambled (scrb) control cells (n=50, *p<0.001), arrows indicate enlarged LAMP1 positive vesicles. **B)** Primary cortical neurons labeled with LysoTracker Green (right panel). Quantification of the number of LysoTracker positive puncta (left panel) (n=25, *p<0.001). **C)** Graph represents evaluation of ATP13A2 knockdown by quantitative PCR. GAPDH and 18S were used as control genes (n=3, *p<0.001). In all graphs error bars represent SEM. Scale bars=10 μm.

Importantly, overexpression of wild-type ATP13A2 tagged with GFP (WT-ATP13A2-GFP), but not GFP alone resulted in a significant decrease of LAMP1 staining in mutant fibroblasts (Figure 11).

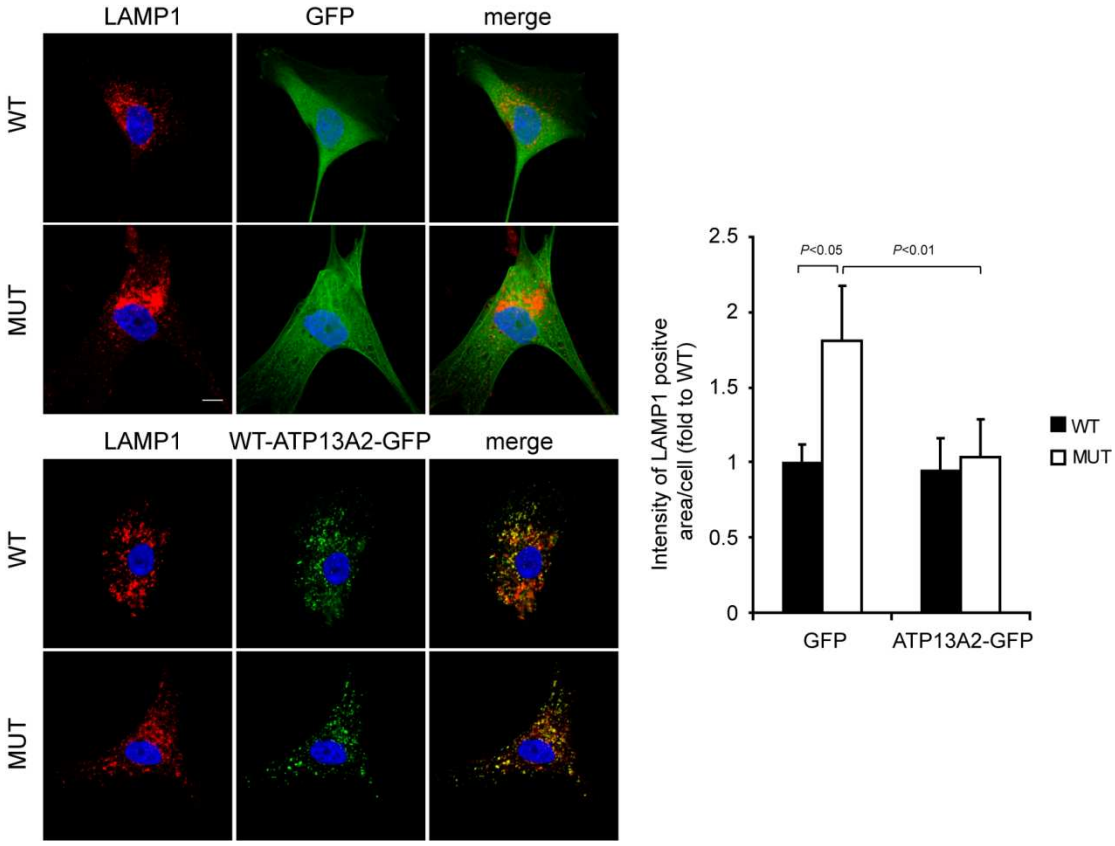


Figure 11. Overexpression of WT ATP13A2 rescues the lysosomal alterations in mutant fibroblasts
Mutant or wild-type fibroblasts overexpressing GFP (top) or WT-ATP13A2-GFP (bottom) and immunostained with LAMP1. Quantification of staining intensity is represented as fold change compared to WT line (right panel) (n=25). Error bars represent SEM. Scale bar=10 μm.

Together, these results suggest that the loss-of-function of ATP13A2 in patient fibroblasts and in primary cortical neurons results in up-regulation of lysosomal size and number, and that restoration of ATP13A2 can rescue these lysosomal alterations in mutant fibroblasts.

4.1.2 ATP13A2 loss-of-function impairs lysosomal degradation in patient fibroblasts and primary neurons

In light of the observation that Cathepsin B activity was decreased, while the lysosomal number was increased, we hypothesized that depletion of ATP13A2 may affect lysosomal degradation capacity. As a first step, we examined proteolysis of long-lived proteins in fibroblasts by radioactive leucine (L-^{3}H-leucine) during the time course of 24 hours. These experiments revealed that lysosomal proteolysis was significantly decreased in patient fibroblasts compared to healthy controls (Figure 12A). Treatment of cells with well-established lysosomal inhibitors ammonium chloride (NH_4Cl) and leupeptin did not exhibit additional decrease in the proteolysis indicating that the lysosomal degradation pathway is primarily affected by loss of ATP13A2 (Figure 12A).

To further investigate if loss of ATP13A2 function also compromises lysosomal degradation in neurons, we measured degradation of epidermal growth factor receptor (EGFR), an established readout of lysosomal degradation efficiency (Liang et al., 2008). Upon binding with epidermal growth factor (EGF), EGFR gets internalized and subsequently degraded in lysosomes. Using this method, we found significantly decreased average EGFR degradation rate in ATP13A2-silenced neurons compared to shRNA scrambled controls (Figure 12B). Similarly, lower degradation rate of EGFR was observed in fibroblasts with mutant ATP13A2 (Figure 12C). Together, these results suggest that loss of function of ATP13A2 leads to decreased lysosomal degradation capacity in both patient fibroblasts and in neurons.

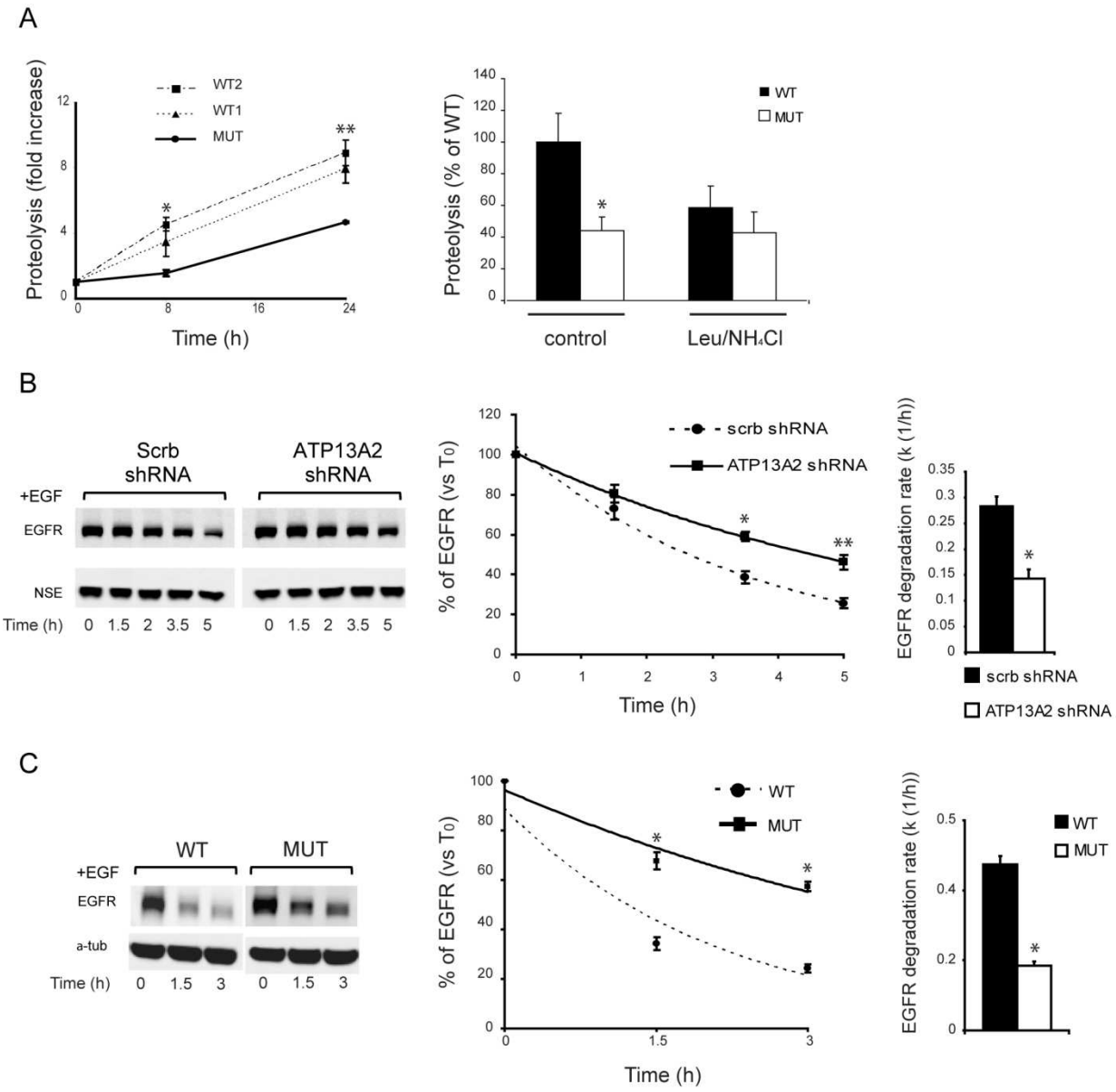


Figure 12. Decreased lysosomal degradation in patient fibroblasts and ATP13A2-depleted neurons
A) Degradation of long-lived proteins in wild type and mutant ATP13A2 fibroblasts at 8 and 24 hours after incorporation of [3H]-leucine (n=4, *p<0.05, **p<0.005). Values are expressed as fold increase from the initial time point. The effect of leupeptin and ammonium chloride was examined at 8 hours (left graph; n=4, *p<0.05). **B)** EGFR degradation in primary cortical neurons was followed for 5 hours. Levels of EGFR were normalized to NSE levels and expressed as % levels at initial time point (T_0) (n=6, *p=0.019, **p=0.008). Average of degradation rates of EGFR is shown (right graph) (*k* (1/h)) (n=6, *p<0.001). **C)** EGFR degradation rate in patient fibroblasts compared to wild-type fibroblasts (n=3, *p<0.001). In all panels error bars represent SEM.

4.1.3 Accumulation of LC3 positive vesicles in ATP13A2 KD MEF cells and neurons

Since our data so far suggest that lysosomal degradation capacity is decreased in presence of mutant ATP13A2, we next examined the status of macroautophagy, a lysosomal degradation pathway involved in removal of bulky cytoplasmic material including long-lived proteins and entire organelles (Martinez-Vicente and Cuervo, 2007). Macroautophagy includes the increased lipidation of Atg8/LC3 and the formation of autophagosomes that fuse with lysosomes to release the breakdown products. As a first step, we examined LC3-II, membrane associated marker of autophagosomes (Kabeya et al., 2000) that is degraded by lysosomes and accumulates when autophagy is impaired. Western blot analysis showed a significant increase in LC3-II to LC3-I ratio in ATP13A2 KD neurons compared to scrb control (Figure 13A). Consistent with these results, immunostaining revealed higher levels of LC3 positive puncta in KD neurons than in control cells (Figure 13B). Using leupeptin as additional control, a similar increase in LC3 staining was observed in leupeptin-treated as in ATP13A2 KD neurons. These results suggested that silencing of ATP13A2 leads to accumulation of autophagosomes in neurons.

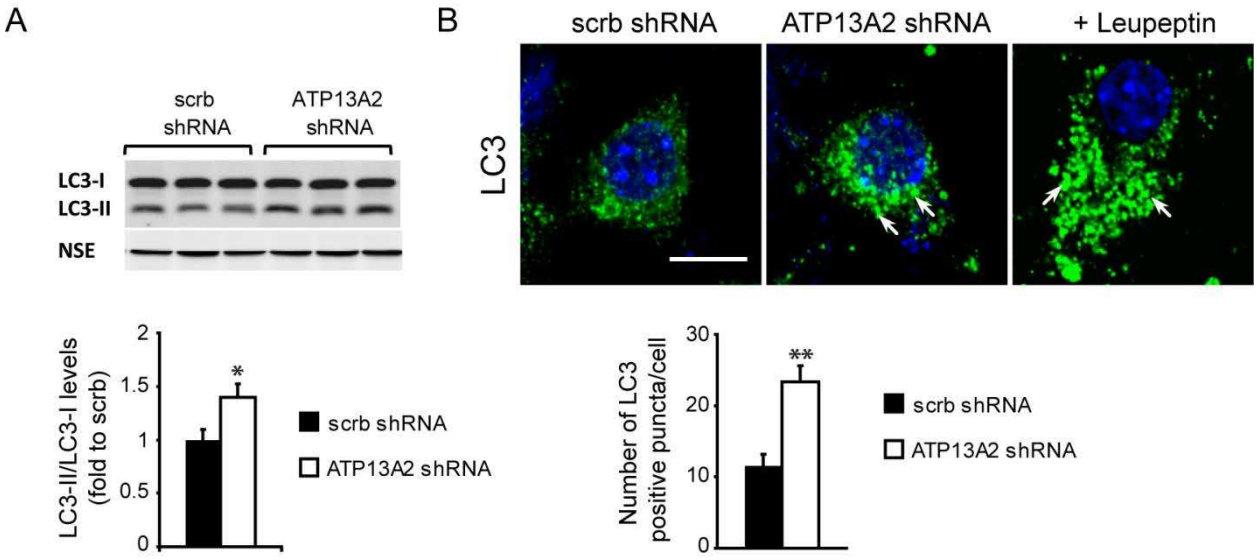


Figure 13. Silencing of ATP13A2 results in increased levels of LC3 positive vesicles
A) Protein levels of LC3-I and LC-II were quantified and normalized to NSE levels. The ratio between LC3-II over LC3-I was represented as fold change over scrb shRNA (n=8, *p<0.05). **B)** ATP13A2 shRNA neurons showed increase in LC3 immunostaining compared to scrb shRNA (n=50, **p<0.001). As a positive control, non-infected neurons were treated with leupeptin. Arrows indicate enlarged LC3 positive vesicles. In all graphs error bars represent SEM. Scale bar=10 μm.

In order to examine the turnover of autophagosomes we used mouse embryonic fibroblasts (MEFs) that stably express tandem-tagged fluorescence reporter mCherry-EGFP-LC3b. This reporter is useful for distinguishing immature autophagosomes (emitting yellow signal) from mature autophagolysosomes (emitting red signal) (Tresse et al., 2010). This is because both mCherry and EGFP emit fluorescence in the neutral environment of the immature autophagosome, but EGFP fluorescence is quenched by the acidification that occurs upon fusion with lysosomes. MEFs cells that were transfected with ATP13A2 siRNA exhibited a significantly increased number of immature autophagosomes (yellow puncta) and a decrease in the number of mature autophagolysosomes (red puncta) compared to cells transfected with non-targeting (scrambled) siRNA (Figure 14). As a positive control, we used siRNA mediated KD of VCP/p97 in MEFs cells that is known to cause accumulation of immature autophagosomes due to a failure in autophagosome-lysosome fusion (Tresse et al., 2010).

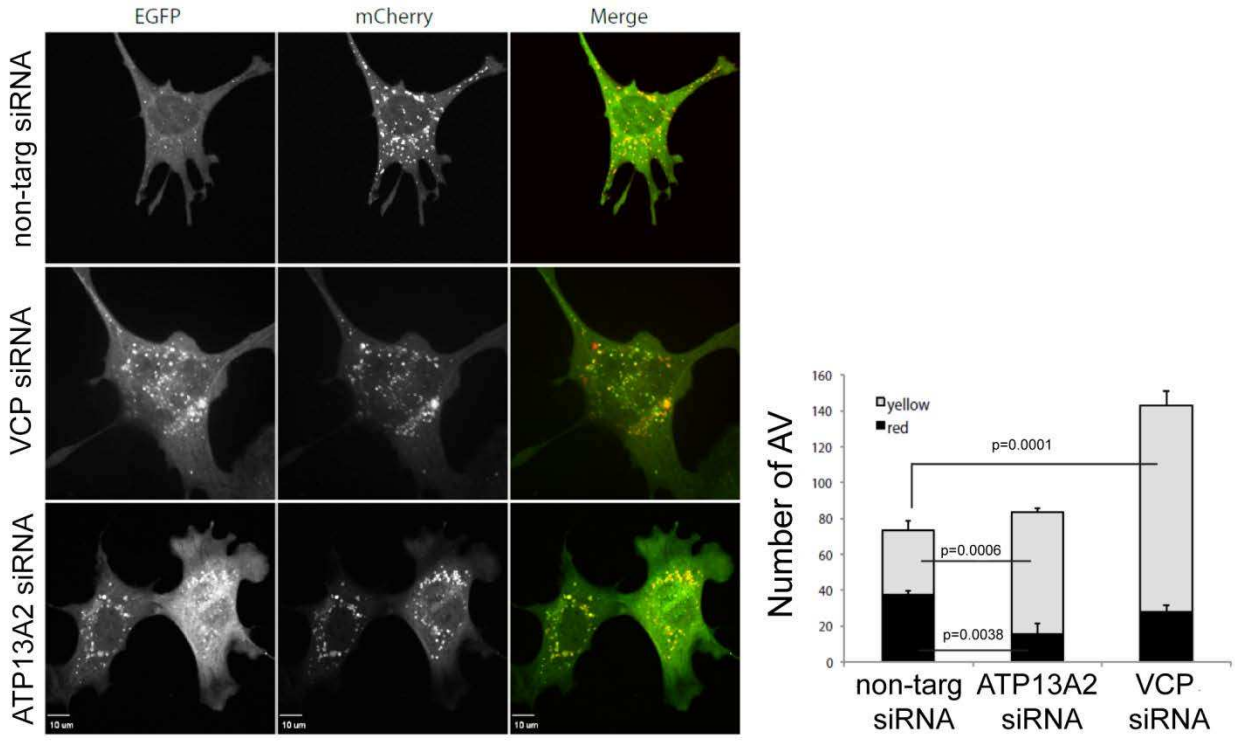


Figure 14. Depletion of ATP13A2 impairs turnover of autophagosomes
MEF cells stably expressing mCherry-EGFP-LC3b fluorescence reporter were transfected with siRNA for ATP13A2, non-targeting control and VCP/p97. Graph presents quantifications of autophagosomes (yellow puncta) and autophagolysosomes (red puncta). In all panels error bars represent SEM. AV, autophagic vesicle.

These data suggest that depletion of ATP13A2 might affect fusion of lysosomes with autophagosomes and this way impaired lysosomal clearance of autophagic vesicles and their cargo.

4.1.4 ATP13A2 KD in neurons causes accumulation of α–syn and toxicity

Since previous data in *C. elegans* suggested a link between ATP13A2 and α–syn (Gitler et al., 2009a), we examined if deficient lysosomal function in ATP13A2 KD neurons causes accumulation of α-syn. Indeed, we found significantly increased levels of endogenous α-syn in ATP13A2 KD compared to control neurons (Figure 15A). Interestingly, we did not observe an accumulation of another aggregation-prone protein tau (Figure 15B), suggesting that impaired lysosomal function in ATP13A2 KD neurons preferentially affects α-syn.

A previous report demonstrated that expression of ATP13A2 rescued the toxicity of α-syn (Gitler et al., 2009a). Based on our observation that loss of ATP13A2 function leads to decreased lysosomal function and accumulation of α-syn, we next examined if silencing of ATP13A2 in primary cortical neurons resulted in neurotoxicity. Neuronal toxicity was evaluated by neurofilament staining (NF), release of lactate dehydrogenase (LDH) in cell medium and by nuclear condensation. NF staining allows for detection of early neuronal toxicity that consistently precedes more severe nuclear cell death (Zala et al., 2005). While no changes in neurofilament staining were seen at day post-infection 5 (DPI 5), ATP13A2 KD neurons exhibited a significant decrease in NF at DPI 12 (Figure 15C). To validate these findings, we used LDH, an enzyme that is released into culture medium when plasma membrane is damaged. Knockdown of ATP13A2 resulted in significantly increased release of LDH compared to control at DPI 9 and DPI 12 (Figure 15D). In addition, knockdown of ATP13A2 in neurons resulted in increased nuclear condensation (Figure 15E). Together, these data suggest that loss-of-function of ATP13A2 leads to neuronal toxicity.

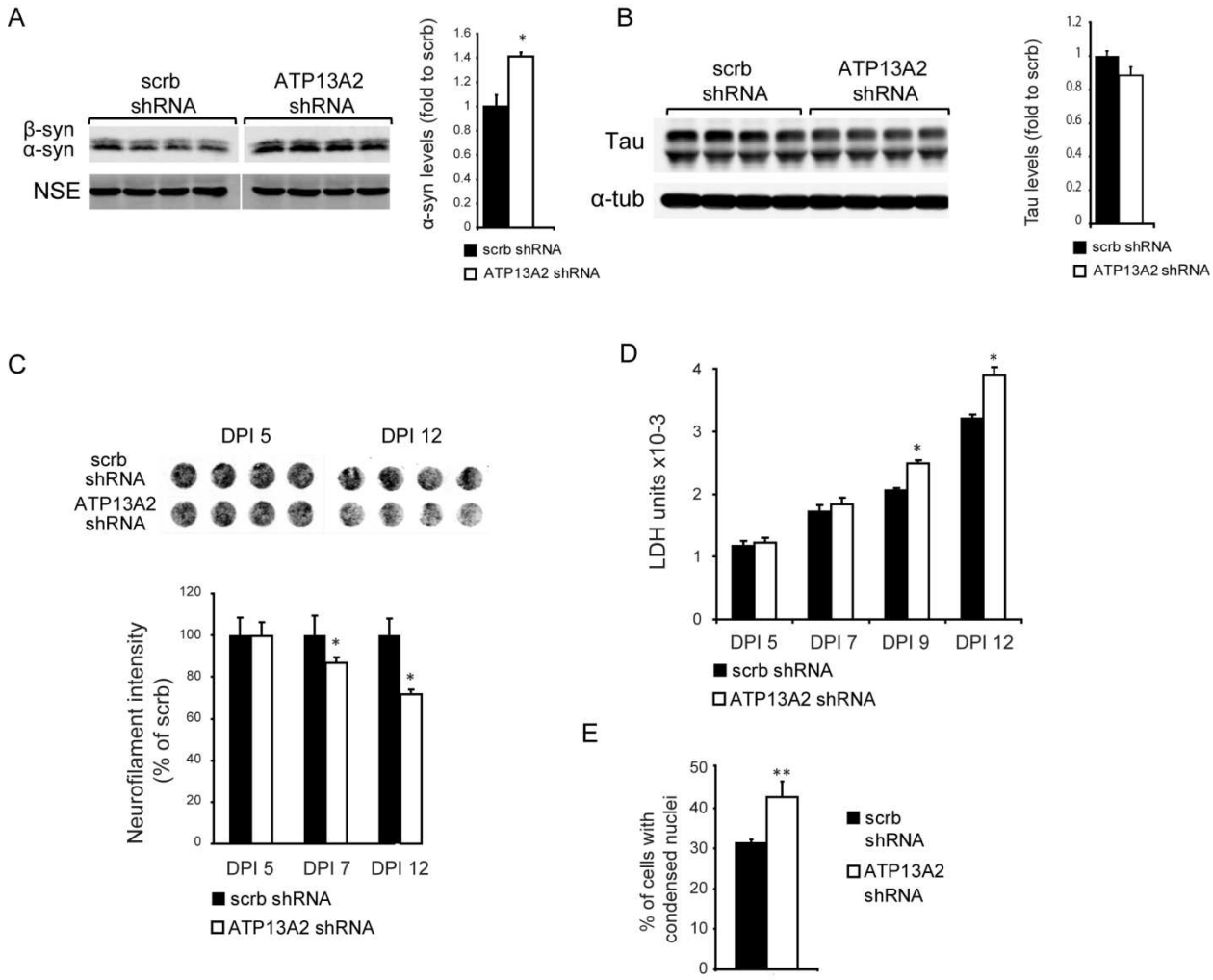


Figure 15. Knockdown of ATP13A2 causes accumulation of α-syn and neurotoxicity
A) Western blot showing α-syn protein levels normalized to NSE and presented as fold change over scrb shRNA levels (n=6, *p=0.002). **B)** Western blot analysis of Tau protein levels in scrb shRNA and ATP13A2 shRNA; α-tubulin was used as loading control (n=10, p= 0.578). **C)** *In-cell* western blot for neurofilament staining of neurons infected with lentivirus carrying ATP13A2 shRNA and scrb shRNA at DPI 5 and DPI 12. Graph shows quantification of neurofilament intensity expressed as a percentage of scrb shRNA (n=4, *p<0.001). **D)** LDH activity in neuronal culture medium at indicated time points (n=4, *p<0.001). **E)** Percentage of neurons with condensed nuclei stained with DAPI [n(cells)=180, four different fields, **p<0.05]. In all panels error bars represent SEM.

Having shown that KD of ATP13A2 causes accumulation of α–syn and neurotoxicity we wanted to examine if observed toxicity is α–syn mediated. We developed lentiviral transduction system to successfully silence endogenous α–syn (Figure 16A). Interestingly, knockdown of α–syn in neurons with silenced ATP13A2 rescued neuronal toxicity observed as an increase in the neurofilament staining to the levels of controls (Figure 16B). These data suggest that loss of

ATP13A2 mediates neurotoxicity at least partially via lysosomal dysfunction and consequent accumulation of α-syn.

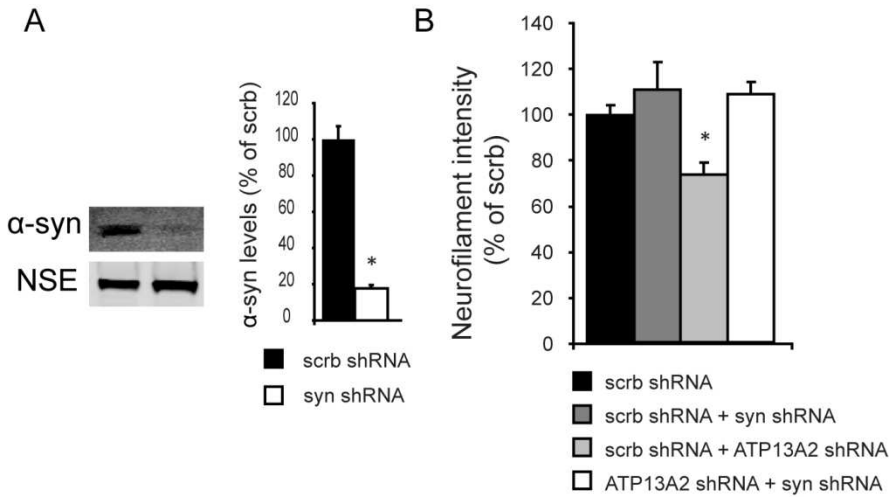


Figure 16. Depletion of endogenous a-syn attenuates neurotoxicity in ATP13A2-deficient neurons
A) Western blot and quantification of silencing of endogenous α-syn (n=3,*p<0.001). **B)** Quantification of neurofilament intensity in neurons with knockdown of α-syn and ATP13A2. In all panels error bars represent SEM.

Together, these results suggest that loss-of-ATP13A2 function leads to accumulation of enlarged lysosomes, impaired lysosomal turnover of autophagic vesicles and impaired lysosomal degradation capacity. This lysosomal dysfunction results in preferential accumulation of α-syn and toxicity in neurons. Importantly, depletion of endogenous α-syn attenuated the toxicity in ATP13A2 deficient neurons, suggesting that loss of ATP13A2 mediates neurotoxicity primarily via the accumulation of α-syn.

4.2 ATP13A2 interactors and their role in α-syn accumulation and toxicity in *C. elegans*

4.2.1 Novel interacting proteins of ATP13A2 identified by membrane yeast two-hybrid screen

Since ATP13A2 is a transmembrane protein, we utilized a split-ubiquitin membrane yeast two hybrid system (MYTH) to identify its interactors (Stagljar et al., 1998; Thaminy et al., 2004; Iyer et al., 2005; Gisler et al., 2008; Snider et al., 2010). This method uses split-ubiquitin approach, in which reassociation of two ubiquitin halves is mediated by a specific protein-protein interaction (Johnsson and Varshavsky, 1994). Protein of interest (bait) is fused to C-terminal fragment of ubiquitin (Cub) followed by artificial transcription factor (TF). The putative interactor (prey) is fused to N-terminal fragment carrying an Ile13Gly mutation (NubG). If the bait and prey interact, active ubiquitin (Ub) molecule is reconstituted and is recognized by cytoplasmic ubiquitin proteases (UBPs) that cause proteolytic cleavage of the TF, allowing its translocation to the nucleus and activation of yeast reporter genes that result in growth of yeast on selective media [Figure 17, (Stagljar et al., 1998; Iyer et al., 2005; Snider et al., 2010)].

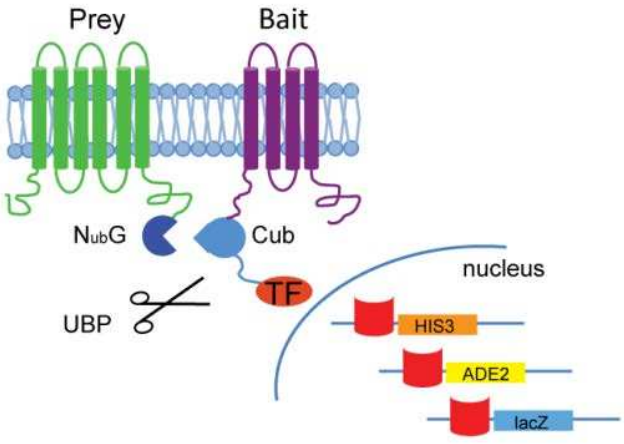


Figure 17. Main principle of MYTH method
Protein of interest-bait (purple) and putative interactor-prey (green) are tagged with Ub halves, Cub-TF and NubG, respectively. If bait and prey interact, active Ub molecule will be reassembled and recognized by UBPs. This will result in cleavage of TF and its translocation into nucleus, and activation of reporter genes HIS3/ADE2/*lacZ*.

One of the prerequisites of a successful screen is to establish that the bait construct gets correctly expressed but does not self-activate the reporter genes. To this end, bait construct ATP13A2-Cub-TF was tested on selective media in the presence of two commonly used non-interacting yeast integral membrane proteins, Ost1 (ER membrane) and Fur4 (plasma membrane). These two proteins were fused to either NubI (Ost1-NubI and Fur4-NubI) or NubG (Ost1-NubG or Fur4-NubG). NubI positive control constructs activate the yeast reporter system independently of a bait-protein interaction, because of high affinity of NubI (wild type form of N-terminus half of Ub) for Cub, resulting in active Ub molecules (Iyer et al., 2005). Activation of the reporter system in yeast cells coexpressing ATP13A2-Cub-TF and either of the NubI positive controls indicated that the bait protein was expressed, correctly inserted into the membrane, and the TF properly cleaved upon the formation of active Ub (Figure 18). In contrast, non-interacting yeast membrane proteins fused to the mutant NubG were used as negative controls, since they should not interact with human ATP13A2 bait fused to Cub. Lack of growth of yeast cells co-expressing ATP13A2-Cub-TF and non-interacting control prey (Ost1-NubG or Fur4-NubG), indicated that the bait construct was not self-activating (Figure 18).

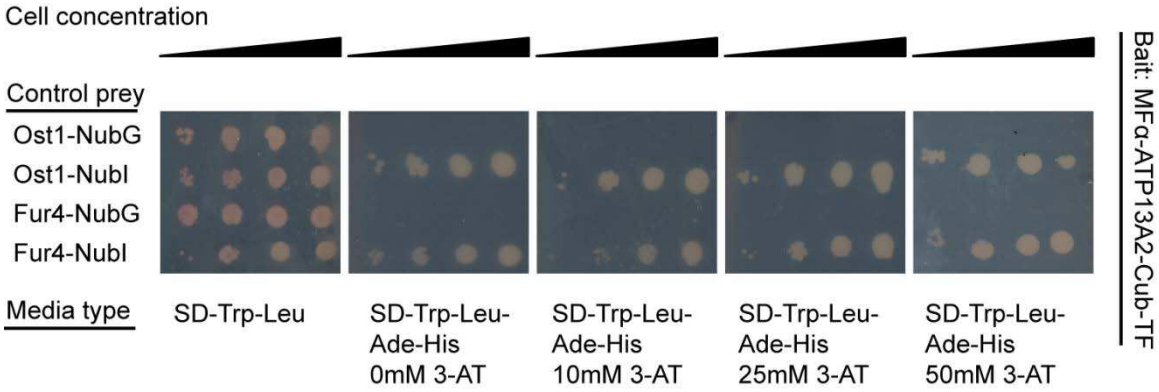


Figure 18. Validation of bait MFα-ATP13A2-Cub-TF
Transformed yeast cells growing on SD-Trp-Leu media confirmed the presence of both control prey and bait proteins. Cells carrying MFα-ATP13A2 and positive prey controls, Ost1-NubI or Fur4-NubI grew on selective media (SD-Trp-Leu-Ade-His), indicating the correct insertion of bait in the membrane so that the Cub-TF portion is oriented toward the cytoplasm. The absence of growth of yeast cells coexpressing negative prey controls (Ost1-NubG or Fur4-NubG) and the bait indicates the lack of MFα-ATP13A2-Cub-TF self-activity. Selective media were supplemented with different concentrations of 3-AT to increase stringency of HIS3 selection.

Following confirmation that our bait construct was suitable for MYTH, we performed a large scale screen using a human brain library with cDNAs fused C-terminally to the NubG moiety (DualSystems Biotech Inc, Zurich, Switzerland). We screened approximately $4x10^6$ transformants and identified 43 novel ATP13A2 interactors (Table 4, Figure 20A).

Table 4. ATP13A2 interactors

Gene name	Gene symbol	Function
Signal peptidase complex subunit 2 homolog	SPCS2	Component of the microsomal signal peptidase complex which removes signal peptides from nascent proteins as they are translocated into the ER
Lectin, mannose-binding 2	LMAN2	Protein which recycles between the Golgi and the ER; binds high manosse glycoproteins facilitating their sorting, trafficking and quality control
Protein disulfide isomerase family A, member 6	PDIA6	Catalyzes the rearrangement of -S-S- bonds in proteins; chaperone that inhibits aggregation of misfolded proteins
Sec61 beta subunit	SEC61B	Part of a complex necessary for protein translocation in the endoplasmic reticulum
Ubiquitin-conjugating enzyme E2, J2	UBE2J2	E2 ubiquitin-conjugating enzyme located in the membrane of the ER
Yip1 interacting factor homolog A	YIF1A	Protein involved in trafficking between ER and Golgi
Vesicle-associated membrane protein 2 (synaptobrevin 2)	VAMP2	Component of SNARE complex responsible for vesicle fusion
AP2 associated kinase 1	AAK1	Serine/threonine kinase involved in clathrin dependent vesicular trafficking
Cyclin G associated kinase	GAK	Protein involved in clathrin dependent vesicular trafficking
Neurexin 1	NRXN1	Adhesion molecule involved in synaptic vesicle fusion
FK506 binding protein 8, 38kDa	FKBP8	Co-chaperone implicated in ER to Golgi and plasma membrane trafficking
Synaptotagmin XI	SYT11	Protein involved in calcium-dependent vesicular trafficking and fusion
Heat shock 70kDa protein 8	HSPA8	Cytosolic chaperone involved in protein folding, vesicular trafficking
Chemokine (C-X-C motif) receptor 4	CXCR4	Chemokine receptor involved in cell interaction and migration; G-protein coupled receptor 1 family
Chemokine (C-X-C motif) receptor 7	CXCR7	Chemokine receptor involved in cell interaction and migration; G-protein coupled receptor 1 family
Intercellular adhesion molecule 2	ICAM2	Protein involved in cellular adhesion
Protocadherin beta 10	PCDHB10	Potential calcium-dependent cell-adhesion protein
Cholecystokinin B receptor	CCKBR	G-protein coupled receptor that regulates dopamine activity in the brain
Tetraspanin 14	TSPAN14	Member of tetraspanin family involved in diverse biological processes including membrane fusion, adhesion and motility
Neuropeptide Y receptor Y1	NPY1R	Receptor for neuropeptide Y that is involved in appetite regulation, memory and seizure; G-protein coupled receptor 1 family
Coagulation factor II (thrombin) receptor	F2R	G-protein coupled receptor involved in various processes in the nervous system

Low density lipoprotein receptor-related protein 6	LRP6	Transmembrane cell surface protein involved in receptor-mediated endocytosis of lipoprotein and protein ligands
Histone deacetylase 6	HDAC6	Plays role in microtubule-dependent cell motility via deacetylation of tubulin, facilitates clearance of polyubiquitinated misfolded proteins by autophagy
Actin, beta	ACTB	Part of cytoskeleton; involved in cell motility, structure and integrity
BCL2/adenovirus E1B 19kDa interacting protein 3-like	BNIP3L (NIX)	Protein responsible for mitochondrial clearance
ATP synthase, H+ transporting, mitochondrial F0 complex, subunit C2 (subunit 9)	ATP5G2	Subunit of mitochondrial ATP synthase
FUN14 domain containing 2	FUNDC2	Belongs to the FUN14 family; localized to the mitochondia
Homeodomain interacting protein kinase 1	HIPK1	Belongs to the Ser/Thr family of protein kinases; regulates gene transcription
Mex-3 homolog B	MEX3B	RNA-binding protein
MYC binding protein 2	MYCBP2	E3 ubiquitin ligase, mediates mTOR activation
Oligosaccharyltransferase complex subunit	OSTC	Component of the oligosaccharyltransferase (OST) complex
WD repeat domain 5B	WDR5B	Belongs to the WD repeat WDR5/wds family
WD repeat domain 68	WDR68	Belongs to the WD repeat DCAF7 family, involved in craniofacial development
Oculocutaneous albinism II	OCA2	Protein involved in melanin synthesis, regulates the pH of melanosome and the melanosome maturation
Protein-L-isoaspartate (D-aspartate) O-methyltransferase domain containing 2	PCMTD2	Part of methyltransferase family, predicted to incited protein repair
Cytoglobin	CYGB	Plays protective function during conditions of oxidative stress
Collagen, type I, alpha 1	COL1A1	The fibrillar collagen found in most connective tissues
G protein-coupled receptor 21	GPR21	Plays a role in regulating body weight and glucose metabolism
Family with sequence similarity 111, member B	FAM111B	Unknown function
Family with sequence similarity 8, member A1	FAM8A1	Unknown function
NEFA-interacting nuclear protein NIP30	NIP30	Unknown function
Chromosome 15 open reading frame 24	C15orf24	Unknown function
Chromosome 19 open reading frame 56	C19orf56	Unknown function

Gene name and gene symbol were sourced from Entrez Gene, NCBI (http://www.ncbi.nlm.nih.gov/gene). Functions of interactors were sourced from PubMed/ NCBI (http://www.ncbi.nlm.nih.gov/pubmed/) and UniProt database (http://www.uniprot.org/). Abbreviations: ER, endoplasmic reticulum.

The specificity of these interactions was assessed with a bait dependency test in which 480 selected prey plasmids were re-transformed into yeast cells expressing ATP13A2 bait, as well as artificial bait MFα-CD4-Cub-TF. The preys that activate the reporter system in the presence of artificial bait are considered spurious because they activate the reporter system in a manner independent of bait identity (Snider et al., 2010; Petschnigg et al., 2012). Interactions are considered specific (bait dependent) only if the prey interacts with the bait but not with the artificial construct. As shown in Figure 19A, yeast cells were selected for growth on selective medium (SD-Trp-Leu), confirming the presence of both ATP13A2 bait and prey vectors, and for growth and development of blue color on X-gal medium (SD-Trp$^-$Leu$^-$Ade$^-$His$^-$ + X-gal) confirming the interactions. Yeast cells expressing artificial bait and prey that did not grow on the same selective media were considered bait-specific interactors (Figure 19B). Using this approach, we identified 43 interactors (Table 4) that specifically associated with ATP13A2 (Figure 19A), but not with the artificial bait construct (Figure 19B).

A subset of these interactors was then confirmed by co-immunoprecipitation (Figure 19C). Based on the availability of constructs, we selected 7 interactors and expressed them together with V5-tagged ATP13A2 in HEK293. Using immunoprecipitation of whole cell lysates with V5 antibody, we reconfirmed all 7 proteins as ATP13A2 interacting partners (HDAC6, NIX, HSPA8, STY11, GAK, AAK1L, YIF1A), validating the reliability of the MYTH method.

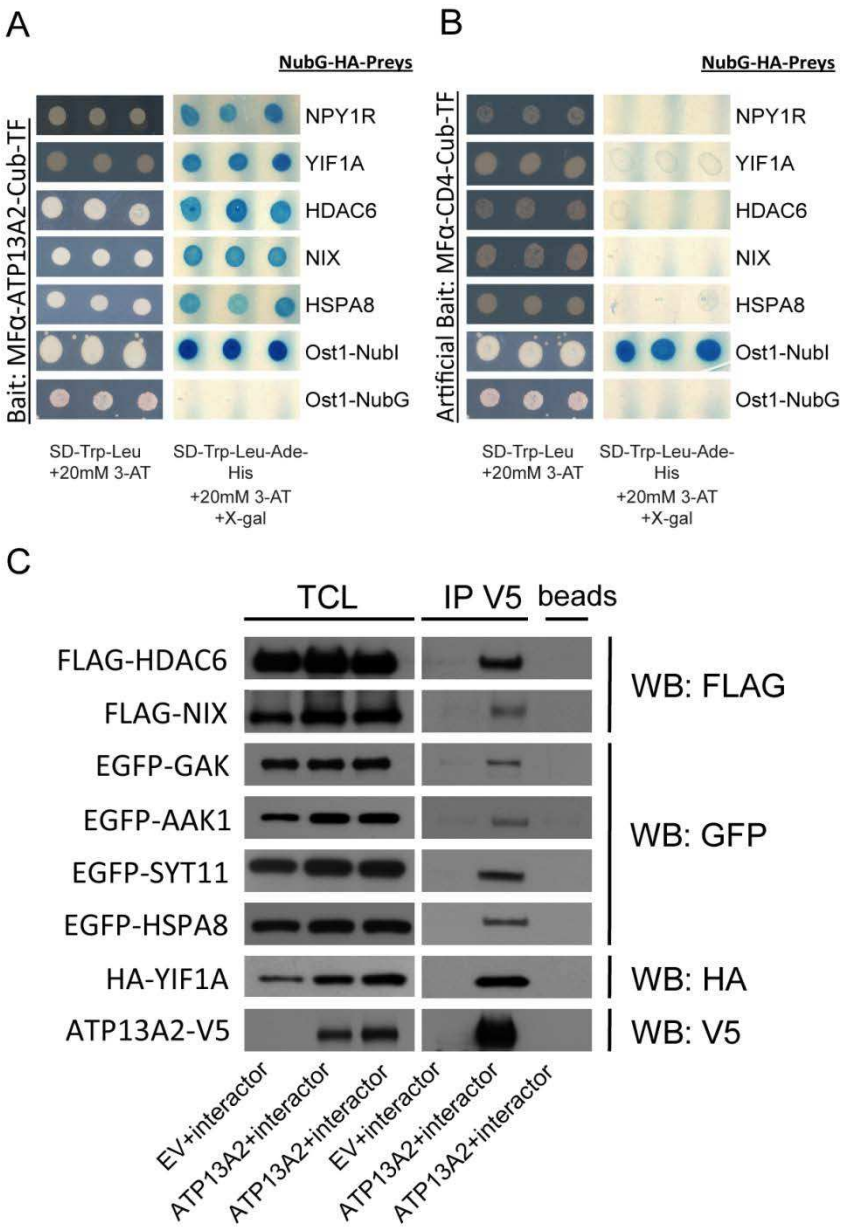


Figure 19. Verification of ATP13A2 interactors

A) Representative pictures of yeast strain THY.AP40 containing interacting bait–prey pairs grown on selective plates with X-gal. **B)** Bait dependency test was applied to determine the specificity of the interactions using the artificial bait construct MFα-CD4-Cub-TF as a negative control. Yeast strain THY.AP40 carrying this artificial bait was transformed with the same NubG-HA preys used in the screen in panel A. Interactors incapable of activating the reporter system in this strain were considered for further analysis. To confirm the correct expression and lack of self-activation of both bait and artificial bait constructs, Ost1-NubI and Ost1-NubG were used as positive and negative control, respectively. Selection medium was supplemented with 20 mM 3'AT to increase the stringency of the selection. **C)** Co-immunoprecipitation of HEK293FT cells co-expressing ATP13A2 and selected interactors. ATP13A2 tagged with V5 was immunoprecipitated from cell lysates using V5 antibody. Samples were immunoblotted and probed with antibodies against indicated tags of selected interactors. Specificity of the interaction between selected proteins and ATP13A2 can be seen by comparing total cell lysate (TCL) with immunoprecipitated lanes (IP V5). EV, empty vector.

4.2.2 Bioinformatic analysis of MYTH data

In order to assess the possible functions of ATP13A2 interactors, gene ontology (GO) terms were used to sort the interactors based on their suggestive or known biological function (Ashburner et al., 2000). First MYTH-identified interactors with related GO terms were presented as a network (Figure 20A) with indicated known and predicted interactions from two online databases (for details see Materials and Methods). In addition, we used a functional annotation tool (DAVID) that clusters related GO terms and provides a score representing enrichment (Huang et al., 2009). This analysis resulted in 18 clusters (Figure 20B) with one of the top scoring clusters (referred to Endoplasmic reticulum in Figure 20B) that included proteins involved in ER transport, ER-Golgi trafficking and protein folding (OSTC, SEC61B, BCAP31, ARV1, HSPA8, LMAN2, YIF1A, HSPA8, FKBP38, PDIA6, SPCS2, UBE2J2, LRP6). Other predominant functional categories included clathrin-mediated endocytosis (HSPA8, GAK, AAK1), vesicular trafficking and fusion (STY11, VAMP2, NRXN1) and cell migration and adhesion (CXCR4, CXCR7, ICAM2 and PCDHB10). Also, the fourth-highest scoring cluster is consisted of interactors involved ion homeostasis (CXCR4, NPY1R, F2R, CCKBR), emphasizing the previously suggested involvement of ATP13A2 in cation homeostasis (Gitler et al., 2009a; Tan et al., 2011; Ramonet et al., 2012). Since the majority of our ATP13A2 interactors cluster with cellular processes such as ER translocation, ER to Golgi trafficking, vesicular transport and fusion, this analysis suggested the involvement of ATP13A2 in vesicular trafficking.

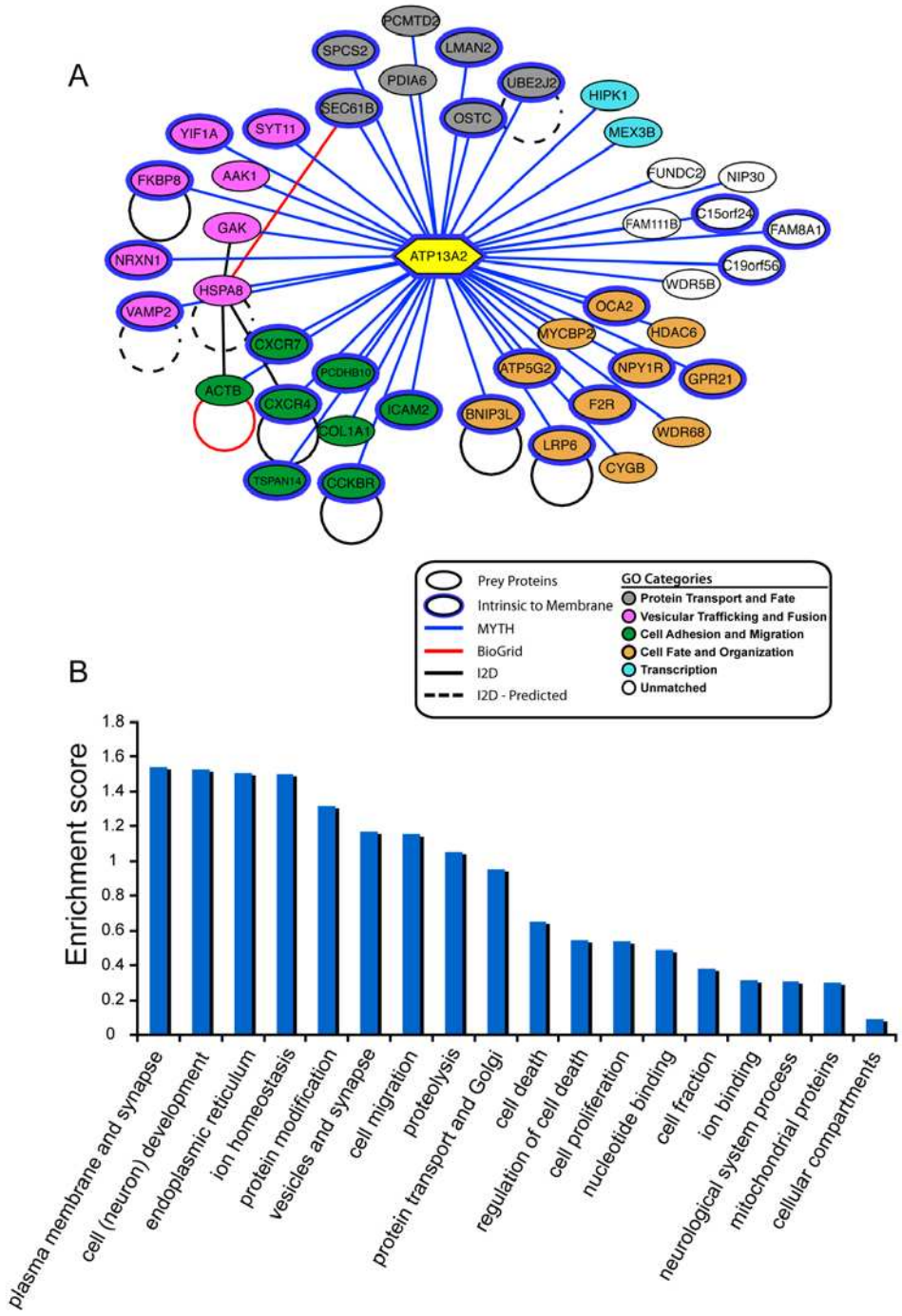
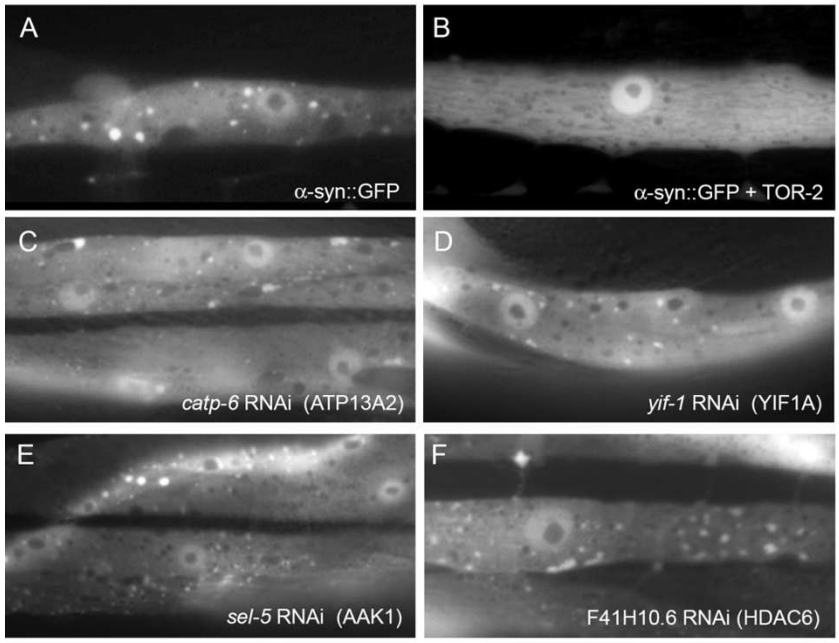


Figure 20. Functional assignment of ATP13A2 interactors

A) The network represents ATP13A2 interactome that was identified using MYTH technology and visualized using the NAViGaTOR graph visualization software. Edges, which represent physical protein-protein interactions, were colored according to the interaction source. Node color represents the functional category each gene was manually assigned to, based on its Gene Ontology (GO) annotations. Blue node highlights indicate genes annotated by GO to be intrinsic to the plasma membrane. **B)** Graph represents clusters of GO terms obtained by DAVID functional annotation tool.

4.2.3 Deficiency of ATP13A2 interactors exacerbates α-syn misfolding in *C. elegans*

Since ATP13A2 was recently identified as a modifier of α-syn toxicity and misfolding in yeast (Gitler et al., 2009a; Yeger-Lotem et al., 2009) and *C. elegans* (Hamamichi et al., 2008; Gitler et al., 2009a), we wanted to further examine the link between α-syn and ATP13A2. We tested the ability of our ATP13A2 interactors to modify α-syn aggregation in *C. elegans.* We focused on 22 interactors that had well-defined worm orthologs and were available in the *C. elegans* RNA interference (RNAi) library (Kamath and Ahringer, 2003). Also two candidates had two different worm orthologs, bringing the total number of gene candidates available for screening in *C. elegans* to 24 (Table 5). RNA interference (RNAi) was used to knock down each of the candidate genes, in *C. elegans* to examine whether depletion enhanced α-syn misfolding. This nematode model consists of overexpression of human α-syn fused to GFP at the C-terminus, wherein expression of this construct in the body wall muscle cells (P_{unc-54}:: α-syn::GFP) of the animal (Hamamichi et al., 2008) exhibits age-dependent α-syn misfolding in the cytoplasm, seen as punctuated pattern of GFP signal (Figure 21A). When chaperone TOR-2 is co-expressed with α-syn- GFP it attenuates α-syn misfolding and pattern of GFP signal changes from punctuated to diffused (Figure 21B). These animals provide a genetic background in which enhancement of α-syn misfolding is easily visualized. Using this model, we silenced the worm orthologs of 24 ATP13A2 interactor candidates using RNAi. Depletion of *C. elegans catp-6* (ATP13A2) resulted in increased misfolding of α-syn, as previously described (Gitler et al., 2009a), and served as a positive control (Figure 21C). The depletion of 8 out of 24 candidate genes by RNAi also significantly enhanced α-syn misfolding (Figure 21 table). Representative images of enhanced α-syn misfolding in worms expressing α-syn::GFP + TOR-2 with RNAi targeting candidate genes are shown in Figure 21D-F.



C. elegans **gene ID**	**Description**
C16D6.2	neuropeptide Y receptor Y1 (NPYIR)
F20B6.8a (*hpk-1*)	homeodomain interacting protein kinase 1 (HIPK1)
F35G12.3b (*sel-5*)	AP2 associated kinase 1 (AAK1)
K09G1.4 (*dop-2*)	coagulation factor II (thrombin) receptor (F2R)
R106.2	coagulation factor II (thrombin) receptor (F2R)
F41H10.6 (*hdac-6*)	histone deacetylase 6 (HDAC6)
Y37D8A.10 (*hpo-21*)	signal peptidase complex subunit 2 (SPCS2)
F57A8.2 (*yif-1*)	YIP1 interacting factor homolog A (YIF1A)

Figure 21. Knockdown of specific gene targets using RNAi enhances misfolding of α-syn in *C. elegans*

A) Nematodes expressing the α -syn::GFP transgene alone in body wall muscle cells of *C. elegans* (P_{unc-54}::α-syn::GFP) display misfolded protein, seen as GFP puncta. **B)** In the presence of TOR-2 (P_{unc-54}::α-syn::GFP + P_{unc-54}::tor-2), a protein with chaperone activity, the misfolded α-syn protein is attenuated and puncta are no longer visible. **C)** When worms expressing α-syn::GFP + TOR-2 are treated with *catp-6* (ATP13A2) RNAi, the misfolded α-syn::GFP returns. (**D – F)** Knockdown of specific candidate genes, that interact with ATP13A2, also causes misfolding of α-syn::GFP in worms expressing α-syn::GFP + TOR-2. Representative images are shown. Genes that were knocked down are: *yif-1* **D**), *sel-5* **E**), and F41H10.6 **F**). The corresponding human orthologs are YIF1A, AAK1, and HDAC6, respectively. The listing below summarizes the ATP13A2 interactors that, when knocked down via RNAi, caused α-syn misfolding in *C. elegans* body wall muscle cells. The human orthologs corresponding to these 8 interactors are also provided.

Table 5. *C. elegans* orthologs of human ATP13A2 interactors

Human orthologs	Description	*C. elegans* gene ID	E-value
SPCS2	Homo sapiens signal peptidase complex subunit 2 homolog	Y37D8A.10 (*hpo-21*)	5.2e-29
HIPK1	Homo sapiens homeodomain interacting protein kinase 1	F20B6.8a (*hpk-1*)	4e-143
YIF1A	Homo sapiens YIP1 interacting factor homolog A	F57A8.2 (*yif-1*)	1.3e-50
AAK1	Homo sapiens AP2 associated kinase 1	F35G12.3b (*sel-5*)	2.1e-63
F2R	Homo sapiens coagulation factor II (thrombin) receptor	R106.2	9e-12
F2R	Homo sapiens coagulation factor II (thrombin) receptor	K09G1.4 (*dop-2*)	3e-11
HDAC6	Homo sapiens histone deacetylase 6	F41H10.6 (*hdac-6*)	3.1e-169
PCMTD2	Homo sapiens protein-L-isoaspartate O-methyltransferase domain containing protein 2	R119.5	5.3e-26
CXCR4	Homo sapiens chemokine receptor 4	AC7.1b (*tag-49*)	4.3e-41
GAK	Homo sapiens cyclin G associated kinase	F46G11.3 (*tag-257*)	1.5e-52
FKBP8	Homo sapiens FK506 binding protein 8	F31D4.3 (*fkb-6*)	1.3e-87
SYT11	Homo sapiens synaptogamin XI	F31E8.2a (*snt-1*)	1.5e-116
NPY1R	Homo sapiens neuropeptide Y receptor Y1	C53C7.1 (*npr-10*)	3.9e-40
NPY1R	Homo sapiens neuropeptide Y receptor Y1	C16D6.2	3.1e-40
ATP5G2	Homo sapiens ATP synthase, H+ transporting, mitochondrial F0 complex, subunit C2	Y82E9BR.3	7e-34
LMAN2	Homo sapiens lectin, mannose-binding 2	T04G9.3 (*ile-2*)	1.9e-72
HSPA8	Homo sapiens heat shock protein 8	F26D10.3 (*hsp-1*)	1.9e-292
WDR5B	Homo sapiens WD repeat domain 5B	C14B1.4 (*wdr-5.1*)	1.4e-124
CXCR7	Homo sapiens chemokine (C-X-C motif) receptor 7	Y116A8B.5	9.9e-21
LRP6	Homo sapiens low density lipoprotein receptor-related protein 6	F29D11.1 (*lrp-1*)	0
MYCBP2	Homo sapiens MYC binding protein 2	C01B7.6 (*rpm-1*)	0
NIP30	Homo sapiens NEFA-interacting nuclear protein NIP30	C25A1.1	6.4e-24
NRXN1	Homo sapiens neurexin 1	C29A12.4 (*nrx-1*)	3.2e-135
CCKBR	Homo sapiens cholecystokinin B receptor	Y39A3B.5c (*ckr-2*)	3.2e-48

C. elegans orthologs were sourced from wormbase.org.

4.2.4 α-syn-induced dopaminergic neurodegeneration is enhanced when ATP13A2 interactors are depleted in *C. elegans*

Having discovered a set of ATP13A2 interactors that modify α-syn misfolding we hypothesized that these 8 candidates might also have an effect in the toxicity of dopamine (DA) neurons. Previously it was established that overexpression of wild-type human α-syn cDNA under control of a DA transport-specific promoter [P_{dat-1}:: α-syn + P_{dat-1}::GFP] results in age- and dose-dependent degeneration of DA neurons in comparison to neurons expressing GFP alone (Cao et al., 2005) (Figure 22A, B). By day 6 of adulthood most animals are missing at least one anterior DA neuron (Figure 22B). We wanted to examine the 8 positive candidates in this α-syn-induced degeneration model, via RNAi knockdown, to determine if candidate depletion would change the amount of degeneration. However, until recently, silencing genes with RNAi technique was an experimental challenge. We utilized newly developed method (Calixto et al., 2010) where worms exhibited selective RNAi silencing pan-neuronally. These worms were crossed into our P_{dat-1}:: α-syn + P_{dat-1}::GFP strain which allowed the impact of candidates knocked down via RNAi to be examined exclusively in the DA neurons.

An advantage of *C. elegans* is that detailed quantitative analyses of neurons are achievable. There are precisely six DA neurons within the anterior region of the worm that consistently display degenerative characteristics. We examined worms for changes in the cell bodies as well as the neuronal processes for normal appearance vs. degenerative changes (Figure 22). Using the pan-neuronal, cell-specific RNAi strain, expressing α-syn + GFP in the DA neurons, we knocked down the 8 candidate genes and analyzed the DA neurons for degeneration. A total of 30 animals, in triplicate, were analyzed per RNAi condition, for a total of 90 animals per gene knockdown. In order to examine the neuronal damage in this population we analyzed the percentage of the worm population that exhibited any neuronal degeneration (that is, if even one of six DA neurons was degenerating, the entire animal was considered mutant). This reporting method may be akin to the clinical situation wherein a patient (or worm or rat) is scored as affected or unaffected. When the empty vector (EV) control RNAi was examined, 74% of the worm population displayed degeneration at day 6 of development. Within the worm population treated with RNAi for the positive control, *catp-6*, 94% displayed enhanced neuron degeneration [Figure 22C, Figure 22I ($p<0.05$ One-way ANOVA)]. This increase in α-syn toxicity resulting from the depletion of *catp-6* was consistent with the RNAi result obtained from the body wall

muscle cells where the depletion of *catp-6* caused enhanced α-syn misfolding (Figure 21C). These results were also complementary to those previously published where ATP13A2 overexpression rescued α-syn-induced DA neuron degeneration (Gitler et al., 2009a).

Depletion of 5 *catp-6* (ATP13A2) interactors (out of 8 candidates) resulted in significant DA neurodegeneration. These candidates were C16D6.2 (96% degeneration), F41H10.6 (95%), *yif-1* (85%), *dop-2* (90%), and *sel-5* (88%). The corresponding human orthologs are NPY1R, HDAC6, YIF1A, F2R and AAK1, respectively [Figure 22D-H, Figure 22I ($p<0.05$ One-way ANOVA)].

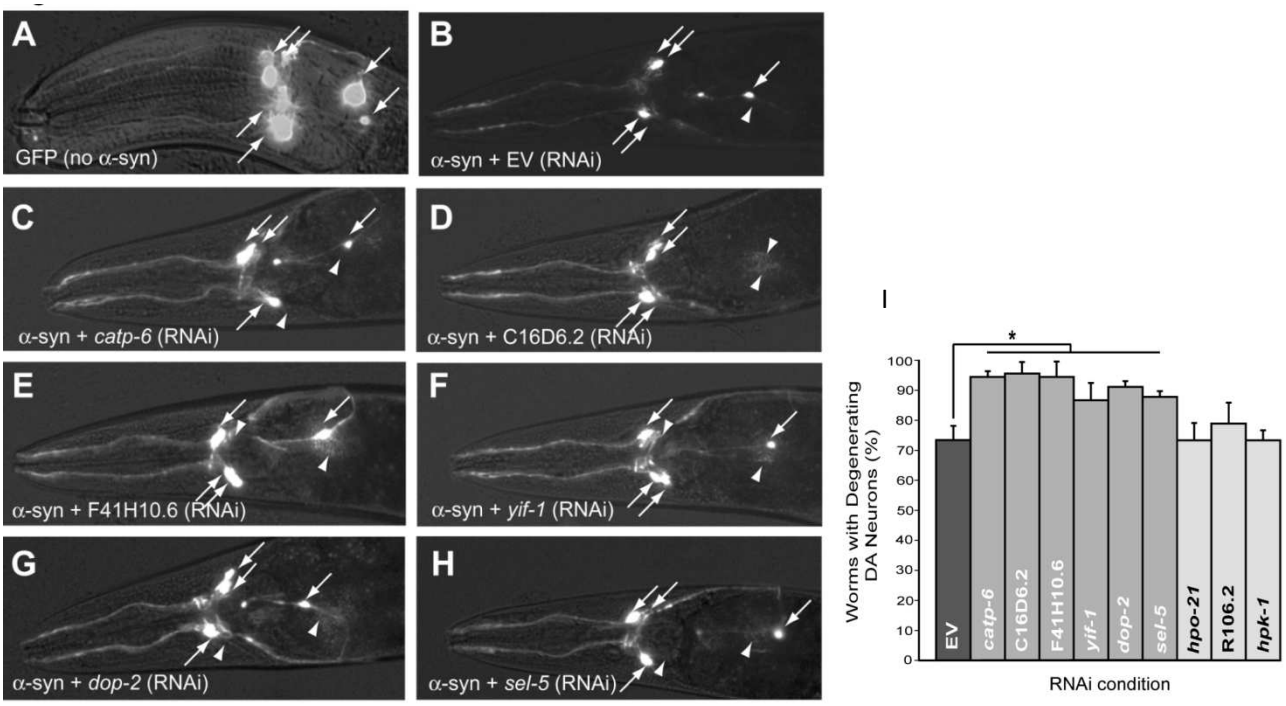


Figure 22. Knockdown of positive *catp-6* (ATP13A2) interactors enhances α-syn-induced DA neurodegeneration

A) The anterior DA neurons of a *C. elegans* are selectively illuminated with GFP driven from the DA transporter promoter (P_{dat-1}::GFP). The six cell bodies are indicated with arrows. **B)** A representative worm co-expressing both GFP and α-syn in DA neurons displaying neurodegeneration. An arrowhead indicates the approximate location of a missing neuron. These worms were also treated with empty vector (EV) RNAi as a negative control. **C)** A representative worm co-expressing GFP and α-syn in DA neurons, and RNAi silenced *catp-6*/ATP13A2, used as a positive control, demonstrating enhanced neurodegeneration compared to EV (RNAi) where 2 neurons are missing in this representative animal (indicated with arrowheads). (**D-H)** Knockdown of five positive candidates also yielded significantly greater numbers of missing DA neurons compared to the EV control RNAi. These candidates are C16D6.2 (**D**), F41H10.6 (**E**), *yif-1* (**F**), *dop-2* (**G**), and *sel-5* (**H). I)** The graph represents quantitative analysis of worm populations exhibiting enhanced α-syn-induced DA neurodegeneration upon RNAi knockdown of positive ATP13A2 interactors. RNAi knockdown of five positive candidates significantly enhanced the DA neurodegeneration with in the worm population, compared to the worms treated with control EV (*$p<0.05$ by one-way-ANOVA). These five candidates are: C16D6.2, F41H10.6, *yif-1*, *dop-2*, and *sel-5*. The corresponding human orthologs are NPY1R, HDAC6, YIF1A, F2R, and AAK, respectively. Values are expressed as mean ± standard deviation (SD) from 3 independent replicates.

To determine if the 5 positive candidates were specific to α-syn-mediated DA neurodegeneration or if they represented a general effect on DA neuron toxicity, we examined knockdown of these candidates in a background lacking α-syn. For this analyzes, we utilized a pan-neuronal, cell specific RNAi strain expressing GFP only in the DA neurons (P_{dat-1}::GFP only). Notably, DA

neurodegeneration was not observed following knockdown of any of these 5 candidates when α-syn was absent, indicating that these modifiers are indeed specific to α-syn (Figure 23A, B and C).

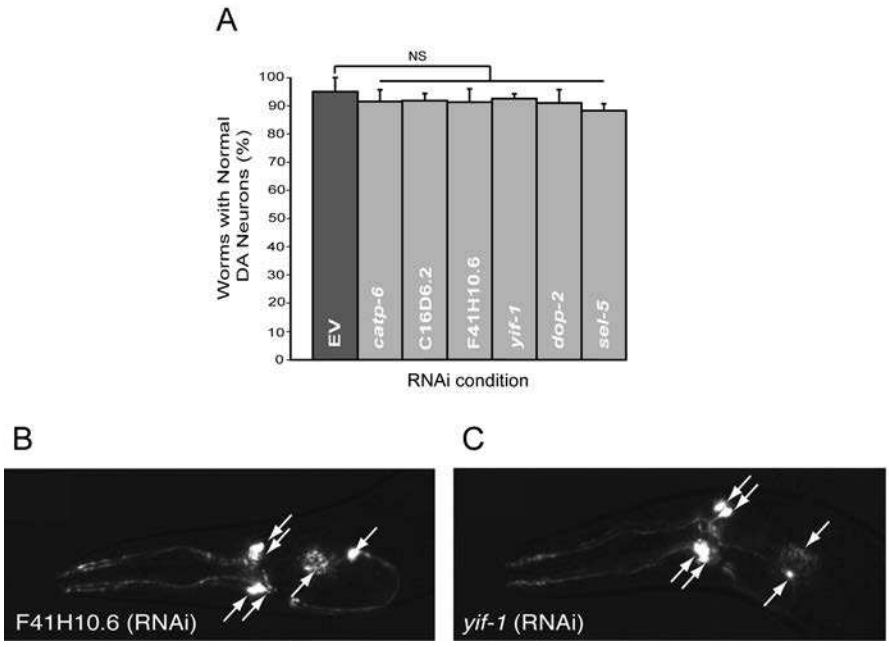


Figure 23. *catp-6* (ATP13A2) positive interactors are specific to a-syn mediated DA neurodegeneration
A) The 5 candidates that enhanced α-syn -induced neurodegeneration in *C. elegans* were examined in the absence of α-syn for effects on neurodegeneration (P_{dat-1}::GFP only). None of the five positive candidates caused significant degeneration when knocked down compared to control RNAi (EV). **B-C)** Representative images of the DA neurons following knockdown of F41H10.6 (HDAC6) and *yif-1*(YIF1A) in *C. elegans* in a background lacking α-syn (P_{dat-1}::GFP only). Arrows indicate the intact six anterior DA neurons. Values are expressed as mean ± standard deviation (SD) from 3 independent replicates.

The existence of ATP13A2 interactors that enhance α-syn aggregation and α-syn-induced neurodegeneration suggests an overlap of genetic networks between ATP13A2 and α-syn, further strengthening the connection between these two proteins. The modifiers of α-syn misfolding and neurotoxicity belong to groups responsible for ER and Golgi transport (YIF1A), clathrin mediated vesicular transport (AAK1), and lysosomal fusion and degradation of aggregated proteins (HDAC6) suggesting the importance of these processes in α-syn-mediated toxicity.

5. DISCUSSION

In this study we discovered that loss of ATP13A2 function leads to impaired autophagy-lysosomal degradation pathway. This lysosomal dysfunction results in preferential accumulation of α-syn and toxicity in neurons. Importantly, depletion of endogenous α-syn attenuated the toxicity in ATP13A2-deficient neurons, suggesting that loss of ATP13A2 mediates neurotoxicity at least partially via lysosomal dysfunction and consequent accumulation of α-syn. In addition, silencing of ATP13A2 in *C. elegans* model of Parkinson disease enhanced α-syn mediated degeneration of dopaminergic neurons, further underscoring the functional link between ATP13A2 and α-syn in neurodegeneration. Furthermore, in order to elucidate a normal biological function of ATP13A2 protein we identified its interacting partners that revealed putative role for ATP13A2 in vesicular trafficking and fusion. Importantly, a subset of these interactors was modifiers of α-syn aggregation and neurotoxicity in *C. elegans,* further supporting a functional link between ATP13A2 and α-syn.

5.1 ATP13A2 and lysosomal dysfunction

Mutations in ATP13A2 result in misfolding and destabilization of the protein in the endoplasmic reticulum that presumably results in deficiency of ATP13A2 in lysosomes, but the mechanisms through which mutations in ATP13A2 are mediating neurodegenerative processes remain elusive (Ramirez et al., 2006; Park et al., 2011; Tan et al., 2011; Ugolino et al., 2011). To model ATP13A2 loss of function, we used RNAi to silence the protein in mouse primary neurons and examined lysosomal function. We found that depletion of ATP13A2 in neurons resulted in accumulation of enlarged lysosomes. These results were consistent with data obtained in fibroblasts from patients with Kufor-Rakeb disease, suggesting that loss of ATP13A2 function accurately models disease pathogenesis. Importantly, restoration of ATP13A2 partially reversed these lysosomal alterations, further suggesting the importance of ATP13A2 function for lysosomal function. Increased lysosomal size and number could be the result of cellular compensation for insufficient degradation. In support of this we found lower rate of proteolysis of long-lived proteins and impaired degradation of EGFR. We also found decreased lysosomal turnover of autophagic vesicles lending further support to impaired lysosomal degradation capacity in the presence of silenced ATP13A2. In agreement with our data, ATP13A2 involvement in autophagy-lysosomal dysfunction has been independently demonstrated in a parallel study of another research group (Dehay et al., 2012).

Moreover, mutations in ATP13A2 identified to cause NLS, a lysosomal storage disorder, further suggested a connection between ATP13A2 and lysosomal dysfunction (Farias et al., 2011; Bras et al., 2012).

Precise mechanism through with ATP13A2 mutations affect lysosomal function remains obscure. It was hypothesized that ATP13A2 as an ion pump might be responsible for the transport of cofactors essential for the activity of lysosomal enzymes (Covy et al., 2012). Recent studies suggested that ATP13A2 is involved in maintenance of cation homeostasis (e.g. calcium and manganese) (Tan et al., 2011; Ramonet et al., 2012). These experiments were performed in non-controlled cell systems; therefore additional studies to identify ion substrates of the ATP13A2 should be done in *in vitro,* maybe with isolated protein and incorporated in membrane lipid layer.

Second non answered question is how lysosomal dysfunction due to deficiency in ATP13A2 leads to neurotoxicity. One possibility is that insufficient lysosomal degradation in ATP13A2-depleted cells causes protein accumulation and increase in numerous vesicles that could consequently cause molecular crowding of cytosolic space and effect major trafficking of autophagy-endocytosis-lysosomal pathway. Similar consequence has been observed in other neurodegenerative disorders with affected lysosomal function (Schultz et al., 2011). We provide a potential explanation for ATP13A2 mediated neurotoxicity thought the lysosomal dysfunction and consequent accumulation of α-syn. In addition, ATP13A2 interactors gave an insight into potential role for ATP13A2 in vesicular trafficking and fusion, which might be compromised due to ATP13A2 mutations and affect cellular homeostasis.

5.2 ATP13A2 network

Observed accumulation of immature autophagic vesicles and decreased number of mature one, suggested impaired fusion of lysosomes with autophagosomes in ATP13A2 depleted cells. Involvement of ATP13A2 in vesicular trafficking and fusion was further supported by ATP13A2 interacting proteins, newly identified with MYTH screen. For example, one of the identified interactors, the cytosolic chaperone heat shock 70kDa protein 8 (HSPA8), and its co-chaperone FK506 binding protein 8 (FKBP38) are implicated in ER to Golgi and plasma membrane trafficking (Walker et al., 2007). HSPA8 also functions as an ATPase in the uncoating of clathrin-coated vesicles during transport of membrane components through the cell (Schlossman et al., 1984). Interactors cyclin G-associated kinase (GAK/auxilin 2) and adaptor–associated

kinase 1 (AAK1) are also involved in clathrin dependent vesicular trafficking (Conner and Schmid, 2002; Eisenberg and Greene, 2007; Lee et al., 2008). Furthermore, vesicle-associated membrane protein 2 (VAMP2/ synaptobrevin 2) is a component of SNARE (soluble N-ethylmalemide-sensitive factor activating protein receptor) complexes that play a role in membrane fusion during synaptic vesicle exocytosis (Duman and Forte, 2003; Südhof and Rizo, 2011). SYT11 is a member of the synaptotagmin family of transmembrane proteins (SYTs) that functions in calcium-dependent vesicular trafficking/fusion and lysosomal exocytosis. Synaptotagmin binds to SNAREs in Ca^{2+}-dependent manner and triggers membrane fusion machinery (Bai et al., 2004; Sudhof, 2004; Südhof and Rizo, 2011). Interactor HDAC6 was found to be responsible for proper fusion of autophagosomes and lysosomes (Lee et al., 2010b). These interactors suggest that, in addition to its presumable function as ion pump (Kühlbrandt, 2004), ATP13A2 might be involved in membrane fusion. There are other examples of ATPases with this type of dual functionality such as V-ATPase that primarily functions as a proton pump and also interacts with VAMP2 that it is involved in SNARE dependent membrane fusion (El Far and Seagar, 2011). In another recent study, genome-wide screens were performed in yeast to define the cellular function of yeast homolog of ATP13A2 (Ypk9) and the mechanism by which Ypk9 protects cells from manganese toxicity (Chesi et al., 2012). This work suggested that Ypk9 plays a role in vesicle-mediate transport and vacuolar fusion and organization, further supporting the notion that ATP13A2 regulates vesicular trafficking.

Our newly-identified ATP13A2 protein partners and data obtained from lysosomal function studies in ATP13A2-depleted cells provide insights into putative role of ATP13A2 in vesicular trafficking/fusion as well in lysosomal degradation pathway. Even though the localization studies of ATP13A2 protein suggest its predominant function at the lysosomes (Ramirez et al., 2006; Park et al., 2011; Tan et al., 2011; Ugolino et al., 2011), our data show that it can also have roles in dynamic vesicles that interact with lysosomes during processes of fusion.

Identified ATP13A2 interactors provide a platform for further mechanistic studies of normal and mutant ATP13A2 in the context of neurodegeneration. In order to found out in which conditions and how ATP13A2 is involved in lysosomal pathway and vesicular trafficking/fusion requires additional studies of the nature of the interaction between ATP13A2 and its binding partners. Particularly it will be interested to see how disease-causing ATP13A2 mutations effect these

interactions. Since the mutant ATP13A2 is unstable and degraded fast, we assume that most of the interactions will be compromised thus affecting ATP13A2-involved pathways.

NIX protein (BNIP3L, BCL2/adenovirus E1B 19kDa interacting protein 3-like) emerges as an interesting interactor since it is a mitophagy receptor (Novak et al., 2010). Further, recent study demonstrated that loss-of-function mutations in ATP13A2 cause accumulation of fragmented mitochondria and increased ROS production (Grünewald et al., 2012; Gusdon et al., 2012). These data suggest that ATP13A2 is involved in mitochondrial clearance via lysosomes and how mutations in ATP13A2 might compromise the interaction between ATP13A2 and NIX leading to impaired mitophagy. This hypothesis requires additional studies; however together these findings implicate loss of ATP13A2 function in mitochondrial maintenance and oxidative stress, lending a support to converging lysosomal and mitochondrial pathways in KRS pathogenesis.

5.3 ATP13A2 and α-syn

Increasing evidence implicates lysosomal pathway in degradation of protein aggregates that represent pathological hallmark of neurodegenerative diseases. In PD and related synucleinopathies accumulation of α-syn protein in Lewy bodies (intracellular protein inclusions) plays a key role in disease pathogenesis (Spillantini et al., 1997). Interestingly, recent studies implicated ATP13A2 in α-syn misfolding and toxicity in yeast and *C. elegans*, but the underlying mechanism has not been established. We found that diminished lysosomal degradation in ATP13A2-depleted neurons resulted in preferential accumulation of endogenous α–syn, highlighting the importance of intact lysosomal function for turnover of α–syn. Furthermore, we found that depletion of endogenous α–syn diminishes the toxicity in ATP13A2-deficient neurons, suggesting that neurotoxicity due to loss of ATP13A2 function is at least partially mediated by lysosomal dysfunction and consequent accumulation of α–syn. In addition to this, we found that *C. elegans* depleted from ATP13A2 exhibited α–syn dependent neurodegeneration of dopaminergic neurons, further suggesting that ATP13A2 and α–syn have overlapping pathways in process of neurodegeneration.

Recent genetic and clinical studies demonstrated that subtle changes in levels of α–syn protein correlate with clinical presentation. For example multiplications of the wild type α-syn gene, in familial PD, result in increased expression of α–syn and its enhanced propensity to aggregate (Singleton et al., 2003; Chartier-Harlin et al., 2004). Over-expression of α–syn protein in various

animal models of PD results in accumulation and neurotoxicity of α–syn (Masliah et al., 2000; Auluck et al., 2002; Cao et al., 2005), while lowering the expression of α–syn in conditional mouse models leads to partial reversal of the observed pathological and behavioral phenotypes. Moreover, variations in α–syn locus that result in increased levels of α–syn are the major genetic risk factor for sporadic disease (Simón-Sánchez et al., 2009). Although these studies strongly suggest that α–syn accumulation plays a key role in the pathogenesis of synucleinopathies and highlight α–syn clearance as a key therapeutic target, the molecular mechanisms of α–syn accumulation and clearance have not been well understood.

Studies of lysosomal genes that are linked to neurodegeneration and α–syn have offered an opportunity to examine these pathways in more detail. Recent findings demonstrated that mutations in lysosomal enzyme glucocerebrosidase (GC) that cause Gaucher disease result in decreased lysosomal degradation capacity and accumulation of α–syn (Mazzulli et al., 2011). Deficiency of lysosomal GC results in accumulation of its lipid substrates that in turn interact with α–syn and enhance its aggregation (Mazzulli et al., 2011). While both ATP13A2 and GC loss-of-function mutations result in lysosomal dysfunction and accumulation of α–syn the mechanisms responsible for α–syn accumulation are likely distinct, since ATP13A2 presumably functions as an ion pump that regulates cation homeostasis (Tan et al., 2011; Ramonet et al., 2012). Therefore, the precise mechanism responsible for neurodegeneration in KRS remains unknown. However, our data suggest that lysosomal dysfunction and consequent α–syn accumulation play a key role in ATP13A2-mediated toxicity. This view is supported by our observation that ATP13A2-deficient mouse cortical primary neurons and *C. elegans* DA neurons require the presence of α–syn to exhibit maximal toxicity. The unique primary amino acid sequence of α–syn, containing a stretch of mostly hydrophobic residues between positions 71-82, renders it more likely to form amyloid fibrils and aggregates compared to most other cellular proteins (Chiti and Dobson, 2006). This property also precludes degradation of the misfolded conformer by the proteasome and could explain its preferential accumulation in neurons that exhibit lysosomal dysfunction.

Another characteristic of α–syn is that it forms oligomers upon oxidation that might be cytotoxic (Butterfield and Kanski, 2001; Glaser et al., 2005). Oxidative stress has been previously reported as one of the underlying mechanisms of neurodegeneration in PD (Henchcliffe and Beal, 2008; Zhou et al., 2008). Accumulated dysfunctional mitochondria represent the source of excessive

amount of ROS. Recent data suggested that decreased autophagy associated with ATP13A2 deficiency affects mitochondrial clearance and quality control, resulting in increased ROS production (Grünewald et al., 2012; Gusdon et al., 2012). These findings propose that preferential α–syn accumulation in ATP13A2-deficient cells might also be due to its oxidation in ROS-increased cell environment.

Recent data provided additional connection between ATP13A2 and α-syn showing that overexpression of yeast ATP13A2 (Ypk9) rescued α-syn-mediated toxicity and α-syn-induced blockade of ER-Golgi vesicular trafficking (Gitler et al., 2009a). These data, together with our newly identified interactors, suggested that ATP13A2 may play a role in vesicular trafficking and indicated the involvement of this pathway in α-syn-mediated toxicity. We extend these observations by demonstrating that a subset of interactors of ATP13A2 act as modifiers of α-syn aggregation and neurotoxicity in *C. elegans*. These interactors/modifiers further suggest a role for vesicular trafficking in α-syn-mediated toxicity. For example, AAK1 regulates clathrin-mediated endocytosis (Conner and Schmid, 2002), Yip1 interacting factor homolog A (YIF1A) cycles between ER and Golgi to maintain the structure of the Golgi apparatus (Yoshida et al., 2008), and its yeast homolog (Yip1p) is responsible for ER to Golgi transport and vesicular fusion (Matern et al., 2000; Barrowman et al., 2003). We also identified histone deacetylase 6 (HDAC6) as a modifier of α-syn misfolding and DA neurodegeneration in *C. elegans*. These results are consistent with previous studies in a *Drosophila* model of PD, in which depletion of HDAC6 resulted in accumulation of α-syn inclusions and loss of DA neurons (Du et al., 2010). Interestingly, HDAC6 has previously been implicated in the clearance of aggregation-prone proteins through autophagy, by recruiting and transporting aggresomes via the microtubule network and the dynein motor complex (Kawaguchi et al., 2003; Pandey et al., 2007). Taken together, the interaction of HDAC6 with ATP13A2 suggests the involvement of ATP13A2 in the lysosomal degradation pathway, and emphasizes the importance of protein clearance in mediating α-syn aggregation and toxicity.

Recently, transcription factor EB (TFEB), which regulates expression of majority of lysosomal proteins involved in lysosomal biogenesis and function, was discovered (Sardiello et al., 2009). Overexpression of TFEB restores disrupted lysosomal levels and attenuated cell death in cellular model of PD (Dehay et al., 2010). It also promotes autophagy-lysosomal pathway and clearance of accumulated Htt protein (Settembre et al., 2011). This discovery indicates the activation of

lysosomal system as a potential therapeutic mechanism in neurodegenerative disorders characterized with protein accumulations.

6. CONCLUSION

Our studies suggest that ATP13A2 plays a role in lysosomal function and vesicular trafficking/fusion, and that these processes can get disrupted due to ATP13A2 loss of function and contribute to the α-syn accumulation and α-syn-mediated neurodegeneration (Figure 24). These data implicate lysosomal dysfunction and α-syn accumulation in pathogenesis of KRS, and suggest that enhancing the lysosomal function may be neuroprotective for this syndrome. From a broader perspective these findings, together with other recent studies of lysosomal dysfunction in neurodegeneration, suggest that strategies to upregulate lysosomal function in neurons represent a promising therapeutic approach for neurodegenerative disorders.

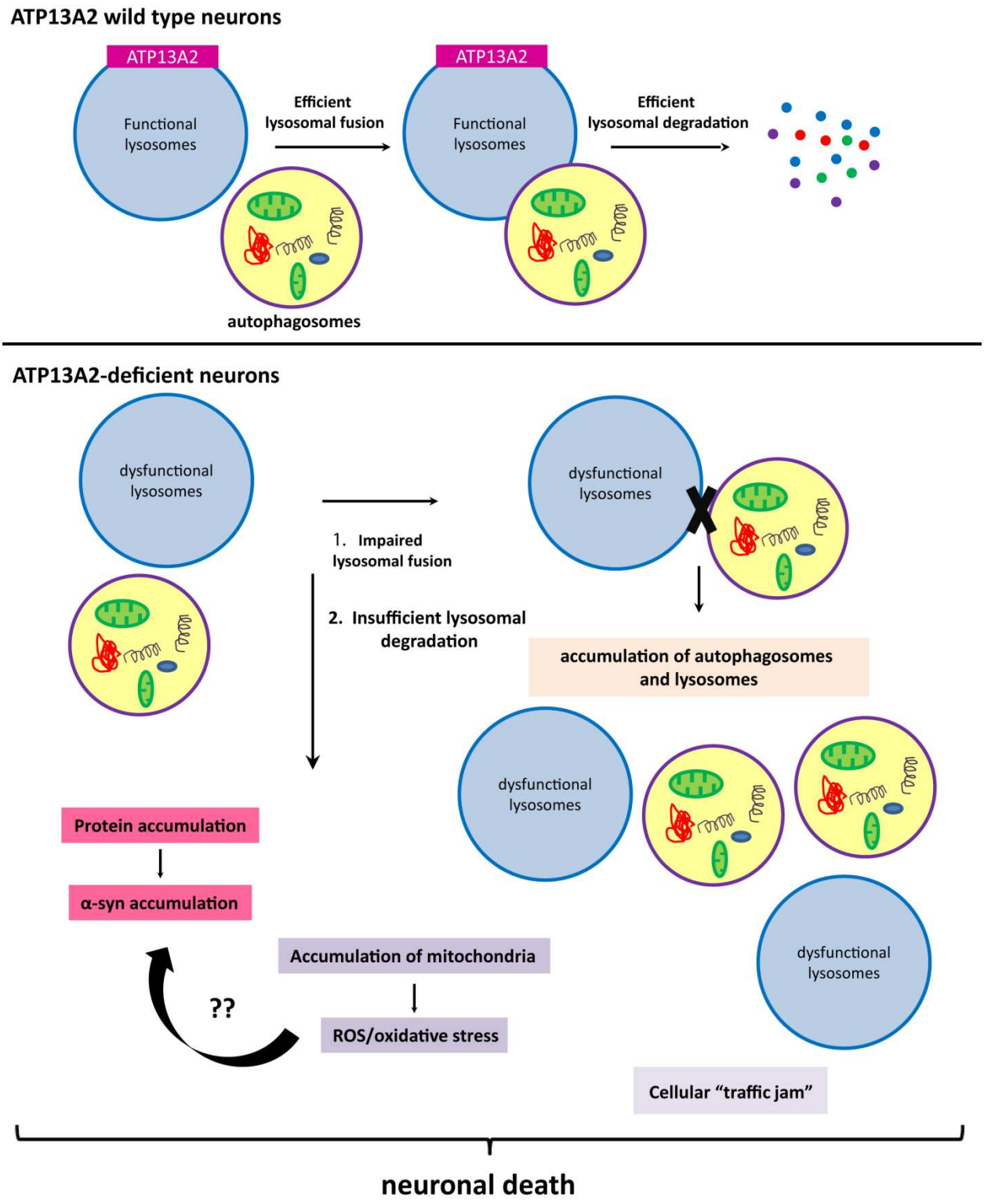


Figure 24. Pathogenic consequence of ATP13A2 deficiency
Loss of ATP13A2 function causes 1) impaired lysosomal fusion with autophagosomes and 2) insufficient lysosomal degradation. This lysosomal dysfunction results in accumulation of autophagosomes and lysosomes, and also in accumulation of α-syn and mitochondria. Increased amount of ROS previously identified in ATP13A2-deficient cells could promote preferential accumulation of α-syn. Together; these processes might disrupt homeostasis and cause neuronal death.

7. SUMMARY

Lysosomes are essential for maintaining neuronal homeostasis through degradation of damaged organelles and misfolded and accumulated proteins. Increasing evidence implicates lysosomal dysfunction in several neurodegenerative disorders, including Parkinson, Alzheimer and Huntington disease. In addition, more than two-thirds of lysosomal storage diseases are exhibiting dysfunction in the central nervous system, emphasizing how neurons are particularly vulnerable to lysosomal impairment. In order to more directly examine the lysosomal function in neurodegeneration it is particularly informative to study neurodegenerative diseases that are caused by mutations in lysosomal proteins. One such disease is Kufor-Rakeb syndrome that is caused by loss-of-function mutations in the lysosomal protein ATP13A2/PARK9 and characterized by early-onset parkinsonism with pyramidal degeneration and dementia. While previous data implicate ATP13A2 in α-synuclein misfolding and toxicity (a protein involved in pathogenesis of Parkinson disease and related synucleinopathies), the underlying mechanism and biological function of ATP13A2 have not been established. We found that loss of ATP13A2 function leads to accumulation of enlarged lysosomes, impaired lysosomal turnover of autophagic vesicles and impaired lysosomal degradation capacity. This lysosomal dysfunction results in preferential accumulation of α-synuclein and toxicity in neurons. Importantly, depletion of endogenous α-synuclein attenuated the toxicity in ATP13A2-deficient neurons, suggesting that loss of ATP13A2 mediates neurotoxicity at least partially via lysosomal dysfunction and consequent accumulation of α-synuclein. In addition, silencing of ATP13A2 in the *Caenorhabditis elegans* model of Parkinson disease has enhanced α-synuclein mediated degeneration of dopaminergic neurons, further underscoring the functional link between ATP13A2 and α-synuclein in neurodegeneration. Furthermore, in order to elucidate a normal biological function of ATP13A2 protein we identified its interacting partners that revealed putative role for ATP13A2 in vesicular trafficking and fusion. Importantly, a subset of these interactors was modifiers of α-synuclein aggregation and neurotoxicity in *Caenorhabditis elegans,* further supporting a functional link between ATP13A2 and α-synuclein.

Our findings implicate lysosomal dysfunction and α-synuclein accumulation in the pathogenesis of Kufor-Rakeb syndrome and suggest that upregulation of lysosomal function and downregulation of α-synuclein represent promising therapeutic strategies for this disorder.

8. SAŽETAK

Uloga proteina ATP13A2/PARK9 u lizosomskom putu razgradnje u neurodegeneraciji

Lizosomi su važni za održavanje neuronske homeostaze kroz razgradnju oštećenih organela i nakupljenih proteina. Brojni dokazi ukazuju na postojanje lizosomske disfunkcije u nekoliko neurodegenerativnih bolesti, uključujući Parkinsonovu, Alzheimerovu i Huntingtonovu bolest. Nadalje, više od dvije-trećine lizosomskih bolesti odlaganja okarakterizirane su poremećajima središnjeg živčanog sustava, što pokazuje da su neuroni posebno osjetljivi na lizosomske nepravilnosti. Kako bi smo što detaljnije ispitali lizosomsku funkciju u neurodegeneraciji vrlo je značajno istražiti neurodegenerativne bolesti uzrokovane mutacijama u lizosomskim proteinima. Jedna od tih bolesti je i Kufor-Rakeb sindrom koji je uzrokovan mutacijama u lizosomskom proteinu ATP13A2/PARK9 i okarakteriziran kao juvenilni parkinsonizam s piramidalnom degeneracijom i demencijom. Prijašnja otkrića sugeriraju da ATP13A2 ima važnu ulogu u nepravilnom smatanju i toksičnosti α-sinukleina (proteina uključenog u Parkinsonovu bolest i slične sinukleinopatije), no biološka funkcija proteina ATP13A2 kao ni mehanizam povezanosti proteina ATP13A2 i α-sinukleina još nisu poznati. Našim istraživanjem otkrili smo da gubitak funkcije ATP13A2 dovodi do nakupljanja povećanih lizosoma, poremećene lizosomske razgradnje proteina i autofagosoma. Ova lizosomska disfunkcija rezultira nakupljanje α-sinukleina i smrću neurona. Ujedno, utišavanje endogenog α-sinukleina smanjilo je toksičnost u neuronima s utišanim ATP13A2 genom, što sugerira da je neurotoksičnost uzrokovana gubitkom ATP13A2 jednim dijelom posredovana disfunkcijom lizosoma i akumulacijom α-sinukleina. Osim toga, utišavanje ATP13A2 u *Caenorhabditis elegans* modelu za Parkinsonovu bolest pojačalo je α-sinuklein-posredovanu degeneraciju dopaminskih neurona, što dodatno upućuje na funkcionalnu povezanost između ATP13A2 i α-sinukleina u procesu nerodegeneracije. Nadalje, kako bismo rasvijetlili znanje o normalnoj biološkoj funkciji ATP13A2 proteina, identificirali smo njegove inter-reagirajuće partnere koji su pokazali da ATP13A2 igra ulogu u procesima prijenosa i stapanja vezikula. Značajno je spomenuti i da su pojedini interaktori povećali nakupljanje i neurotoksičnost α-sinukleina u *Caenorhabditis elegans,* što je pružilo daljne dokaze o funkcionalnoj povezanosti ATP13A2 i α-sinukleina.

Naša otkrića impliciraju lizosomsku disfunkciju i nakupljanje α-sinukleina kao važne elemente u patogenezi Kufor-Rakeb sindroma te ukazuju na to da pojačavanje funkcije lizosoma i smanjivanje razine α-sinukleina predstavljaju obećavajuće terapijske strategije za ovu bolest.

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Zhou C, Huang Y, Przedborski S (2008) Oxidative stress in Parkinson's disease: a mechanism of pathogenic and therapeutic significance. Ann N Y Acad Sci 1147:93–104.

10. RESUME

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EDUCATION

October 2008-present

Ph.D. in Neuroscience, expected date: November 2012

Research and thesis advisor: Dimitri Krainc, M.D., Ph.D., Massachusetts General Hospital / Harvard Medical School.
Degree granting institution: University of Split, School of Medicine, Croatia
Thesis Title: "The role of PARK9 protein in lysosomal degradation pathway in neurodegeneration"

M.S. in Molecular Biology; 2007

University of Zagreb, Faculty of Natural Sciences, Croatia

WORK EXPERIENCE

October 2008-present

Visiting graduate student; Department of Neurology, Massachusetts General Hospital, Harvard Medical School, Boston, USA
Mentor: Dimitri Krainc, M.D., Ph.D., Professor of Neurology

Project: "Lysosomal degradation pathway in neurodegeneration; the role of PARK9"

May 2007-October 2008

Research assistant; Mediterranean Institute for Life Sciences, Split, Croatia
Advisors: Dimitri Krainc, M.D., Ph.D. and Janos Terzic M.D., Ph.D.

October 2006-January 2007

Undergraduate Research Internship; Department of Oncology and Surgical Science, University of Padua, Italy
Project: "Dissecting the role of L-arginine metabolism in tumor-induced immune suppression"
Principle investigator: Prof. Paola Zanovello, Ph.D.

January 2006-May 2006

Undergraduate Research Internship; Croatian Institute for Brain Research, Zagreb, Croatia
Project: "Genetical and Epidemiological Research of Muscular Dystrophies in Croatia"
Principle Investigator: Nina Canki-Klain M.D., Ph.D.

SKILLS AND EXPERTISE

MOLECULAR BIOLOGY: DNA cloning, site-directed mutagenesis, PCR, DNA and RNA extraction, quantitative PCR, gel electrophoresis, Western blotting, microarray analysis-DNA chips, shRNA gene silencing, lentivirus generation

PROTEIN BIOCHEMISTRY: denaturing protein gel electrophoresis, subcellular fractionation, degradation assays (long-lived protein degradation assay, pulse-chase experiment), cytotoxicity assays (neurofilament staining, LDH activity), ELISA, flow cytometry, protein interaction assays: immunoprecipitation and membrane yeast two-hybrid method

IMAGING: confocal microscopy, live cell imaging, immunocytochemistry, immunohistochemistry

CELLULAR BIOLOGY: immortalized cell cultures, primary cultures of cortical neurons, primary cultures of dermal fibroblasts, cell transfections, electroporation

STEM CELLS: generation of midbrain dopaminergic neurons from induced pluripotent stem (iPS) cells

FELLOWSHIPS AND AWARDS

April 2012	"Poster of Distinction" Award at MGH Research Day Symposium
October 2008-January 2009	European Molecular Biology Organization (EMBO) Fellowship for International Research Exchange
May 2007-May 2011	Scholarship from City of Split, Croatia
September 2006-January 2007	Student Exchange Fellowship from University of Padua, Italy
October 2001-March 2006	Croatian Ministry of Science, Education and Sports Scholarship

PROFESSIONAL MEMBERSHIPS

2011-present Member of Society for Neuroscience (SfN)

2008-present Member of Croatian Society of Biochemistry and Molecular Biology

LANGUAGE SKILLS

Fluent in English, Croatian, and Italian. Basic knowledge of French.

PUBLICATIONS

Usenovic, M., Knigh, A.L., Ray, A., Wong, V., Brown, K.R., Caldwell, G.A., Caldwell, K.A., Stagljar, I. & Krainc, D. Identification of novel ATP13A2 interactors and their role in α-synuclein misfolding and toxicity. *Hum. Mol. Genet. (*Advance online publication, May 2012, 10.1093/hmg/dds206, PMID: 22645275).

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Conference abstracts

Usenovic, M., Tresse, E., Mazzulli, J. R., Taylor, J. P. & Krainc, D. Lysosomal dysfunction in neurodegeneration: the role of PARK9. *Gordon Research Conference, Lysosomes and Endocytosis, June 2012.*

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Usenovic, M., Knigh, A.L., Ray, A., Wong, V., Brown, K.R., Caldwell, G.A., Caldwell, K.A., Stagljar, I., Krainc, D. Identification of novel ATP13A2 interactors and their role in α-synuclein misfolding and toxicity. *Society for Neuroscience, Z19. 885.10. October 2011.*