Loss of P-type ATPase ATP13A2/PARK9 function induces general lysosomal deficiency and leads to Parkinson disease neurodegeneration

Benjamin Dehay[a,1], Alfredo Ramirez[b], Marta Martinez-Vicente[c], Celine Perier[c], Marie-Hélène Canron[a], Evelyne Doudnikoff[a], Anne Vital[a], Miquel Vila[c,d,e], Christine Klein[b], and Erwan Bezard[a]

[a]Insitute of Neurodegenerative Diseases, University of Bordeaux Segalen, Centre National de la Recherche Scientifique Unité Mixte de Recherche 5293, 33076 Bordeaux, France; [b]Section of Clinical and Molecular Neurogenetics, Department of Neurology, University of Lubeck, 23538 Lubeck, Germany; [c]Neurodegenerative Diseases Research Group, Vall d'Hebron Research Institute, Centro Investigación Biomédica en Red Enfermedades Neurodegenerativas, 08035 Barcelona, Spain; [d]Department of Biochemistry and Molecular Biology, Autonomous University of Barcelona, 08193 Bellaterra, Barcelona, Spain; and [e]Catalan Institution for Research and Advanced Studies, 08010 Barcelona, Spain

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Parkinson disease (PD) is a progressive neurodegenerative disorder pathologically characterized by the loss of dopaminergic neurons from the substantia nigra pars compacta and the presence, in affected brain regions, of protein inclusions named Lewy bodies (LBs). The *ATP13A2* gene (locus PARK9) encodes the protein ATP13A2, a lysosomal type 5 P-type ATPase that is linked to autosomal recessive familial parkinsonism. The physiological function of ATP13A2, and hence its role in PD, remains to be elucidated. Here, we show that PD-linked mutations in ATP13A2 lead to several lysosomal alterations in ATP13A2 PD patient-derived fibroblasts, including impaired lysosomal acidification, decreased proteolytic processing of lysosomal enzymes, reduced degradation of lysosomal substrates, and diminished lysosomal-mediated clearance of autophagosomes. Similar alterations are observed in stable ATP13A2-knockdown dopaminergic cell lines, which are associated with cell death. Restoration of ATP13A2 levels in ATP13A2-mutant/depleted cells restores lysosomal function and attenuates cell death. Relevant to PD, ATP13A2 levels are decreased in dopaminergic nigral neurons from patients with PD, in which ATP13A2 mostly accumulates within Lewy bodies. Our results unravel an instrumental role of ATP13A2 deficiency on lysosomal function and cell viability and demonstrate the feasibility and therapeutic potential of modulating ATP13A2 levels in the context of PD.

autophagy | lysosome | neurodegeneration

Parkinson disease (PD) is characterized by extensive cell loss in the substantia nigra pars compacta (SNpc) in conjunction with the formation of intraneuronal proteinaceous cytoplasmic inclusions, named Lewy bodies (LBs) (1). Genetic studies have enabled the identification of 18 gene loci, named *PARK1–18*, that result in autosomally dominant or recessive inherited forms of PD or are associated with an increased risk for developing PD (2). Among these, the *ATP13A2* gene (*PARK9*) has been linked to autosomal recessive, levodopa-responsive, nigrostriatal-pallidal-pyramidal neurodegeneration (Kufor–Rakeb syndrome) as well as to some juvenile and young-onset forms of PD (3–7). The *PARK9* gene encodes the protein ATP13A2, a transmembrane lysosomal type 5 P-type ATPase protein consisting of 1,180 amino acid residues (4).

Both the cellular function of human ATP13A2 and its role in PD are yet to be elucidated. Genetic studies in yeast suggest that ATP13A2 yeast ortholog is involved in protecting cells against divalent heavy metal cations (8). ATP13A2 has been suggested to protect against α-synuclein misfolding and toxicity in *Caenorhabditis elegans* and in primary dopaminergic cell cultures (9), suggesting a link between ATP13A2 and α-synuclein pathways. Missense or truncation mutations in ATP13A2 are pathogenic by causing loss of function. For example, cells expressing *ATP13A2* mutations exhibit retention of ATP13A2 in the endoplasmic reticulum (ER) and predispose cells to ER stress-induced cell death followed by degradation by means of the ER-associated degradation-proteasomal pathway (6, 10). Putative lysosomal dysfunction through loss of ATP13A2 function might lead to insufficient lysosomal protein degradation. Impairment of lysosomal function is a subject of growing interest in PD research and is now described as a major contributor to the pathogenesis of this disease (11, 12).

ATP13A2 may represent the first genetic link between lysosomal impairment and PD (13, 14). To this end, we investigated the function of ATP13A2 on lysosomal-autophagy pathways under physiological and pathological conditions in both neuronal cells and PD patient-derived fibroblasts. Our results unravel a pivotal role of ATP13A2 in lysosomal function, which may provide crucial insights into the pathogenic mechanisms of PD.

Results

Lysosomal Alterations in Mutant ATP13A2 Fibroblasts. To determine whether mutations/defects in ATP13A2 may underlie PD-linked lysosomal alterations, we first explored potential defects in the autophagy-lysosomal pathway in cultured fibroblasts derived from patients with PD harboring *ATP13A2* mutations. Fibroblasts from two patients with PD with the L3292 and L6025 *ATP13A2* mutations, respectively, and control fibroblasts from our fibroblast library (4) were used for these studies. Application of the lysosomotropic fluorochrome LysoTracker (Molecular Probes) to ATP13A2 mutant fibroblasts revealed a dramatic increase in the number of lysosomes associated with a reduced intensity of the emitted fluorescence (Fig. 1*A*). Similar results were obtained in these cells by immunostaining for lysosomal proteins cathepsin B (cath-B), cathepsin D (cath-D), and lysosomal-associated membrane protein (LAMP)-2 (Fig. 1*A*). EM examination revealed the presence of abnormally high baseline levels of autophagic vacuoles (AVs) in the cytosol of mutant ATP13A2 fibroblasts, compared with control cells, as identified on the basis of their size and morphology (Fig. 1*B*). In particular, early and late autolysosomes (ALs) containing intact or partly degraded cytoplasmic material were more numerous in ATP13A2 mutant cells compared with control cells under basal conditions (Fig. 1*C*). The presence of abundantly accumulated ALs containing undigested cytosolic material in ATP13A2 mutant cells was further confirmed by immunofluorescence and immunogold-EM analyses using antibodies against LAMP-1, which allowed the identification of lysosome-related vesicles (Fig. 1*D*). Further supporting an accumulation of lysosomal-related vesicles in ATP13A2 mutant fibroblasts, the levels of two lysosomal structural membrane proteins, LAMP-1 and LAMP-2, were markedly increased in these

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[1]To whom correspondence should be addressed. E-mail: benjamin.dehay@u-bordeaux2.fr.

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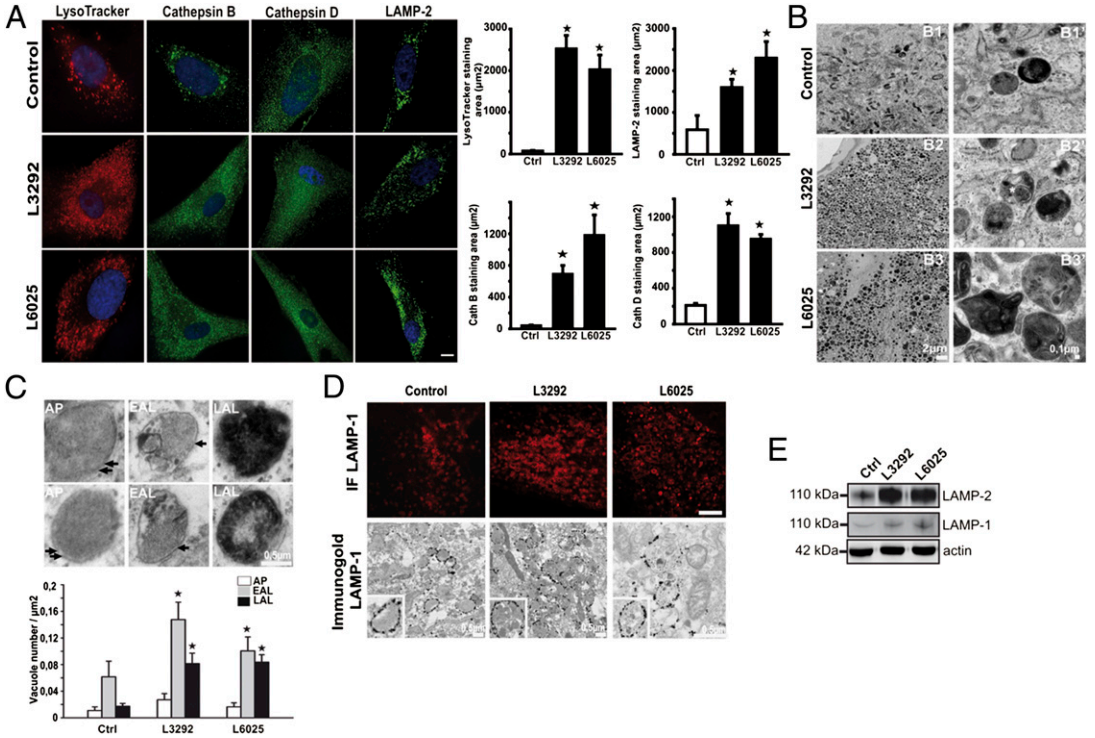

Fig. 1. Accumulation of lysosomal-related structures in ATP13A2 mutant fibroblasts. (*A*) Accumulation of lysosomes and/or ALs in mutant ATP13A2 fibroblasts (L3292 and L6025) under basal conditions, as evidenced by lysosomal marker LysoTracker staining (red; nucleus in blue) and immunostaining for cath-B, cath-D, and LAMP-2 (green; nucleus in blue). *$P < 0.05$ compared with control fibroblasts. (Scale bars, 10 μm.) (*B*) Ultrastructural examination of mutant ATP13A2 fibroblasts by EM. Note the accumulation of AVs/ALs in mutant ATP13A2 fibroblasts (B2, B3) compared with control fibroblasts (B1). B1′–B3′ correspond to high-magnification images. (*C*) AVs were subcategorized based on their morphology. AP, autophagosome-double membrane; EAL, early AL-single membrane with relatively undigested material; LAL, late AL-single membrane with amorphous electron-dense material. Double arrows represent double membranes, and single arrows represent single membranes. (Scale bars, 500 nm.) *$P < 0.05$ compared with control fibroblasts. (*D*) (*Upper*) Immunofluorescent (IF) labeling for LAMP-1 in control and mutant ATP13A2 fibroblasts. (Scale bars, 1 μm.) (*Lower*) EM-immunogold labeling for LAMP-1 in control and mutant ATP13A2 fibroblasts. (*Insets*) Higher magnification images of single vesicles. (Scale bars, 500 nm.) (*E*) Representative immunoblot levels of LAMP-2 and LAMP-1 in mutant ATP13A2 and control (Ctrl) fibroblast under basal conditions.

cells (Fig. 1*E*). Taken together, these data indicate that lysosomal-mediated clearance of AVs is impaired in ATP13A2 mutant fibroblasts.

To determine the potential mechanisms underlying impaired lysosomal-mediated degradation in ATP13A2 mutant fibroblasts, we next analyzed cath-D maturation in these cells. Procath-D (52 kDa) is slowly processed into a 44-kDa form and finally into a 32-kDa mature form in the acidic milieu of late endosomes and lysosomes. In control fibroblasts, procath-D 52-kDa and 44-kDa bands were barely visible by immunoblot (Fig. 2*A*). In mutant ATP13A2 fibroblasts, however, the total amount of cath-D was increased and the relative amounts of immature and mature cath-D forms were changed, with the 52-kDa and 44-kDa forms being significantly more abundant than the 32-kDa mature form (Fig. 2*A*). The maturation process of cath-B was also similarly impaired in mutant ATP13A2 fibroblasts (Fig. 2*B*). In vitro assays of cath-D activity in lysosomal fractions of mutant ATP13A2 fibroblasts confirmed a markedly reduced proteolytic activity of this lysosomal enzyme relative to control fibroblasts (Fig. 2*C*). In addition, measurement of average lysosomal pH with LysoSensor Yellow/Blue DND-160 (Invitrogen)-dextran unraveled that lysosomal pH acidification was compromised in mutant ATP13A2 fibroblasts (Fig. 2*D*). Overall, these data indicate that loss-of-function mutations of ATP13A2 impair proper lysosomal acidification, cathepsin activity, and proteolytic capacity.

We next attempted to examine the extent of impairment of proteolytic activity by studying the rate of intracellular protein degradation. We found that both control and mutant ATP13A2 fibroblasts were able to increase total rates of degradation of long-lived proteins under basal conditions in a similar manner (Fig. 2*E* and Fig. S1*A*). However, when the percentage of protein degradation occurring within lysosomes [sensitive to inhibition of lysosomal proteolysis by ammonium chloride (NH_4Cl) and leupeptin] was assessed, the mutant L6025 cell line presented 90% lower lysosomal activity (Fig. 2*F*). Overexpression of WT ATP13A2 completely restored lysosomal proteolytic deficiency in mutant L6025 cells (Fig. 2*F*). These results indicate the occurrence of a dramatic impairment of lysosomal proteolytic activity in the homozygous mutant L6025 cell line that may be compensated for by other nonlysosomal forms of protein degradation. In contrast to homozygous mutant L6025 fibroblasts, compound heterozygous fibroblasts (L3292) showed a normal rate of lysosomal proteolysis (Fig. S1*B*), which might suggest that the effect of the L3292 mutation on lysosomal proteolytic capacity is not as drastic as that seen in the L6025 mutant cells.

To corroborate the occurrence of impaired lysosomal-mediated degradation in PD-derived ATP13A2 fibroblasts further, we then assessed whether the accumulation of AVs observed in these cells was secondary to increased AV formation or to defective lysosomal-mediated clearance. To do so, we blocked the fusion of AVs with lysosomes in these cells with the lysosomal H^+-ATPase inhibitor bafilomycin A_1 (15). We first confirmed by immunoblot that ATP13A2 mutant fibroblasts exhibit an accumulation of AVs at basal conditions, compared with control cells, as indicated by increased levels of the AV marker LC3-II in these cells (Fig. 2 *G* and *H*). Following bafilomycin A_1 treatment, ATP13A2 mutant fibroblasts did not exhibit further increases in LC3-II levels, in contrast to control cells (Fig. 2 *G* and *H*), thereby indicating that the accumulation of AVs observed at basal conditions in ATP13A2 mutant cells is secondary to

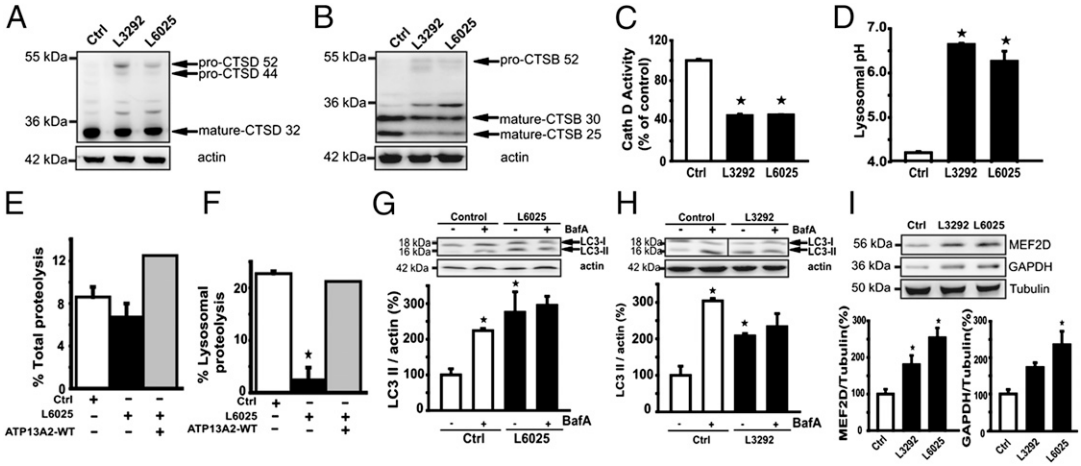

Fig. 2. Impaired lysosomal degradation in ATP13A2 mutant fibroblasts. (*A* and *B*) Cath-D (CTSD) and Cath-B (CTSB) immunoblot levels in mutant ATP13A2 and control (Ctrl) fibroblasts. (*C*) In vitro assay of cath-D enzyme activity in lysosomal fractions of control and mutant ATP13A2 fibroblasts. (*D*) Lysosomal pH values as measured ratiometrically using Lysosensor Yellow/Blue DND-160 in control and mutant ATP13A2 fibroblasts. (*E* and *F*) Degradation of long-lived proteins in control, mutant L6025, and WT ATP13A2-transfected L6025 fibroblasts. After incorporation of [^{3}H]-valine, cells were incubated in serum-supplemented or -deprived medium with or without lysosomal inhibitors (20 mM NH_4Cl, 100 μM leupeptin) during the chase period (up to 24 h). (*F*) Lysosomal degradation calculated as degradation sensitive to NH_4Cl/leupeptin. LC3 immunoblot levels in mutant ATP13A2 L6025 (*G*) and L3292 (*H*) fibroblasts were treated or not treated with 5 nM bafilomycin A_1 for 1 h. (*I*) MEF2D and GAPDH immunoblot levels in mutant ATP13A2 and control fibroblasts. In all panels, n = 3–5 per experimental group. *$P < 0.05$ compared with control untreated cells.

impaired autophagic flux. Further supporting the occurrence of impaired lysosomal-mediated degradation in ATP13A2 mutant fibroblasts, these cells also exhibited an accumulation of undegraded chaperone-mediated autophagy (CMA) substrates (Fig. 2*I*). Taken together, these results indicate that although the delivery of substrates to lysosomes by either macroautophagy or CMA translocation may not be affected in ATP13A2 mutant cells, the degradation process inside the lysosome is impaired.

Finally, we explored whether lysosomal integrity or stability was affected in ATP13A2 mutant cells by assessing their sensitivity to lysosomal membrane permeabilization (LMP) (16). These experiments were performed by measuring the ectopic activities of two lysosomal enzymes, β-hexosaminidase and acid phosphatase, in cytosolic lysosomal-free fractions from mutant ATP13A2 fibroblasts exposed to various lysosomal stress drugs (Fig. S2). Compared with control fibroblasts, ATP13A2 mutant fibroblasts displayed an increased susceptibility to LMP induced by either NH_4Cl or chloroquine (CQ), two inhibitors of intralysosomal catabolism (Fig. S2).

Overall, the results obtained with mutant ATP13A2 PD patient-derived fibroblasts indicate that loss-of-function mutations in ATP13A2 lead to several lysosomal alterations, including impaired lysosomal acidification, decreased proteolytic processing of lysosomal enzymes, reduced degradation of lysosomal substrates, and diminished lysosomal-mediated clearance of AVs.

Stable ATP13A2 Knockdown in Dopaminergic Cell Lines Recapitulates Phenotypes Observed in Fibroblasts Derived from Patients with PD. The effects of a loss of function of ATP13A2 on the lysosomal-autophagy system were further explored using human dopaminergic BE-M17 neuroblastoma cells. First, we performed experiments to identify shRNA sequences against human ATP13A2 that provide maximum silencing efficiency in BE-M17 cells (Fig. S3*A*). Using sh403 as the most efficient sequence, we then generated a panel of BE-M17 clonal cell lines that stably exhibited decreased expression of endogenous ATP13A2 (Fig. S3*B*). From there, we selected for subsequent experiments the ATP13A2 shRNA clone 1, which displayed a 95% reduction in ATP13A2 levels (Fig. S3*B*).

Lysosomal staining revealed that control shScr-1 cells exhibited a normal lysosomal profile, whereas ATP13A2-knockdown sh403-1 cells showed a dramatic increase in the number of lysosome-related vesicles (Fig. 3*A*). Similarly, LAMP-1 and LAMP-2 immunoblot levels were increased in ATP13A2-knockdown cells (Fig. 3*B*). Furthermore, ATP13A2-knockdown cells also exhibited an impaired cathepsin maturation process (Fig. 3 *C–E*) and a higher lysosomal pH (Fig. 3*F*), compared with control shScr-1 cells. Although both control and ATP13A2-knockdown cell lines showed a similar percentage of total rates of protein degradation under basal conditions (Fig. 3*G*), lysosomal-mediated protein degradation was decreased by 80% in ATP13A2-knockdown cells (Fig. 3*H*). Restoration of ATP13A2 levels in ATP13A2-knockdown cells by overexpression of WT ATP13A2 rescued lysosomal-mediated proteolytic activity (Fig. 3*H*). Comparable to ATP13A2 mutant fibroblasts, ATP13A2-knockdown cells displayed decreased lysosomal-mediated clearance of AVs, because LC3-II immunoblot levels were increased in sh403-1 cells at basal levels and did not further change in the presence of bafilomycin A_1 (Fig. 3*I*). Further supporting the occurrence of impaired lysosomal-mediated degradation in ATP13A2-knockdown cells, these cells exhibited a marked accumulation of undegraded macroautophagy and CMA substrates, such as increases in p62, Myocyte enhancer factor 2 D (MEF2D), GAPDH, and α-synuclein (Fig. 3*J*). Of note, there were no changes in the expression of beclin-1, a positive regulator of autophagy, in these cells (Fig. S4*A*). ATP13A2-knockdown cells also displayed an increased susceptibility to LMP after lysosomal stress caused by NH_4Cl and CQ (Fig. S4 *B* and *C*), which resulted in a further accumulation of undegraded AVs (Fig. S4*D*). All these lysosomal alterations were also confirmed in an additional ATP13A2 shRNA clone (Fig. S5).

Our results in BE-M17 cells indicate that knockdown of endogenous ATP13A2 in dopaminergic cell lines results in several lysosomal alterations, as in the mutant ATP13A2 fibroblasts derived from patients with PD. Moreover, in BE-M17 cells, lysosomal alterations were associated with cell death (Figs. S4*E* and S5*F*). Further confirming a deleterious effect of ATP13A2 loss of function on cell viability, lentiviral vector-mediated ATP13A2 knockdown in primary mesencephalic dopaminergic neurons also resulted in neurodegeneration (Fig. S6 *A–D*). In addition, cell death induced by ATP13A2-knockdown was greatly enhanced by (*i*) additional lysosomal stress caused by NH_4Cl and CQ (Figs. S4*E* and S5*F*) and (*ii*) overexpression of α-synuclein (Fig. S6*E*). Altogether, these data highlight the striking sensitivity of dopaminergic cells to loss of ATP13A2 function, either on its own or in combination with toxic or genetic stressors.

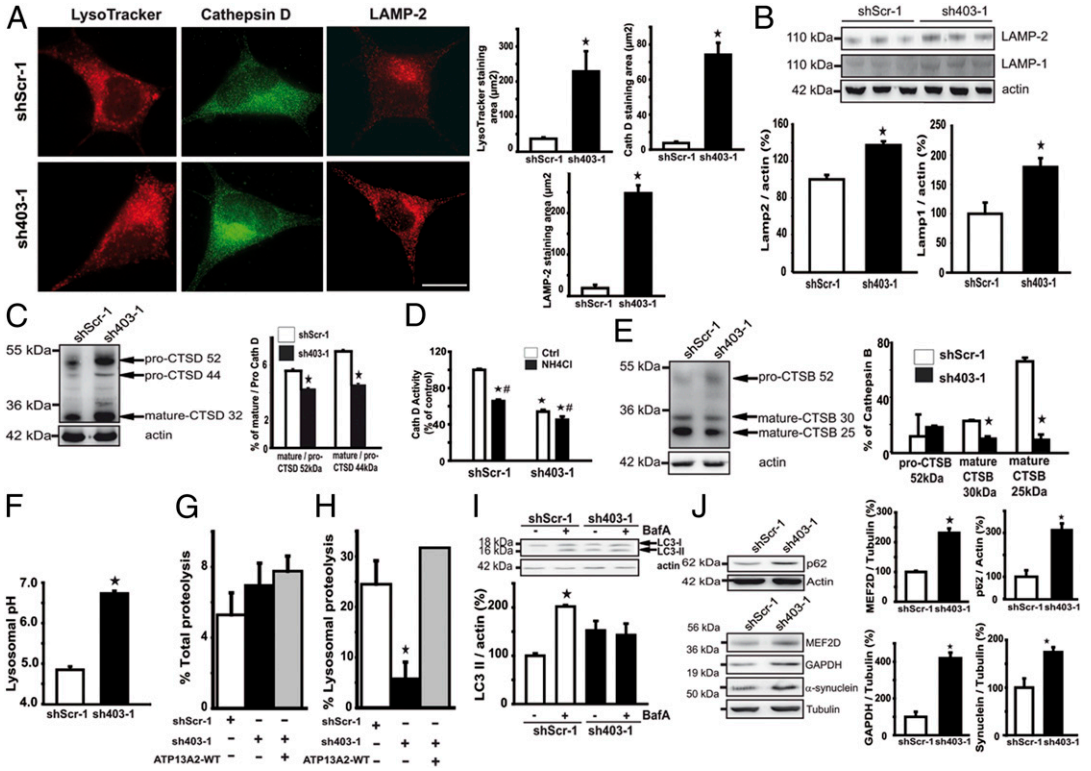

Fig. 3. Characterization of ATP13A2-knockdown cell lines. (*A*) Accumulation of lysosomes and/or ALs in M17 cells with stable knockdown of ATP13A2 (sh403-1) under basal conditions, as evidenced by LysoTracker staining and immunohistochemistry for cath-D and LAMP-2. (Scale bars, 10 μm.) (*B* and *C*) Representative immunoblot levels of LAMP-2, LAMP-1, and cath-D in sh403-1 and control (Ctrl) cells (shScr-1). (*D*) In vitro assay of cath-D enzyme activity in lysosomal fractions of sh403-1 and shScr-1 cells. (*E*) Cath-B (CTSB) immunoblot levels in sh403-1 and shScr-1 cells. (*F*) Lysosomal pH values in sh403-1 and shScr-1 cell lines. (*G* and *H*) Degradation of long-lived proteins in shScr-1 and sh403-1 cells and in lentiviral (LV)-transduced sh403-1 cells. (*H*) Lysosomal degradation (i.e., sensitive to NH_4Cl/leupeptin) in shScr-1 and sh403-1 cells and LV-transduced sh403-1 cells. (*I*) LC3 immunoblot levels in M17 cells treated or not treated with 5 nM bafilomycin A_1 for 1 h. (*J*) Immunoblot levels of p62, MEF2D, GAPDH, and α-synuclein in control and ATP13A2-knockdown cells. In all panels, $n = 3–5$ per experimental group. $^*P < 0.05$ compared with shScr-1.

ATP13A2 Restores Lysosomal Function and Attenuates Cell Death in ATP13A2-Deficient Cells. Because loss of ATP13A2 function is associated with lysosomal deficiency and cell death, we next explored whether restoration of ATP13A2 levels could rescue some of the pathogenic changes associated with ATP13A2 deficiency. Already confirming a pathogenic role for ATP13A2 defects, overexpression of WT ATP13A2 is indeed able to rescue impaired proteolytic activity in both L6025 PD-derived mutant fibroblasts (Fig. 2*F*) and ATP13A2-knockdown dopaminergic cell lines (Fig. 3*H*). Here, we determined whether restoration of the WT ATP13A2 form would rescue cathepsin activity, attenuate LMP, and prevent cell loss. Overexpression of ATP13A2-GFP in ATP13A2-knockdown stable cell lines increased ATP13A2 levels up to 35% of those of control cells (Fig. 4*A*). Overexpressed ATP13A2-GFP was appropriately localized in lysosomes, as shown by colocalization studies with LysoTracker (Fig. 4*B*). ATP13A2 overexpression was able to restore impaired cath-D activity and attenuate drug-induced LMP in both ATP13A2-knockdown cells and ATP13A2 mutant fibroblasts (Fig. 4 *C–H* and Fig. S5*G*). In ATP13A2-knockdown cells, restoration of ATP13A2 levels resulted in an attenuation of cell death (Fig. 4*I*). These results confirm an instrumental role of ATP13A2 deficiency on lysosomal function and cell viability. They also demonstrate the feasibility of restoring ATP13A2 levels in vitro, and hence the therapeutic potential of ATP13A2.

ATP13A2 Is a Constituent of LBs in the SNpc of Patients with PD. After having experimentally demonstrated a pathogenic role for ATP13A2 deficiency in vitro, we next determined whether defects in ATP13A2 may occur in patients with PD. Supporting the latter hypothesis, postmortem nigral tissue samples from sporadic patients with PD exhibited marked decreases in ATP13A2 protein levels by immunoblot, compared with control subjects (Fig. S8*A*). Immunohistochemical examination of the SNpc of patients with PD indicated that reductions in ATP13A2 occurred in the cytosol of melanized dopaminergic neurons (Fig. S8 *B* and *C*). Quantification of the ATP13A2 immunofluorescent signal in postmortem PD samples confirmed the reduction in ATP13A2 levels within dopaminergic SNpc neurons (Fig. S8*C*). For both immunoblot and immunohistochemical analyses in human tissue, the specificity of the ATP13A2 antibody was validated by immunizing peptide adsorption experiments (Fig. S7 *A–D*). Remarkably, immunohistochemical studies identified ATP13A2 as a component of LBs (Fig. S8 *D–I*). In the SNpc of patients with PD, more than 90% of LBs were strongly immunoreactive for ATP13A2, especially in their core, whereas the localization of α-synuclein was peripheral to that of ATP13A2 (Fig. S8 *D–I*).

The postmortem PD data indicate that the overall levels of ATP13A2 are decreased in SNpc dopaminergic neurons from patients with PD, where ATP13A2 appears to be mostly trapped within LBs. These observations support the relevance of our experimental results to PD and indicate that ATP13A2 defects contribute to the lysosomal alterations occurring in this disease.

Discussion

Here, we show that PD-linked mutations/defects in ATP13A2 lead to major lysosomal alterations in both mutant ATP13A2 fibroblasts from patients with PD and ATP13A2-knockdown dopaminergic cells, with the latter being associated with cell death. Restoration of ATP13A2 levels in ATP13A2-mutant/

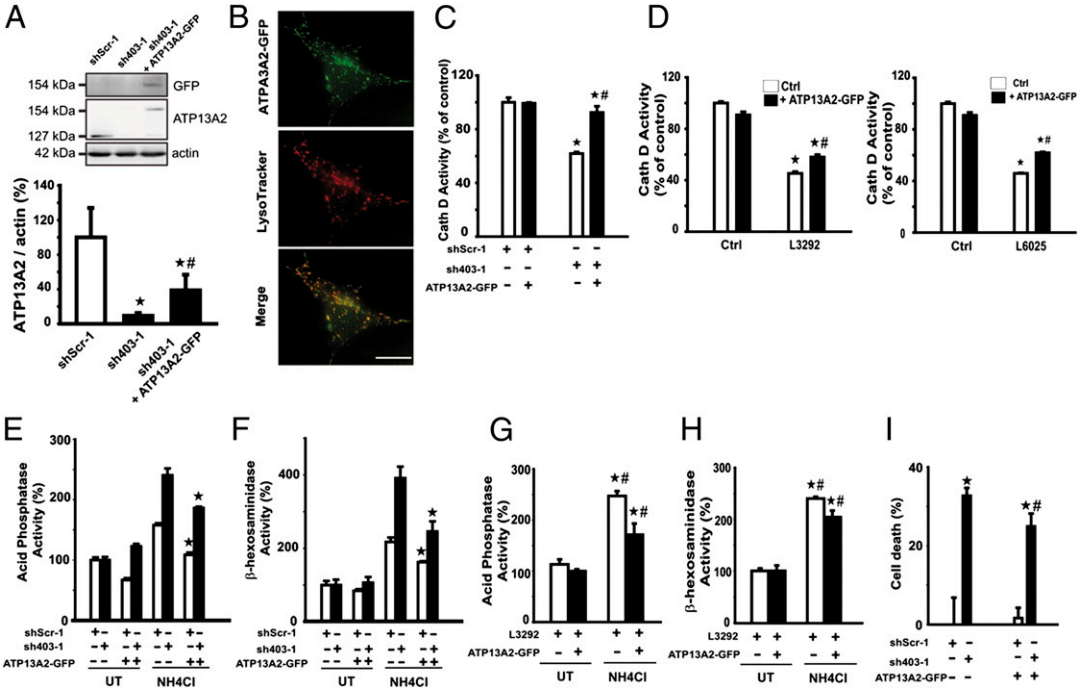

Fig. 4. Overexpression of WT ATP13A2 in ATP13A2-deficient cells rescues lysosomal deficiency. (*A*) Overexpression of ATP13A2 assessed by Western blot in stable cell lines. (*B*) Colocalization of LysoTracker staining and GFP immunofluorescence in sh403-1 cells transfected with ATP13A2-GFP. (Scale bars, 10 μm.) (*C* and *D*) Cath-D activity in lysosomal fractions from sh403-1 cells and mutant ATP13A2 fibroblasts overexpressing or not overexpressing WT ATP13A2. Effects of ATP13A2 overexpression on lysosomal stress susceptibility to lysosomal inhibitor NH_4Cl (40 mM for 24 h) in sh403-1 cells and mutant ATP13A2 fibroblasts (L3292), as evidenced by the presence of acid phosphatase (*E* and *G*) and β-hexosaminidase (*F* and *H*) activities in the cytosol of NH_4Cl-treated cells after removing lysosomes by differential centrifugation. (*I*) Cell death in sh403-1 cells overexpressing or not overexpressing ATP13A2. In all panels, $n = 3–5$ per experimental group. $^{*}P < 0.05$ compared with control (Ctrl) untreated (UT) cells; $^{\#}P < 0.05$ compared with control treated cells.

depleted cells restores lysosomal function and attenuates cell death. Relevant to PD, we also show that ATP13A2 levels are decreased in SNpc dopaminergic neurons from patients with PD and that ATP13A2 mostly accumulates within LBs in these patients. Overall, our results unravel an instrumental role of ATP13A2 deficiency on lysosomal function and cell viability, and demonstrate the feasibility and therapeutic potential of restoring ATP13A2 levels.

Loss of ATP13A2 function in human fibroblasts and dopaminergic cells affects lysosomal degradation capacity. This failure of lysosomal function was manifested here as (*i*) an increased intralysosomal pH, (*ii*) impaired cathepsin maturation, (*iii*) increased sensitivity to lysosomal permeabilization, and (*iv*) defective clearance and subsequent accumulation of undegraded AVs/ALs. Lysosomal acidification is necessary to complete the proteolytic maturation and activation of cathepsins (17). All these functions were impaired in ATP13A2 mutant or defective cells, resulting in impaired proteolytic clearance of autophagic substrates and their accumulation in AVs. Restoration of ATP13A2 levels rescued all these deficits. Such pathological processes may be potentially heightened by the continuous formation of defective lysosomes. In addition, undegraded AVs/ALs may eventually contribute to cell dysfunction and death by physically occupying a large portion of the cell body volume, hence interfering with cellular functions (18).

Lysosomes are dynamic organelles receiving many inputs from endocytotic and autophagic pathways. They play a vital role in the clearance of long-lived proteins and in the degradation of cellular organelles in several cell types (19). Degradation is achieved by lysosomal hydrolytic enzymes through a tightly regulated process. The ectopic release of lysosomal proteases into the cytosol, such as cath-B and cath-D, may cause the unwanted digestion of vital proteins; the activation of proteases, including caspases; or the recruitment of proapoptotic proteins (16). Likewise, the release of calcium from lysosomes (20) could lead to cell death. Although mutations in ATP13A2 appear to be linked to PD, impaired lysosomal activity has been implicated in other pathological conditions, ranging from lysosomal storage disorders (21) to other neurodegenerative conditions, such as Alzheimer's disease (22).

Both increased AV formation and impaired lysosomal activity occur in SNpc dopaminergic neurons of patients with PD, along with the deposition of autophagy-related proteins within LBs (11, 23). The identification of ATP13A2 as a component of LBs raises the possibility that LBs, whose mechanisms of formation and significance for the disease process remain unclear, may seed around lysosomes or undegraded ALs and grow in size by the continuous deposition of undegraded AVs as the disease progresses (11). Consistent with this hypothesis, some patients with Gaucher disease, the most common lysosomal storage disorder resulting from the inherited deficiency of the lysosomal enzyme glucocerebrosidase (GBA), exhibit clinical parkinsonism and α-synuclein–immunoreactive LBs (24). GBA is present in the core of LBs in Gaucher-linked PD as well as in sporadic PD cases (25). Depletion of GBA by shRNA in cortical neurons or human Induced pluripotent stem (iPS) cells compromises lysosomal degradation and is associated with accumulation of α-synuclein and neurotoxicity (26). Our data extend these observations by showing enhanced toxicity in dopaminergic cells overexpressing α-synuclein as a result of ATP13A2 deficiency (Fig. S6*E*), similar to what has been previously shown in *C. elegans* (9). α-Synuclein is predominantly degraded by lysosomal pathways, including both macroautophagy and CMA (27, 29). α-Synuclein normally binds to the lysosomal membrane, but PD-linked A30P or A53T α-synuclein mutants block CMA activity (29). A similar effect is observed with dopamine-modified WT α-synuclein (30). PD-linked mutations in *GBA* or *ATP13A2* may result in insufficient clearance of α-synuclein through lysosomes, and hence lead to the accumulation of this protein into the cytosol, eventually contributing

to the formation of LBs. Such a vicious cycle between α-synuclein and GBA has recently been suggested in Gaucher disease (26, 31). Further supporting this notion is the observation that cath-D can cleave α-synuclein, thereby generating carboxy-terminally truncated species (32). In ATP13A2-deficient cells, cath-D maturation is greatly reduced, which may impair α-synuclein degradation, and thus increase α-synuclein accumulation and toxicity. Finally, because lysosomal inhibition has been shown to promote vesicular translocation and extracellular release of α-synuclein (33, 34), lysosomal dysfunction in PD could eventually play a role in the α-synuclein cell-to-cell transfer that is currently believed to underlie the spread and progression of the disease (35).

The present study validates the modulation of lysosomal function as a putative therapeutic strategy for PD. Further supporting this concept, genetic or pharmacological enhancement of lysosomal-mediated degradation has been previously shown to provide neuroprotection in experimental in vitro and in vivo models of PD and other neurodegenerative diseases (36). Here, our results point in particular to lysosomal ATP13A2 as a potential therapeutic target for PD.

Methods

All methods used in this study are routinely used in our laboratories (19), in addition to being described in *SI Methods*. The primary antibodies used for immunoblotting and immunostaining were LC3 (Novus Biologicals), ATP13A2 (Novus Biologicals), LAMP-1 (GeneTex), LAMP-2 (Santa Cruz Biotechnology), cath-B and cath-D (Sigma), beclin-1 (Novus Biologicals), p62 (Progen), GFP (Clontech), MEF2D (BD Transduction Laboratories), α-synuclein (Thermo Scientific), tubulin (Sigma), and actin (Sigma). The different cell lines and postmortem human brain samples used in this study are described in *SI Methods*.

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