Universidade de Lisboa
Faculdade de Medicina de Lisboa

The interplay between alpha-synuclein and ATP13A2: towards the understanding of the molecular basis of Parkinson's disease

Tomás Ribeiro da Silva Lopes da Fonseca

Tese orientada por:
Professor Doutor Tiago Fleming de Oliveira Outeiro

Tese especialmente elaborada para obtenção do grau de Doutor em Ciências Biomédicas, Especialidade em Neurociências

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Abstract

Parkinson's disease (PD) is the most common neurodegenerative disorder with motor impairment. While PD is clinically well characterized, the molecular and cellular basis underlying both the onset and progression of the disorder are still unknown. Gaining deeper knowledge on PD pathophysiology has been hindered by the fact that only a minority of PD patients have a defined genetic cause, with the remaining 90% of the cases being classified as sporadic. Thus far, mutations in more than 20 genes are considered risk factors for developing PD. These PD-related genes are linked to several distinct intracellular pathways, hardening the quest to pinpoint the exact molecular imbalance responsible for PD onset. One of these pathways, the Endolysosomal, has recently gained notoriety due to its importance in alpha-Synuclein homeostasis (α-Syn). α-Syn is a small protein with unknown function and, perhaps, the most extensively studied in PD context. Several point mutations and gene multiplications in α-Syn gene, SNCA, have been linked to PD and the protein is found in Lewy Bodies (LB), the pathological hallmark of the disease. In the Endolysosomal machinery another PD-associated protein has been under the spotlight: ATP13A2. This protein is a transmembrane ATPase located at the late endosomes and lysosomes, with yet unknown function, that is also present in LB. In the last years important steps have been taken towards understanding the interplay between ATP13A2 and α-Syn, with contradictory results reported. Inarguable though is that ATP13A2 can, at least partially, affect the intracellular fate of α-Syn.

Our work confirms that the Endolysosomal pathway plays an important role in α-Syn homeostasis. We start by showing that two proteins members of this pathway, Raba8a and ATP13A2, could alter α-Syn aggregation in a well-established cellular model. Nevertheless, while we found that Raba8a exerts its effect by direct binding to α-Syn, the PD-associated protein ATP13A2 may affect specific cellular mechanisms. We describe that, in human cells, a mutation in ATP13A2, a duplication of 22 base pairs (Dup22), enhances α-Syn aggregation and increases its resistance to proteinase K digestion. The mutated protein could also promote the formation of oligomers and higher α-Syn molecular weight species. In addition, the dynamics between α-Syn and ATP13A2 Dup22 can severely impact cellular homeostasis. Here we report that α-Syn and ATP13A2 Dup22 can be found in a

reticular, membranar structure, which we found to be composed by endoplasmic reticulum (ER). This alteration in the ER morphology was correlated with an unmitigated increase in ER stress that culminates with the activation of apoptotic pathways and cell death. Besides ER changes we also identified significant alterations in mitochondria morphology, along with increased susceptibility to oxidative stress.

Altogether our work provides novel insights into the effect of Endolysosomal proteins on α-Syn. Additionally we describe that the interaction of two PD-associated proteins, ATP13A2 and α-Syn, may trigger a cascade of deleterious intracellular events that involve ER stress and mitochondria alterations, and ultimately lead to cell death. Most importantly, our findings shed new light on the cellular dyshomeostasis that may underlie the development of PD.

Resumo

A doença de Parkinson (PD) é a condição neurodegenerativa com sintomas motores mais comum. Embora o quadro clínico da doença esteja bem caracterizado, os mecanismos moleculares e celulares responsáveis pelo seu aparecimento e progressão são ainda desconhecidos. O estudo aprofundado sobre a patofisiologia de PD tem sido dificultado pelo escasso número de pacientes que apresentam uma causa genética definida, sendo que aproximadamente 90% dos casos são classificados como esporádicos. Atualmente, um total de 20 genes são considerados factores de risco para o aparecimento de PD. O envolvimento desses genes em diferentes mecanismos intracelulares confere complexidade adicional à investigação da causa da doença. Um desses mecanismos é a via endo-lisossomal, a qual tem recentemente ganho relevo devido à sua importância na homeostasia da proteína alpha-sinucleina (α-Syn). A α-Syn é uma proteína com função desconhecida e, provavelmente, a mais estudada no contexto de PD. Várias mutações e multiplicações no gene da α-Syn, SNCA, foram descritas em doentes com PD e a proteína está presente nos Corpos de Lewy (LBs), considerados um dos principais marcos histopatológicos da doença. A via endo-lisossomal é regulada por várias proteínas, sendo que a ATP13A2 tem merecido destaque por estar geneticamente associada a PD. Esta proteína é uma ATPase transmembranar com localização nos endossomas e lisossomas. À semelhança da α-Syn, a ATP13A2 é também encontrada nos LBs e a sua função é ainda desconhecida.

Nos últimos anos têm sido dados passos importantes para melhor compreender a interação entre estas duas proteínas, embora resultados contraditórios tenham sido descritos. Apesar de algumas discrepâncias, é indiscutível que a ATP13A2 afecta, pelo menos parcialmente, o destino intracelular da α-Syn.

O nosso estudo confirma que a via endo-lisossomal desempenha um papel crucial na homeostasia da α-Syn. Inicialmente, mostramos que duas proteínas que intervêm nesta via, ATP13A2 e Raba8a, têm a capacidade de alterar a formação de inclusões intracelulares de α-Syn. No entanto, embora a Raba8a exerça o seu efeito através de uma interação direta com a α-Syn os nossos resultados indicam que a ATP13A2 afecta mecanismos intracelulares específicos. Em células humanas, uma mutação familiar na ATP13A2, que consiste na duplicação de 22

pares bases no gene (Dup22), aumenta a formação de inclusões de α-Syn e a sua resistência à digestão com proteinase K. Esta proteína mutada é também capaz de promover a formação de oligómeros e espécies de peso molecular superior de α-Syn. Paralelamente, a dinâmica de interação entre α-Syn e ATP13A2 Dup22 tem um efeito severo na homeostasia celular. Neste trabalho reportamos que a α-Syn e a ATP13A2 Dup22 co-localizam em estruturas reticulares membranosas, as quais descobrirmos serem compostas por reticulo endoplasmático (ER). Esta alteração da morfologia do ER está associada a um aumento do stress do ER, que culmina na ativação de mecanismos apoptóticos e morte celular. Para além do ER, também observámos alterações significativas na morfologia da mitocôndria acompanhada por um aumento de susceptibilidade a stress oxidativo.

Em resumo, estes resultados oferecem novos conhecimentos sobre o efeito de proteínas associadas à via endo-lisossomal na homeostasis da α-Syn. Adicionalmente descrevemos que a interação de duas proteínas associadas a PD, ATP13A2 e α-Syn, ativa uma cascata perniciosa de eventos intracelulares que envolvem stress do ER e alterações na mitocôndria, e culminam em morte celular. Mais importante, estas descobertas fornecem uma melhor compreensão dos mecanismos celulares que poderão influenciar o aparecimento da doença de PD.

Abbreviations

6-OHDA - 6-hydroxydopamine

α-Syn - Alpha-Synuclein

ATF - Activating Transcription Factor

Atg - Autophagy Related genes

AD - Autosomal Dominant

AR - Autosomal Recessive

BiFC - Bimolecular Fluorescence Complementation

CHOP - C/EBP homologous Protein

CatD - Cathepsin D

Cd^{2+} - Cadmium

CMA - Chaperone Mediataed Autophagy

CSPα - Cysteine-string protein-alpha

DLB - Dementia with Lewy Bodies

Dup22 - Duplication of 22 base pairs

EE - Early Endosome

eIF2α - eukaryotic translation initiation factor 2α

ER- Endoplasmic Reticulum

ERAD - ER-associated protein degradation

ESCRT - Endosomal Sorting Complexes Required for Transport

ILV - Intraluminal vesicles

IRE-1a - inositol-requiring enzyme 1 alpha

KD - Knockdown

KO - Knockout

KRS - Kufor-Rakeb syndrome

LDH - Lactate dehydrogenase

LB - Lewy Body

LE - Late Endosome

LN - Lewy Neurite

Mn^{+} - Manganese

mRNA – messenger Ribonucleic acid

MSA - Multiple System Atrophy

mTor - mammalian target of Rapamycin

MVB - MultiVesicular Body

NAC - non-Aβ component of plaque

NCL - Neuro Ceroid Lipofuscinosis

Ni^{2+} - Niquel

NMR - Nuclear Magnetic Resonance

PAF - Pure Autonomic Failure

PBS – Phosphate buffered saline

PD - Parkinson's Disease

PE - Phosphatidylethanolamine

PERK - protein kinase RNA-like ER Kinase

PK - Proteinase K

PTM - Post-translational modification

ROS - Reactive Oxygen Species

SDS-PAGE - sodium dodecyl sulfate polyacrylamide

Se^{2+} - Selenium

SEC-HPLC - Size Exclusion Chromatography by High-Performance Liquid Chromatography

SN - Substantia nigra pars compacta

SNARE – Soluble NSF Attachment Protein Recpetor

TGN - TransGolgi Network

UPR - Unfolded Protein Response

VAMP2 - Synaptobrevin-2

XBP1 – X-box binding protein

Zn^{2+} - Zinc

1. Introduction

The continuous improvement of life conditions and medical assistance worldwide, but particularly in developed countries, is bringing new challenges to the clinical community in particular, and to modern societies in general. One of these matters is that population aging is drastically increasing the occurrence of neurodegenerative diseases, which many now consider an epidemic. In this picture, it is important to take into consideration the deriving high medical costs that, just in Europe, reached more than 798 billion euros in 2010 (1).
Understanding the micro- and macro-environmental alterations occurring in a neurodegenerative brain has gained enormous interest in the research field over the last century. The ultimate goal is to gather a deep understanding of the many faces of the neurodegeneration process and, ultimately, bring new treatments and therapeutics from bench to clinics.

1.1. Parkinson's Disease

Parkinson's disease (PD) was first described in 1817 by James Parkinson, and is nowadays the second most common neurodegenerative disorder after Alzheimer's disease (2). At the clinical level, PD is classically characterized by motor symptoms that include bradykinesia, resting tremor, postural instability, and muscular rigidity (3) (Fig. 1A). Pathologically the degeneration of dopaminergic neurons in the *substantia nigra (SN) pars compacta*, and the presence of intracellular proteinaceous inclusions in the surviving neurons, known as Lewy Bodies (LBs) and Lewy Neurites (LNs) (3), are the main hallmarks of PD (Fig. 1B). Although initially classified as a movement disorder, it is now well accepted that non-motor symptoms can precede and succeed motor disabilities, and PD is currently regarded as a whole-brain disease (4). In fact, non-motor symptoms are gaining momentum in PD research for possible clinical biomarkers or predictors of the disease onset (5). Furthermore as the understanding on PD symptomatology evolves, the mechanisms underlying the pathological progression of the disease

come under spotlight. Despite lacking full validation, Braak's staging theory is appraised as the most comprehensive data on PD progression. Braak hypothesises that PD pathology might start in either the olfactory bulb or in the lower brainstem. Later on, with the disease progressing, the pathology ascends, affecting other brain regions and promoting the appearance of other symptoms including the motor ones (6-9) (Fig. 1C). Supporting Braak's concept, recent data suggests a "spreading"-like phenomena in PD. In particular it has been reported a time-dependent pathology appearance in normal mesencephalic grafts after being grafted in PD patients. The pathology included LBs and reduced dopamine transporter (10-14).

Phenotypically several other diseases have fallen under the PD umbrella and are commonly denominated as PD-related disorders. Kufor-Rakeb syndrome (KRS) is one of these disorders. KRS patients display a similar clinical chart to severe PD cases with an early disease onset. Besides the common symptomatology, KRS patients also exhibit pallido-pyramidal degeneration and supranuclear upgaze paresis and are levodopa responsive (15).

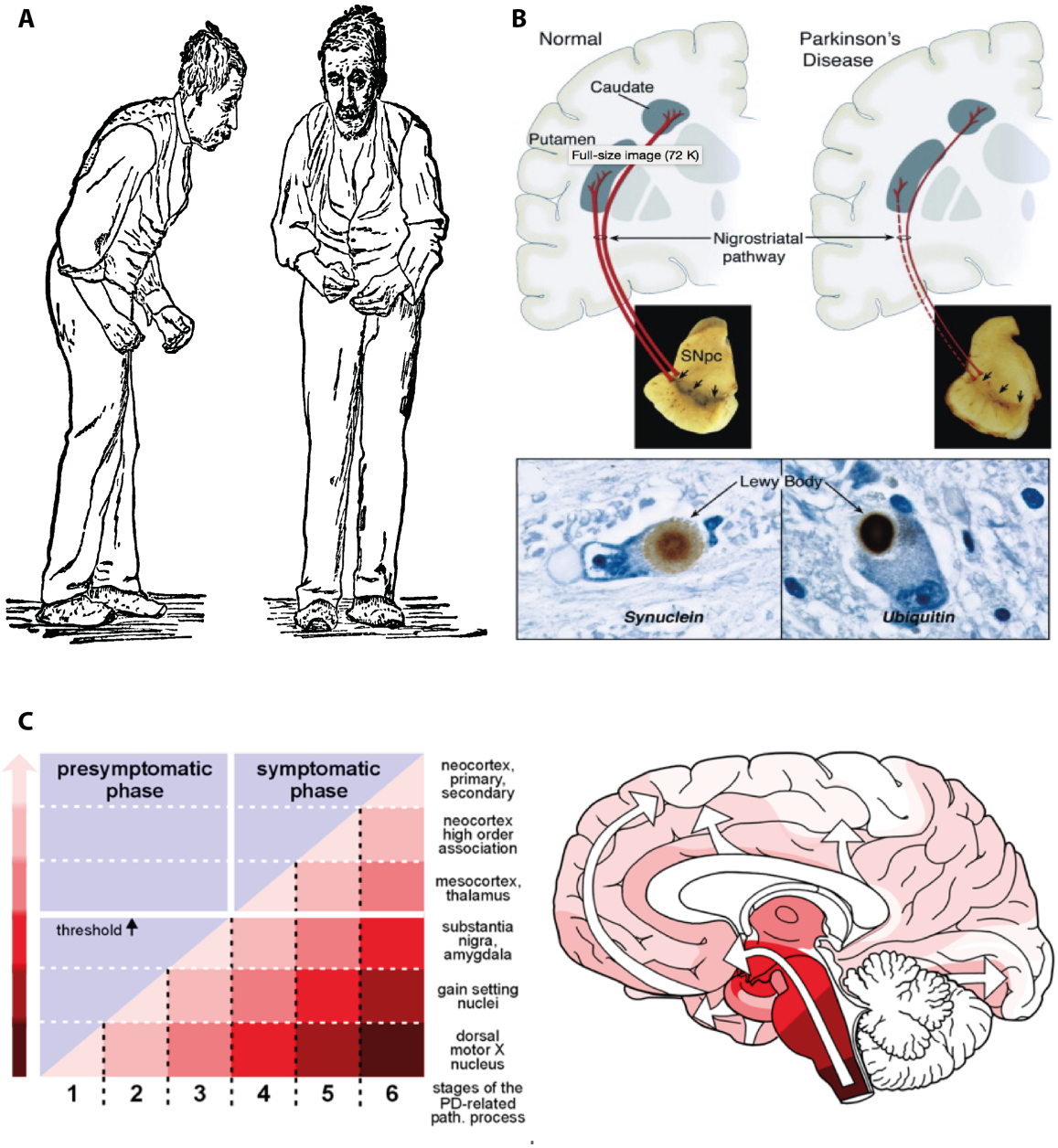


Figure 1. An overview on PD: from patient to pathology. A) The typical posture of a PD patient was first illustrated by James Parkinson in his 1918 essay. **B)** PD has two main pathological hallmarks: the degeneration of dopaminergic neurons at the SN and the presence of intracellular proteinaceous inclusions, known as LBs. **C)** Braak's hypothesis for PD progression suggests that the symptomatic phase appears when the pathology is no longer confined to the brainstem and it ascends to other brain areas. Adapted from (2, 16, 17).

1.1.1. PD Genetics

The cellular triggers underlying PD remain as unknown as the disease progression itself. Only a minority of PD cases, less than 10%, have a defined genetic cause, with the majority of cases being reported as sporadic due to their unknown origin. Thus far alterations in approximately 20 genes have been associated with PD onset (18) (Table 1). The fact that these genes are involved in a broad spectrum of cellular processes, ranging from mitochondrial homeostasis to protein degradation machinery, reflects the complexity of PD pathophysiology. This fact is also a notorious obstacle to understand which alterations in proteins, pathways or organelles are the tipping point that promotes the disease onset and progression. Furthermore, several environmental factors such as metals, pesticides and insecticides are known to cause, or at least increase the risk of developing PD (19-21).

Despite the wide genetic variability, one protein stands out from the crowd and has been extensively studied in the last decades: alpha-Synuclein (α-Syn). Mutations in SNCA, encoding for α-Syn, were the first being associated with the disease, but also duplications and triplications of the gene were later found in PD patients. Besides its involvement in the familial forms of PD, the protein is commonly found in LBs (22) and LNs (23) in sporadic cases.

Table 1. PD associated genes

Locus	Protein	Description	Inheritance	Reference
PARK1/4	Alpha-Synuclein	Presynaptic/ nuclear	AD	(24, 25)
PARK2	Parkin	Ubiquitin ligase	AR	(26)
PARK3	SPR		AD	(27)
PARK5	UCH-L1	Ubiquitin Protease	AR	(28)
PARK6	PINK1	Mitochondrial Kinase	AR	(29)
PARK7	DJ-1	Several functions	AR	(30)
PARK8	LRRK2	Kinase	AD	(31)
PARK9	ATP13A2	P5 ATPase	AR	(32)
PARK10	RNF11 (?)	RING-finger Protein 11	UR	(33)
PARK11	GIGYF2	GRB10 interacting GYF 2	AR	(34)
PARK12	IL-13Ra1 (?)	Interleukin-13	UR	(35)
PARK13	Omi/HTRA2	Serine Protease	UR	(36)
PARK14	PLA2G6	Phospholipase	AR	(37)
PARK15	FBXO7	F-Box Protein	AR	(38)
PARK16	(?)	-	UR	(39)
PARK17	VPS35	Retromer Complex	AD	(40)
PARK18	EIF4G1	Translation Initiation Factor	AD	(41)
PARK19	DNAJC6	HSP40 homolog	AR	(42)
PARK20	SYNJ1	Synaptojanin 1	AR	(43)

AD – Autosomal dominant; AR – Autosomal Recessive; UR – Unknown Relevance.

1.2. Alpha-Synuclein

1.2.1. Structure

Structurally speaking the 140 amino acids of α-Syn can be separated in three distinct domains: a N-terminus prone to mutations, a central domain necessary for aggregation and a C-terminus negatively charged (Fig. 2). Besides all the 6 familial mutations (A30P, E46K, H50Q, G51D, A53E and A53T) that are found in the boundaries of the N-terminal domain (1 to 60), this region is also known for its several imperfect repeats of the consensus motif (KTKEGV) (24, 44-48). These repeats are responsible for the membrane binding and helix folding abilities to α-Syn (49, 50) and they are also found in other proteins that display identical conformational alterations (51). Artificial mutations in this domain block the interaction with membranes and enhance the protein's intracellular toxicity (52). The next 34 amino acids (61 to 95) form the central region, also known as non-Aβ component of plaque (NAC) domain, and represent the main structural difference between the three members of the synuclein family. Highly hydrophobic, this region contains 12 amino acids (71 to 82) essential for α-Syn filament formation (53). The C-terminal part of α-Syn that includes 44 amino acids is rich in glutamates and aspartates imparting a negative charge to the protein. These negative residues can modulate α-Syn aggregation propensity (54) and, according to some reports, this domain may be responsible for the protein's chaperone-like activity (55).

Classically, α-Syn has been considered an unfolded monomeric protein. In 2011, two reports brought controversy to the field by suggesting a native α-Syn tetrameric conformation enriched in alpha-helical structure (56, 57). These findings where rapidly questioned by other groups who found evidence for a predominance of the protein in monomeric and disordered state (58, 59). Nevertheless, this year the tetramer hypothesis was again highlighted with two novel papers reaffirming the presence of α-Syn tetrameric in physiological conditions (60, 61). These two reports put the KTKEGV imperfect repeats as the key mediator of α-Syn tetramerization and describe how some of the familial mutations can shift the equilibrium from tetramer to monomer leading to an increase in cellular toxicity.

α-Syn is highly susceptible to several post-translational modifications (PTMs), which seem to modulate the protein's propensity to aggregate, as well as its cellular behaviour and fate. The protein can be phosphorylated at two serines (S129 and S87) and three tyrosines (Y125, Y133 and Y135). Phosphorylation at S129 is considered a biochemical marker of LBs since approximately 90% of α-Syn present in LBs is phosphorylated in this residue (62, 63). Several kinases including Casein Kinases, Polo-Like Kinases and G protein-clouples receptor Kinases can phosphorylate α-Syn at position 129 (62, 64-68) with some of those enzymes being found up-regulated in PD brains (67), or present in LBs (65, 69). The S129 phosphorylation is described to affect α-Syn fibrillation although it is still unclear if this specific PTM enhances or inhibits this cellular process (63, 70-72).

As for the phosphorylation at position 87 an inhibitory effect on aggregation has been observed (73). Less is known regarding phosphorylation of the tyrosines residues although, in flies, it has been described that Y125 phosphorylation leads to a decrease of α-Syn oligomeric species. Unfortunately, the full functional relevance of α-Syn phosphorylation both in a physiological and disease context is still unknown (74, 75).

In addition to phosphorylation, tyrosine residues (Y39, Y125, Y133, Y136) can also be nitrated, a PTM known to increase α-Syn toxicity. Nitrated α-Syn was found in LBs (76) and nitrated oligomers promoted mitochondrial impairment and cell death in mammalian cells (77). Substantial loss of dopaminergic neurons at the SN, accompanied by down-regulation of striatal dopamine and dopamine receptor D2, was observed upon injection of nitrated α-Syn into SN of rats (78). Interestingly nitration of position Y39 can inhibit α-Syn fibril assembly and reduce monomer degradation via the ubiquitin proteasome system (79).

Truncated α-Syn has also been found in LBs and animal models, with findings suggesting that familial mutations can increase its occurrence (80-82). *In vitro* truncation of α-Syn at the C-Terminus can enhance fibril formation and promote the fibrilization of full-length α-Syn (83, 84). Nevertheless the opposite effect was reported upon calpain 1- or neurosin-mediated α-Syn truncation (85-88). These two proteases cleave α-Syn near the NAC domain highlighting the importance of this region in α-Syn aggregation.

α-Syn can also undergo other PTMs including ubiquitination (82, 89-91), sumoylation (92-94) and acetylation (95-97). Ubiquitinaton occurs mainly at the N-

terminus of α-Syn protein sequence (98) although it has been described that important Ubiquitin ligases require α-Syn C-terminus to proper modify the protein (99). Ubiquitinated α-Syn can be found in LBs and this PTM can affect the protein's degradation via de endolysosomal and autophagy pathways (89, 99, 100). Identical to ubiquitination, sumoylation also targets lysines and, in regards to α-Syn, the lysines 96 and 102 are the main amino acids affected by this PTM (92). The presence of sumoylated α-Syn has been descibred in aggregates of α-Syn although it is believe that this PTM can inhibit the aggregation formation (92, 93). A recent report presented a more functional role for α-Syn sumoylation describing an important role of this PTM on the protein's secretion by exosomes (101).
Lastly, N-terminus acetylation of α-Syn has been has been repetitively associated to the protein's capability for membrane binding and this PTM was also described to increase α-Syn resistance to aggregation (95-97).

1.2.2. Function

α-Syn was initially described in 1988 and its name derives from its intracellular localization: Syn- from synapse and -nuclein from nucleus (102). More than a quarter of a century later, the scientific community has yet to reach a consensus regarding the intracellular function of α-Syn. Several reports have so far have suggested a wide spectrum of hypothetical roles ranging from neurotransmitter release, to DNA binding, and mitochondrial homeostasis (103-105). The strongest evidence points towards a pre-synaptic function. Supporting this line of evidence is the protein's abundance in the brain (106) as well as the described pre-synaptic localization (102, 107) and its co-localization with the reserve pool of synaptic vesicles (108, 109). Initial reports suggested that α-Syn could play several roles in the cycling of synaptic vesicles, modulating the vesicle pool size, mobilization and endocytosis (110, 111). Accordingly, altered synaptic vesicles dynamics, along with decreased striatal dopamine, are the minor alterations described in α-Syn knockout (KO) mice (112, 113). Notorious concerns regarding potential compensatory mechanisms, generated by other members of the synuclein family (β and γ), led to the generation of double and triple KO animals. In KO mice for α-

Syn and β-Syn no alterations in synaptic vesicle dynamics were observed, though the animals exhibited alterations in pre-synaptic protein levels together with a distinct decrease of dopamine levels throughout the brain (114). Likewise the triple-synuclein KO mice also showed changes in the levels of pre-synaptic proteins and a more severe phenotype, including decrease of synaptic terminal size and higher lethality (115).

Important work developed by Südhof and colleagues suggests that α-Syn interacts with synaptobrevin-2 (VAMP2) and with phospholipids via its C- and N-termini, respectively (116). This interaction promotes Soluble NSF Attachment Protein Receptor (SNARE) complex assembly and recent data indicates that α-Syn multimerization ability is required upon binding to membranes (116-118). The role of α-Syn might be extremely specific since this interplay was only reported in docked membranes at the plasma membrane (117) (Fig. 2). Nevertheless VAMP2 has been linked to other intracellular pathways besides neurotransmitter release so it is possible that α-Syn has a broader role in the intracellular trafficking mechanisms (119, 120). Supporting this idea is the fact that both α-Syn and VAMP2 and strongly present in erythrocytes, blood cells that do not perform synaptic vesicles transmission (56, 121).

Nevertheless, reinforcing a putative function of α-Syn on synaptic homeostasis, the protein was also shown to compensate the loss of the pre-synaptic cysteine-string protein-alpha (CSPα) in mice. CSPα KO animals display impairment of SNARE assembly, followed by pronounced neurodegeneration and early lethality. Overexpression of α-Syn in these mice was able to partially rescue the phenotype and increase their life expectancy with the authors suggesting that α-Syn could enhance SNARE-complex assembly acting downstream of CSPα. Additionally KO animals for both α-Syn and CSPα present the most severe phenotype, confirming the involvement of α-Syn in the compensatory mechanism (122, 123).

1.2.3. Toxicity and Disease

Despite many open questions regarding the physiological function of α-Syn, this 14.5 kDa protein has taken a central stage in research on neurodegeneration due to its involvement in several brain-related diseases, commonly described as synucleinopathies. PD is one of these disorders and, as mentioned above, α-Syn

is considered an important component of the LBs and LNs although the positive or negative cellular effect of these inclusions is still elusive. A total of 6 mutations in its 140 amino acids have been found in PD patients and duplication or triplication of the SNCA gene lead to the same clinical phenotype (24, 25, 44-48, 124). Recent studies also revealed that polymorphisms in the SNCA gene lead to an increase of PD risk (39, 125-127).

In addition to PD, the group of synucleinopathies is composed of three other neurological disorders: Multiple Systems Atrophy (MSA), Dementia with Lewy Bodies (DLB) and Pure Autonomic Failure (PAF). Of the three, MSA is the disorder with a strongest connection to α-Syn, although in terms of pathology is the more distinct one. MSA patients present proteinaceous inclusions in oligodendrocytes (128-130). Two recently described mutations in α-Syn (A53E and H50Q) were found in patients displaying MSA pathology, and polymorphisms in the α-Syn gene can also enhance the risk for disease onset (48, 131-133). Patients with DLB exhibit dementia and other Alzheimer-like symptoms due to a stronger pathological effect in the cortex area (134). The clinical discrimination between PD and DLB is challenging since almost half of all PD patients also develop dementia as the disease progresses (135). More distinct from the other disorders is PAF. This disease affects the peripheral nervous system and also presents α-Syn-positive LBs and LNs, although some researchers postulate that PAF in no more than an intermediate disease state that later progresses into other neurological disorders (136-138).

The origin of α-Syn toxicity is still a debatable subject but its deleterious effects have been associated to the majority of the intracellular organelles and pathways. In fact it has been described that α-Syn can impair vesicle and protein trafficking, protein degradation systems, mitochondrial respiration, microtubule polymerization and neurite network (139-145). As mentioned above, the structure of α-Syn confers its aggregation propensity. Thus, understanding the mechanisms underlying this process has become the central quest in the field of synucleinopathies. Three main α-Syn species are commonly represented in the “aggregation pathway” that start with monomers, followed by the formation of intermediate oligomers, and finalized with aggregates/inclusions (Fig. 2). Once again a clear lack of consensus exists regarding the toxic elements of this pathway with some evidence pointing towards the oligomers (146-148), while other suggest

the aggregates (149, 150) (Fig. 2). Knowing that LBs are found in surviving dopaminergic neurons, it has been speculated that they may represent a pro-survival response to the cellular toxicity. Nevertheless a recent work concluded that different α-Syn assemblies could lead to distinct but yet complementary molecular and behavioural patterns (151). These data suggest that more than one culprit species could be underlying the disease onset.

Additionally, since the function of α-Syn might require a multimerization process upon membrane binding, it is likely that the protein is constantly shifting between cytosolic monomer and membrane-bound multimer. It has now become essential to chemically, structurally and biologically distinguish between “functional oligomer” and “toxic oligomer”. Altogether this data points to an extremely sensitive cellular balance between of function and dysfunction that weight towards one of them according to the protein propensity to bind membranes.

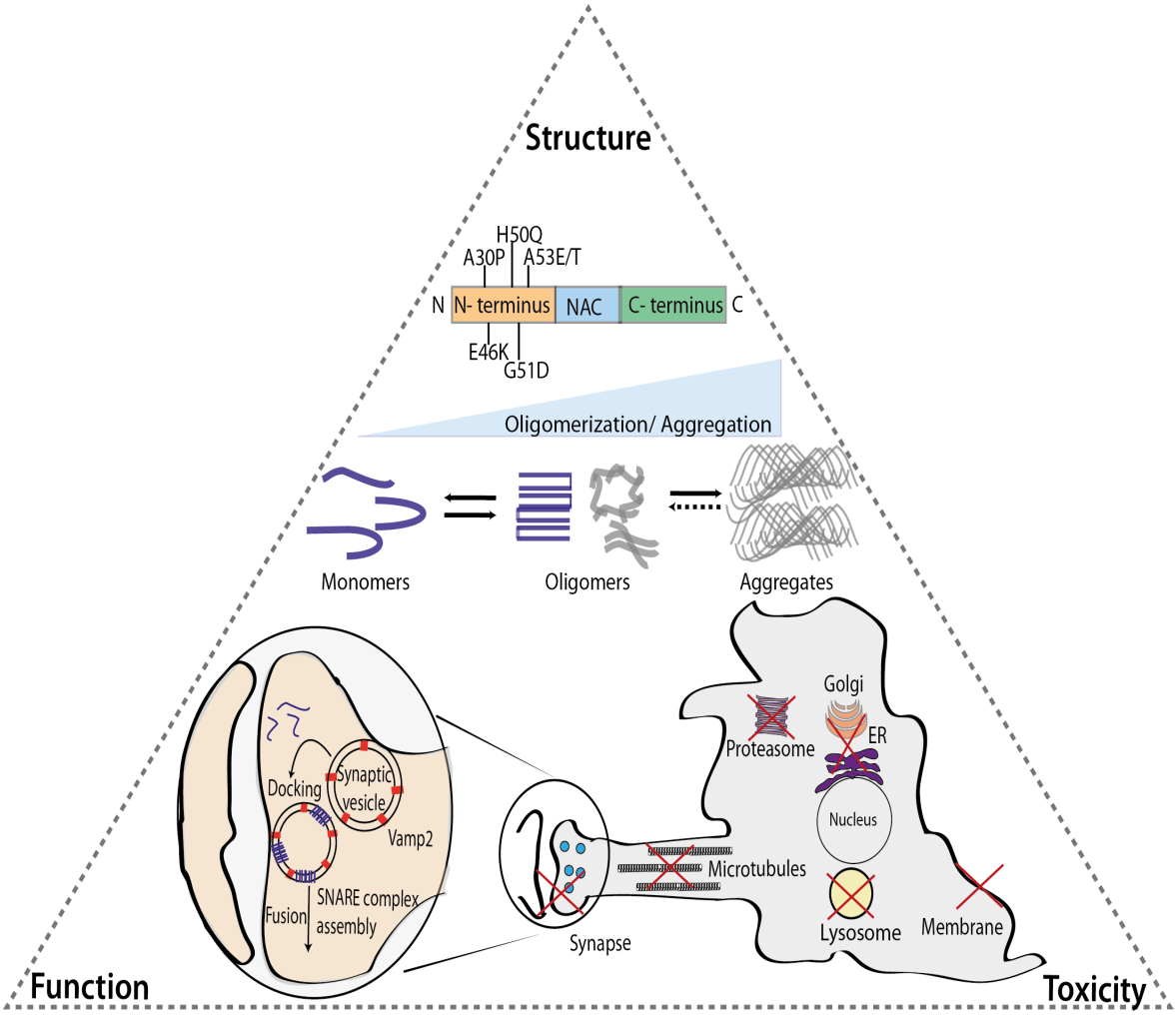


Figure 2. α-Syn: structure, function and toxicity. α-Syn is an intrinsically disordered protein with 6 PD-related point mutations described so far. The protein can undergo oligomerization/multimerization that, under pathological conditions, can lead to the formation of beta-sheet structured aggregates. The outcome of the oligomerization can determine the existence of a functional (blue) or dysfunctional (grey) α-Syn with recent data suggesting a crucial role for the protein's membrane binding domains in this process. In terms of function, α-Syn has been strongly associated with vesicular trafficking, and particularly with synaptic vesicles, interacting with important players in this pathway. Although it is still not fully understood which α-Syn species are toxic, putative deleterious effects on several intracellular organelles and pathways, including proteasomes, lysosomes, synapses, microtubules, ER-to-Golgi trafficking and membranes, have been described (152).

1.3. ATP13A2

1.3.1 Structure

ATP13A2, also known as Park9, is a transmembrane protein of 1180 amino acids that mainly localizes in lysosomes and late endosomes (32). The protein is a member of the P5 type pump ATPases family, together with 4 other proteins, ATP13A1 and ATP13A3-5. Bioinformatic analyses suggest that ATP13A2 has 11 transmembrane domains and four functional domains: an actuator domain (A), a catalytic phosphorylation site (P1), and two nucleotide-binding domains (P2 and N) (153) (Fig. 3A). The protein is highly expressed in the brain, particularly in the SN and is upregulated in the dopaminergic neurons of this region in the brains of PD patients (32, 154). A recent work provided new insights about the intracellular behaviour and homeostasis of ATP13A2. Both C- and N-terminus of the protein are faced towards the cytosol with the latter terminal having a unique conformation that is provided by the eleventh transmembrane domain (155). Additionally it was observed that ATP13A2 could be autophosphorylated in its inactive state with several phosphates being able to induce this process also via the N-terminus (155).

1.3.2. Function

The cellular function of ATP13A2 is still elusive, as in the case of α-Syn. Fibroblasts obtained from patients with ATP13A2 mutations, revealed profound alterations in mitochondrial homeostasis. This impairment was associated with reduced ATP production and increased maximum respiration capacity, due to an impairment of mitochondrial degradation and subsequent accumulation of defective organelles. These phenotypes could be partially rescued upon ATP13A2 overexpression (156). The process of autophagic mitochondria degradation, known as mitophagy, is a crucial quality control mechanism to ensure the proper function of the organelle (157). An imbalance in this process has been associated with PD (158).

ATP13A2 has been directly linked to mitophagy in several studies (156, 159, 160) but little is known about which mechanisms are involved (Fig. 3C).

Apart from mitophagy ATP13A2 has been connected with normal protein autophagy (161-163) and metal/cation homeostasis (154, 160, 163-172) (Fig. 3C). Regarding the latter, ATP13A2 was shown to have a protective effect in manganese (Mn^{+})-mediated α-Syn toxicity, in both yeast and SH-SY5Y cells (166) (Fig. 3C and D). This role in Mn^{+} homeostasis was further explored in yeast and allowed the identification of several genes involved in the process (168). Furthermore some ATP13A2 mutants were unable to rescue Mn^{+} induced toxicity in mammalian cells (170) and two ATP13A2 polymorphisms enhance Mn^{+} neurotoxic effect in patients (169). Considering that this metal has been linked to Parkinsonism (173), and specifically to α-Syn oligomerization and aggregation (174, 175), one can speculate that Mn^{+} could be an intermediate between α-Syn and ATP13A2. This hypothesis is not fully accepted since a recent report concluded that in mammalian cells, ATP13A2 levels had no effect on Mn^{+} sensitivity (164).

Besides Mn^{+}, ATP13A2 was shown to exert a protective effect against niquel- (Ni^{2+}), cadmium- (Cd^{2+}) and selenium- (Se^{2+}) induced toxicity, in yeast and in mammalian cell culture (171, 172), yet little is known about the role of these metals in the context of α-Syn toxicity and PD.

More recently, ATP13A2 was also linked to zinc (Zn^{2+}) homeostasis in a study showing that mitochondrial impairment, due to increased amounts of Zn^{2+}, could be rescued upon overexpression of ATP13A2 (160). The interplay between α-Syn, ATP13A2 and Zn^{2+} has been strongly associated with autophagy and extracellular release and it will be further developed ahead.

The first results with ATP13A2 KO mice showed that the animals exhibit both PD and NCL pathology, including the formation of intracellular aggregates positive for α-Syn and ubiquitin, sensorimotor deficits and lipofuscinosis (161), suggesting that the phenotypes may not be solely gene dependent. A recent report further indicated that, in these mice, α-Syn accumulation was a side effect from a general impairment of the endolysosomal pathway (176).

1.3.3 Toxicity and Disease

Mutations in ATP13A2 have been associated with different diseases including PD, KRS (32, 177-185), and also Neuroid Ceroid Lipofuscinosis (NCL) (186-188). Of the several disease-associated mutations identified in ATP13A2, thus far only a few were investigated in detail. In cells ATP13A2 mutants exhibited loss of protein function, subcellular mislocalization in the endoplasmic reticulum (ER), increased cellular toxicity, and shorter protein half-life (Fig. 3D) (189). In a detailed study of the effects of ATP13A2 missense mutations associated with early-onset parkinsonism several novel phenotypes were identified, including disruption of the protein vesicular localization, impairment of ATPase activity and of neurite outgrowth (190). One of these mutations, commonly known as Dup22, was initially described in a Jordanian family. The patients (four in total) exhibited an early age of onset (12-15 year old) with rapid disease progression. Cardinal PD symptoms like rigidity, bradykinesia and postural instability were present, accompanied by non-motor symptoms such as hallucinations. Genetically the Dup22 mutation consists in a duplication of 22 base pairs (1632-1653) that promotes the formation of 236 erroneous amino acids followed by a stop codon. At a structural level, this mutated protein lacks 6 of the 10 transmembrane domains of ATP13A2 (Fig. 3B) (32, 191, 192). Despite the believe that the lost of transmembrane domains could lead an intrinsic incapability of ATP13A2 to be targeted to its final destination, a recent study revealed that the presence of N-terminus is enough for the protein's colocalization at the late endosomes and lysosomes (155).

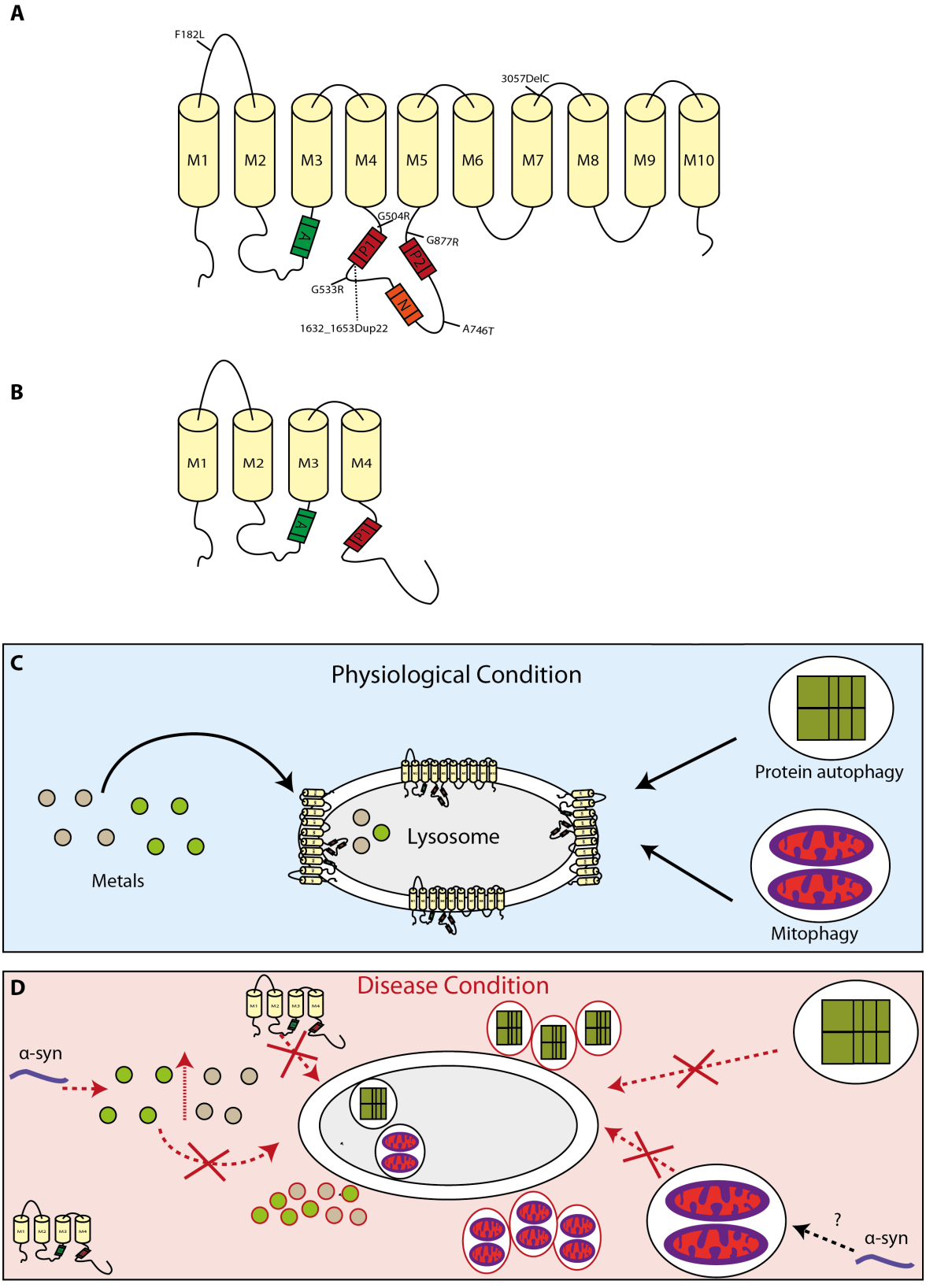


Figure 3. ATP13A2 structure and hypothetic role in physiological and pathological conditions. A) ATP13A2 is an 1180 amino acids protein with 10 transmembrane domains and four functional domains: catalytic phosphorylation (P1), nucleotide binding (P2 and N) and actuator domain. **B)** The familial mutation Dup22 removes 6 of the transmembrane

domains that constitute the ATP13A2 WT **C)** ATP13A2 is thought to play a role on metal homeostasis and autophagy process, including protein and mitochondrial degradation via the lysosome. **D)** A failure in metal processing, caused by mutations or reduced activity of ATP13A2, would lead to the toxic accumulation of metals in the cytoplasm. In disease conditions, α-Syn may increase the intracellular levels of metals, exacerbating cytotoxic effects. Regarding protein and mitochondria degradation it has been reported that deficient ATP13A2 activity can lead to the accumulation of defective mitochondria or proteins (such as α-Syn) that ultimately contribute to increased cytotoxicity. Adapter from (193).

1.4. Protein Degradation

The correct degradation of proteins is an important process to assure the proper intracellular homeostasis and it is ensured by two independent, but complementary, systems. These two mechanisms, the autopaghy-lysosomal pathway and the Ubiquitin Proteasome System are named upon their final destination organelle, the lysosome and the proteasome, respectively. Monomeric α-Syn can be actively degraded by both organelles (194, 195) that can compensate each other upon one's failure (196). When it comes to eliminating higher molecular species the lysosome is the only organelle able to deal with them and promote their correct degradation (197). Considering that both ATP13A2 and α-Syn have been extensively linked to the lysosome, I will further develop the pathways associated with this organelle.

1.4.1. The lysosome

Cell survival requires the constant turnover of its functional machinery, such as proteins and organelles, and the lysosome constitutes the main cellular component responsible for this task. This turnover is crucial for the removal of deleterious intracellular components, and for the recycling of macromolecules to guarantee proteome renewal (198). Autophagy (meaning "self-eating", in Greek) consists in the process of decomposition and degradation of cellular components and organelles via the lysosomal compartment. Autophagy itself serves two main

purposes: the clearance of deleterious intracellular components, and the recycling of macromolecules from functional pre-existing organelles and proteins to guarantee proteome renewal (198).

When it comes to protein and organelle degradation, the lysosome can be the end point of several pathways that can be categorized in two different groups: the autophagy pathway and the Endolysosomal pathway. The first group can be divided in three pathways based on the cargo delivery method: chaperone-mediated autophagy (CMA), macroautophagy and microautophagy. The Endolysosomal pathways, despite also culminating in the lysosome, display distinct molecular players and can have two distinct origins: the ER or endocytic mechanisms.

For the purpose of this thesis, I will focus on macroautophagy and the endolysosomal pathways.

1.4.1.1. Macroautophagy

Macroautophagy is a bulk, content-blind, cellular degradation mechanism that requires the formation of *de novo* double membrane-bound vesicles to sequester intracellular components, including whole organelles, towards the lysosome (199, 200). The entire mechanism deeply relies on the so-called autophagy-related proteins (Atg) that were first described in yeast (201-203). In fact, the formation of the *de novo* membranes mainly depends on the autophagy related protein (Atg) 9, both in yeast and humans (204-207). Macroautophagy is found constitutively active but it can be enhance either via the mTOR pathway, the mammalian target of rapamycin (208), or via the PI3kinase/beclin/vsp34 pathway (209). The autophagosome formation requires two ubiquitination steps highly regulated by Atg proteins (201-203). Initially Atg12 is conjugated with Atg5, a process involving Atg7 and Atg10 (210, 211). The Atg12-Atg5 complex is later targeted to the autophagosome together with Atg16 (212, 213). The localization of this complex at the membrane is required for the second ubiquination step to occur, via Atg8 (also known by LC3) (214, 215). LC3 is C-terminally cleaved by Atg4 to form LC3-I (216, 217), which is then conjugated to the lipid phosphatidylethanolamine (PE) by Atg7 and Atg3 to generate LC3-II (211, 218). LC3-II is the most common marker for

autophagy since it is specific to this pathway and degraded only after the fusion of the autophagosome to the lysosome (217). After formation, the autophagosomes travel along the microtubules in a dynein-dependent manner towards the microtubule organizing centers where the lysosomes are located (219) (Fig. 4A). The release of the autophagosome content to the lysosome can be performed either by fusion of both compartments or just by a transfer of the autophagosome content to the lysosome without loss of the first structure (219). The mechanisms underlying the fusion of both compartments are largely unknown in mammalian cells, except for the fact that Rab7 plays a role on this process (220).

1.4.1.2. The Endolysosomal Pathway

The endosomal pathway is a complex intracellular trafficking mechanism that has been extensively associated to PD (221). In this pathway the early endosomes (EE) are the main sorting pit stops that receive content originating in the plasma membrane via endocytosis, and from the Trans-Golgi network (TGN). Once inside the EE the content may have two destinations: either it is targeted to the lysosome to be degraded or it is recycled towards the plasma membrane. Depending on their faith, the proteins are usually found in different sections of the EE. Specifically, proteins that will be recycled are commonly found in the tubular sorting endosome while the ones targeted for the lysosomes are present in the vacuolar sorting endosome. In the latter the content is stored in intraluminal vesicles (ILV) that originate by inward budding of the EE membrane. The content of the ILVs and their formation is strongly dependent on the endosomal sorting complexes required for transport (ESCRT) (222-224). The cargo identification by the ESCRT typically demands a previous ubiquitination step, although an ESCRT-independent mechanism has also been described (222, 225). The vacuolar endosome suffers a process of continuous maturation that culminates in the formation of late endosome (LE), via multivesicular Bodies (MVBs). At this stage the LE contains few proteins intended for recycling after endocytosis, and the majority of the content being targeted for the lysosome. Importantly, differences are also observed in the protein composition at the membrane of the LE with the most well described alteration being a Rab conversion, consisting in a switch between Rab5, present in the EE, and Rab7 found in LE (226). A second sorting

event takes place at the LE/MVBs complex, enabling the content can either be forwarded towards the lysosome for degradation or targeted to the extracellular environment via exosomes (Fig. 4B). The biological function of exosomes is still elusive, but some speculate that these vesicles may constitute a cellular clearance route to proteins that cannot be degraded by the lysosome (227).

Besides the notorious role in protein degradation, the endolysosomal pathway itself is of an extreme importance for the lysosome biogenesis and function. It is through this pathway that lysosome membrane proteins and intralysosomal hydrolases are delivered to the lysosome. The most studied pathway is the M6PR-dependent transport, used by proteins such as Cathepsin D (CatD), and relies on the addition of an M6P-tag to the hydrolases, though some proteins can undergo different processing (228, 229). Regarding M6P-independent pathways, recent findings point that hydrolases can migrate to the lysosomes bounded to lysosomal membranes, like β-Gluocerebrosidase, a protein which gene, GBA, has been extensively linked to PD (230, 231).

The extensive network of the endosomal pathway requires the constant movement of the vesicles inside the cell. Rab proteins are major regulators of the dynamics underlying these movements, and the overall intracellular trafficking (232, 233). More than 60 members compose the highly conserved Rab family (234) with Rab5, Rab7 and Rab22B being the ones more linked to this endosomal mechanism (235) (Fig. 4B). Nevertheless Rab8a, which has been previously linked to α-Syn (236), has recently emerged as an important protein in the normal function of this pathway (237). In detail, it was shown that normal endolysosomal requires the presence of the complex Rab8 and optineurin at the Golgi membrane (237). A mislocalization of the complex leads to decrease in CatD maturation and impaired lysosomal protein degradation.

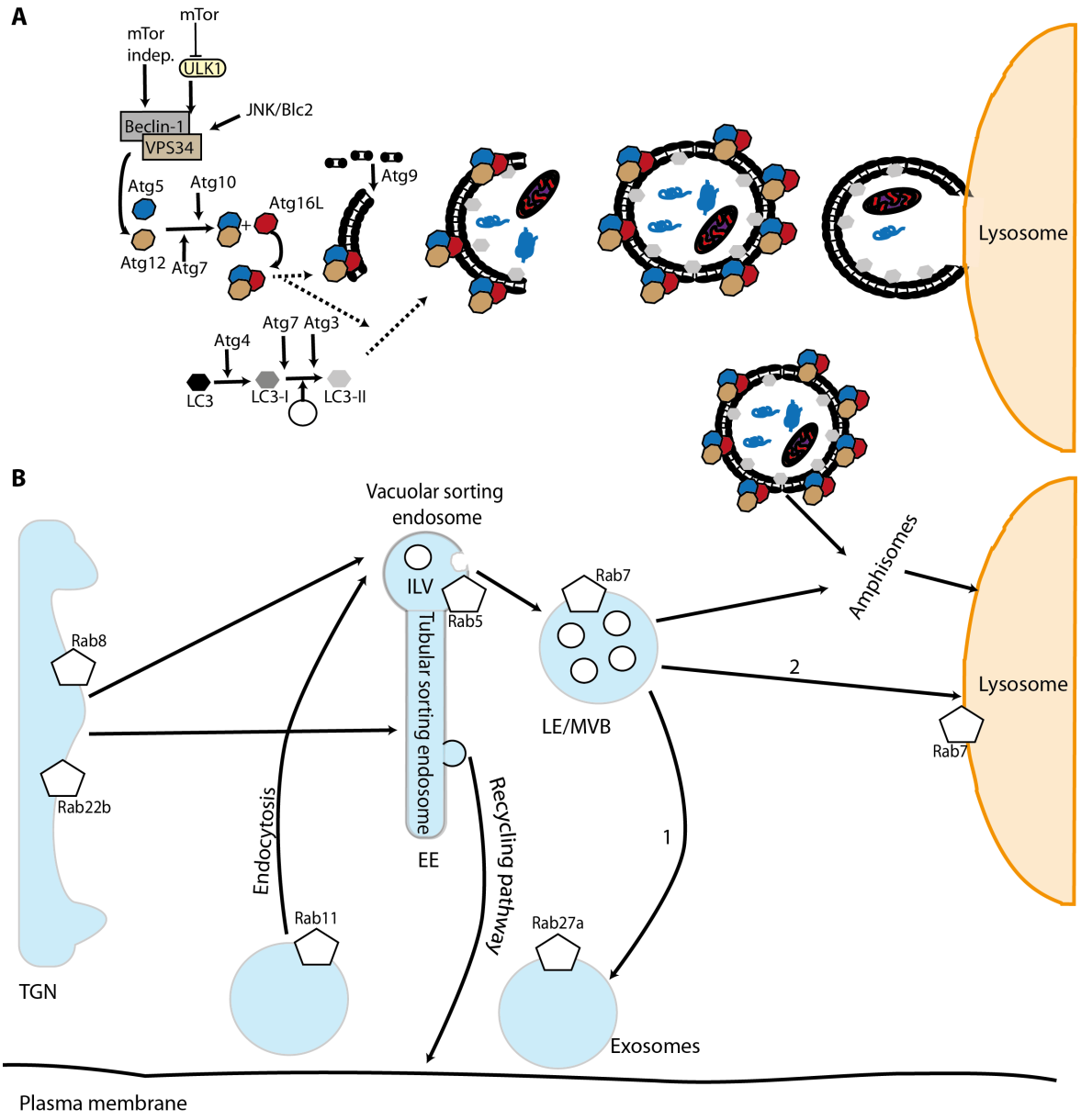


Figure 4. Mechanisms of protein degradation: autophagy and endolysosomal pathways. A) Three main cellular pathways can enhance macroautophagy: mTor dependent, mTor independent and JNK/Blc2. All terminate in the complex Beclin-1/VPS34 that will promote the formation of the autophagosome that surrounds the content targeted to degradation. This autophagosome formation starts with an ubiquitination reaction where Atg12 is conjugated to Atg5, a process mediated by Atg7 and Atg10. After the binding of Atg16L, the Atg12/Atg5/Atg16L complex is targeted to the autophagosome. This complex localization at the membrane is necessary for the second ubiquination and requires LC3. Initially, LC3 is cleaved at the C-terminus by Atg4 to form LC3-I, which is then conjugated to a PE by Atg7 and Atg3 to generate LC3-II. The membrane of the autophagosome is originated from other organelles and transported by Atg9. After closing the autophagosomes is moved towards the lysosomes where it fuses, to deliver the cargo for

degradation. **B)** The endolysosomal machinery involves several intracellular players. The content being delivered into the lysosome can arise from the TGN, where Rab8a is a master player, or through the extracellular environment via endosomes that are positive for Rab11. In either case, the content is targeted to the Vacuolar Sorting component of the EE. This structure will then give origin to the LE/MVBs a process that requires a Rab conversion from Rab5 to Rab7 at the membrane level. These LE/MVBs that can undergo two distinct pathways: 1) form Rab27a positive exosomes and release the content to the extracellular media or 2) fuse with the lysosomes to allow the degradation of proteins or the delivery of lysosomal membrane proteins and hydrolases. Adapted from (238).

1.4.2. The interplay between α-Syn, ATP13A2, and protein degradation pathways

Monomeric α-Syn degradation is divided into two complementary mechanisms, the ubiquitin proteasome system and the CMA (194, 195) that compensate each other upon one's failure (196). When it comes to eliminating higher molecular species the burden shifts entirely to the lysosome (197). Nevertheless, the CMA cannot handle species larger than dimers (239, 240) so another mechanism, macroautophagy, is activated.

The degradation of α-Syn via macroautophagy has been mostly studied by using specific inhibitors and enhancers of this pathway. Macroautophagy can degrade both WT and mutant α-Syn in a Beclin-1 dependent process (99, 241, 242), though some literature points to a mutant-specific degradation (243, 244). Formation of high molecular weight species has been reported upon blockage of this pathway, although the results are controversial (245, 246). No overall consensus has been reached on the effect of macroautophagy in α-Syn homeostasis, although in yeast, a model organism that lacks CMA, it was demonstrated that phosphorylation and sumoylation of α-Syn are able to modulate the protein's degradation via this pathway and, ultimately, inclusion formation (71, 94).

On the other hand, α-Syn also impacts macroautophagy. The overexpression of the protein can inhibit macroautophagy via an interaction with Rab1a that culminates in a mislocalization of Atg9 (141). In DLB patients and αSyn

overexpressing transgenic mice an increase in mTor and decrease in Atg7 levels has been observed (140) (Fig. 5A). The same study also reported the presence of enlarged autophagosomes and lysosomes, as it was first observed in cells overexpressing α-Syn (241). A recent work reported a drastic effect in the presence of α-Syn aggregates, as these species appear to be resistant to macroautophagy and promote a general failure of the pathway with subsequent accumulation of autophagosomes (150) (Fig. 5D). Besides the WT α-Syn, also the familial PD-associated mutations can have a deleterious effect on macroautophagy. The familial mutation E46K was reported to impair macroautophagy via inactivation of the JNK1/Blc2 pathway (247) (Fig. 5B). As for the A53T mutation opposite effects were described, with one group suggesting an enhancement of macroautophagy, especially the mitophagy pathway (248), while other suggested an impairment of degradation leading to the accumulation of autophagosomes (249) (Fig. 5C).

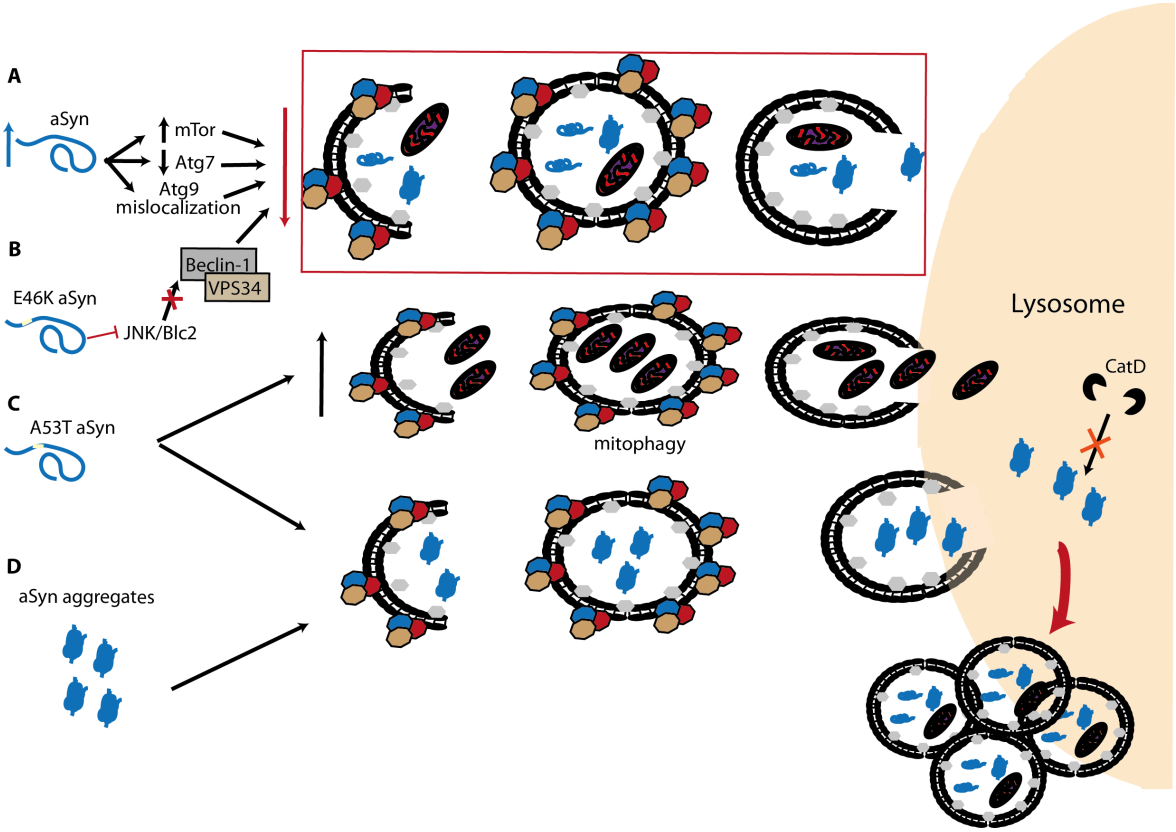


Figure 5. The interplay between α-Syn and macroautophagy. A) Accumulation of α-Syn can increase mTor, decrease Atg7 levels and promote mislocalization of Atg9 that, individually or combined, can alter the delicate homeostasis of macroautophagy. **B)** α-Syn familial mutation E46K was described to inhibit macroautophagy via JNK/Blc2, an

mTor independent pathway. **C)** On the other hand, two opposite effects have been associated with the A53T mutation: increase in mitophagy, and accumulation of autophagosomes due to impaired degradation. **D)** As for α-Syn aggregates, these species cannot be degraded by macroautophagy, leading to the impairment of the pathway. Adapted from (238).

Once inside the lysosome, α-Syn degradation is a task usually performed by CatD (250). Synthetized in the ER, this protease is initially cleaved generating an inactive proCatD (251). The proCatD is then targeted to the lysosome, a process that was found to be endosome-mediated (252, 253). Upon reaching an acidic environment, cysteine proteases are responsible for the cleavage of proCatD at the N-terminus to originate the active form of CatD (254, 255). Confirming the relevance of CatD in α-Syn homeostasis, overexpression of CatD was shown to protect against α-Syn-induced toxicity (256). CatD KO mice exhibit the formation of insoluble α-Syn species while in cell models the expression of an inactive mutant of CatD generated identical effects (257, 258). Increased levels of mutated CatD enhanced the expression of Cathepsin B, a protease that can promote α-Syn aggregate formation (256, 259). Recent findings indicate that treating cells with α-Syn aggregates leads to lysosomal rupture and cathepsin B dependent ROS production (260). At the pathological level, decrease of CatD has been described in neurons of the SN with α-Syn-positive aggregates, in PD patients (261). Nevertheless other groups reported no difference in CatD between PD patients and controls (262, 263).

As mentioned above, ATP13A2 has been strongly associated with autophagy, mainly due to its localization at the LE and lysosomes membranes. ATP13A2 knockdown (KD) and KO models exhibit an impairment of α-Syn degradation (161, 162). Further results pointed towards an overall autophagy impairment due to alterations of lysosomal pH and the levels of hydrolases leading to a failure in autophagosomes clearance, and a decreased proteolytic processing (162, 163, 165). Recently, it was suggested that aggregate formation in ATP13A2 KO mice was α-Syn-independent, and a clear impairment of the endolysosomal pathway was reported. This impairment resulted in a reduction in CatD maturation, and localization at the lysosome (176). These findings are in agreement with a previous report showing a decrease in CatD activity upon ATP13A2 mutations in

Medaka fish (264), and puts both CatD and ATP13A under the spotlight. Curiously mutations in both CatD and ATP13A have been associated to NCL and, alike ATP13A2, CatD KO mice exhibit a NCL phenotype, which may suggest that similar cellular mechanisms are taking place (161, 186, 265, 266) (Fig. 6A and B).

Another strong line of research combines protein clearance with metal homeostasis in the paradigm correlating ATP13A2 and α-Syn. Two recent studies suggest unbalanced Zn^{2+} homeostasis as the starting point of a chain of events responsible for α-Syn clearance. The first study showed that alterations in Zn^{2+} intracellular levels and cellular sub-localization could promote lysosomal dysfunction and α-Syn accumulation. This phenotype was enhanced upon ATP13A2 KD, in cells and in fibroblasts from patients carrying ATP13A2 mutations, and could be rescued after ATP13A2 overexpression (165). On the other hand, an independent study focused upstream of the endolysosomal pathway, in particularly the LE/MVBs. The authors found that MVBs are targeted to exocytosis, instead of autophagy, and this mechanism is the main pathway underlying the decrease of intracellular α-Syn levels (164). In this perspective, ATP13A2 was shown to modulate Zn^{2+} levels, which, in turn, could influence the biogenesis of exosomes (Fig. 6A and B).

Altogether, it becomes obvious that the endolysosomal pathway, either due to metal homeostasis or protein degradation imbalance, plays an important role in neurodegeneration and, more specifically, in PD.

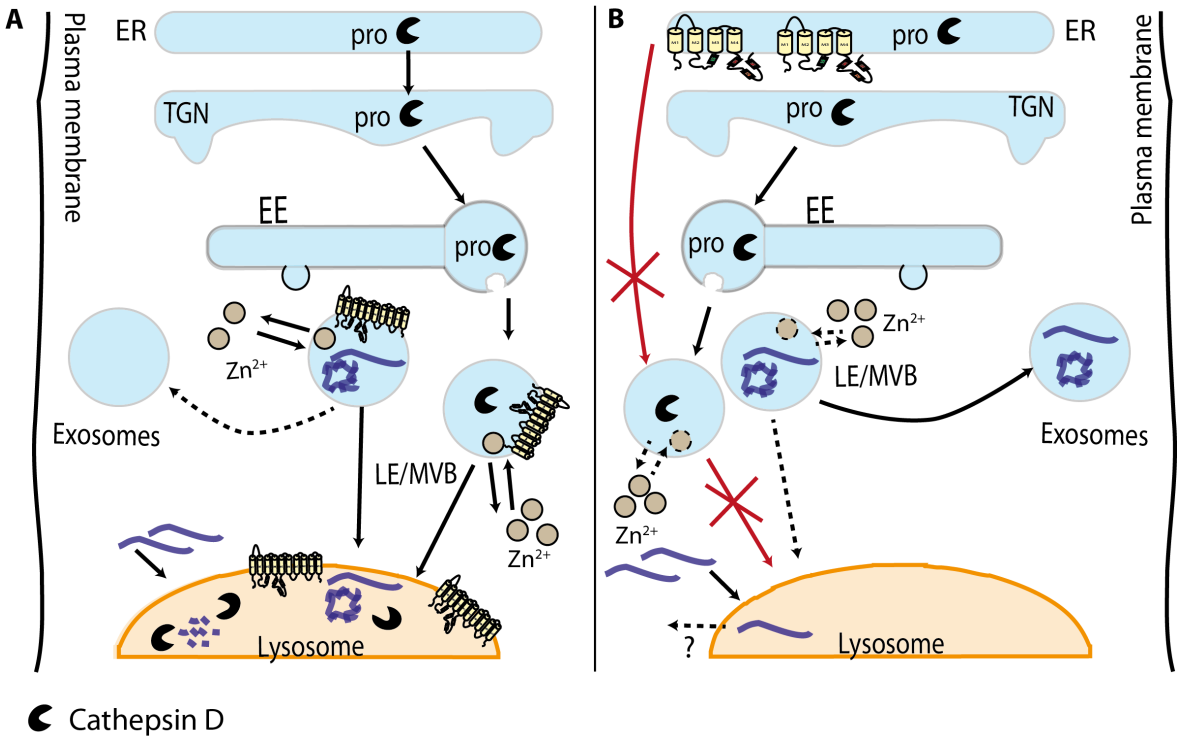


Figure 6. The hypothetical interplay between ATP13A2, Zn^{2+} and α-Syn. A) In normal conditions CatD is processed via the TGN and delivered to the lysosome via the endosomal pathway. Importantly ATP13A2 appears to play an important role in the maturation of CatD. ATP13A2, together with Zn^{2+}, appear to regulate the intracellular fate of α-Syn towards either the lysosome or the extracellular medium via exosomes. **B)** Mutations in ATP13A2 or the absence of the protein can inhibit the maturation of CatD and its localization at the lysosome. In ATP13A2 KO mice the formation of aggregates positive for α-Syn has been described, an identical situation reported in CatD KO mice. In cell models mutations or KD of ATP13A2 promoted the release of α-Syn to the extracellular medium in a process that involved Zn^{2+} homeostasis.

1.5. Endoplasmic Reticulum Homeostasis

To ensure proper protein function, macromolecules need to undergo a meticulous process of synthesis and folding. The ER is one of the main organelles involved in this process, and is also an important fall-back player upon proteostasis impairment.

After production in the cytosol, two main protein types go through ER processing: water-soluble and transmembrane proteins, like ATP13A2. The ER also has a role

in the synthesis of lipids, that will be integrated in the majority of cell organelles such as mitochondria, lysosomes, peroxisomes, Golgi and even the plasma membrane (267). As previously mentioned, the ER also provides *de-novo* membranes to the autophagosome, via Atg 9, and is essential in macrosecretion (268, 269).

The accumulation of misfolded, mutated or aggregated proteins at the ER lumen triggers an organelle response, commonly referred to as ER stress, and the activation of a cascade of cellular pathways, known as Unfolded Protein Response (UPR). Depending on the amount of accumulated misprocessed proteins, and the duration of the stress, two opposite pathways can be activated. More specifically, under transitory stress, pro-survival pathways are initiated but upon prolonged stress, apoptotic cell death mechanisms assume control. Both cellular faiths rely in common initiators that lay on the ER membrane: the inositol-requiring enzyme 1 alpha (IRE1alpha), the protein kinase RNA-like ER kinase (PERK) and the activating transcription factor 6 (ATF6) (270-273). Under resolvable ER stress, the three cascades work in symbiosis by activating or inhibiting several pathways to promote cell survival. Briefly upon phosphorylation, PERK activates its downstream target eiF2α (eukaryotic translation initiation factor 2α), leading to the inhibition of new protein translation mechanisms and activation of an antioxidant, autophagy response (273, 274).

The IRE1alpha pathway is based on an unconventional splicing of the X-Box binding protein 1 (XBP1) that will actively promote the transcription of genes involved in protein folding, autophagy and ER-associated protein degradation (ERAD) (275, 276). IRE1alpha can also upregulate the Regulated IRE1-alpha-Depdendent Decay (RIDD) pathway that is responsible for mRNA degradation. Recent data suggests that RIDD action is crucial to maintaining the Death Receptor 5 (DR5) mRNA levels reduced and avoid the activation of caspase pathways. As for ATF6 the cytosolic fragment generated upon its cleavage will translocate to the nucleus and enhance the transcription of chaperones and ERAD associated genes (277). This latter cascade is the only of the three that is not active in the apoptotic and cell death signalling.

Prolonged (chronic) ER stress leads to an alternative, pro-apoptotic cascade. The majority of ER-mediated death signalling originates from the PERK pathway that, via ATF4, promotes the stable upregulation of the C/EBP-homologous protein

(CHOP) (274), a master player in ER stress. This protein will then upregulate DNA damage-inducible 34 protein (GADD34) inhibiting the pro-survival Bcl2 and upregulating pro-apoptotic Bax, which culminates in increased ROS production and activation of the caspase cascade (278, 279). Another player, which is common to both PERK and IRE1alpha pathways, is Nuclear Factor kappa B (NF-κB) that is activated either via eiF2α or TRAF2 (TNF receptor-associated factor 2), respectively (280-282). The latter protein can also activate the Apoptosis Signal-regulating Kinase 1 (ASK1) that ultimately leads to JUN amino-terminal Kinase (JNK) and caspase activation (283-285).

Activation of pro-survival versus apoptotic pathways requests a fine-tuned machinery and signalling. A recent work provided a deeper understanding on the mechanisms underlying the molecular shift between short-term and chronic ER stress (286). Under resolvable stress a survival cascade mediated by IRE1alpha compensates the pro-apoptotic cascade, initiated by PERK, that ultimately lowers DR5 mRNA levels. Nevertheless, in case of unmitigated stress the IRE1alpha pathway is somehow attenuated and the PERK cascade, via CHOP, increases the levels of DR5 mRNA. This leads to increased DR5 oligomerization and accumulation in the ER/Golgi, and promotes a Caspase 8-mediated programmed cell death (286) (Fig 7).

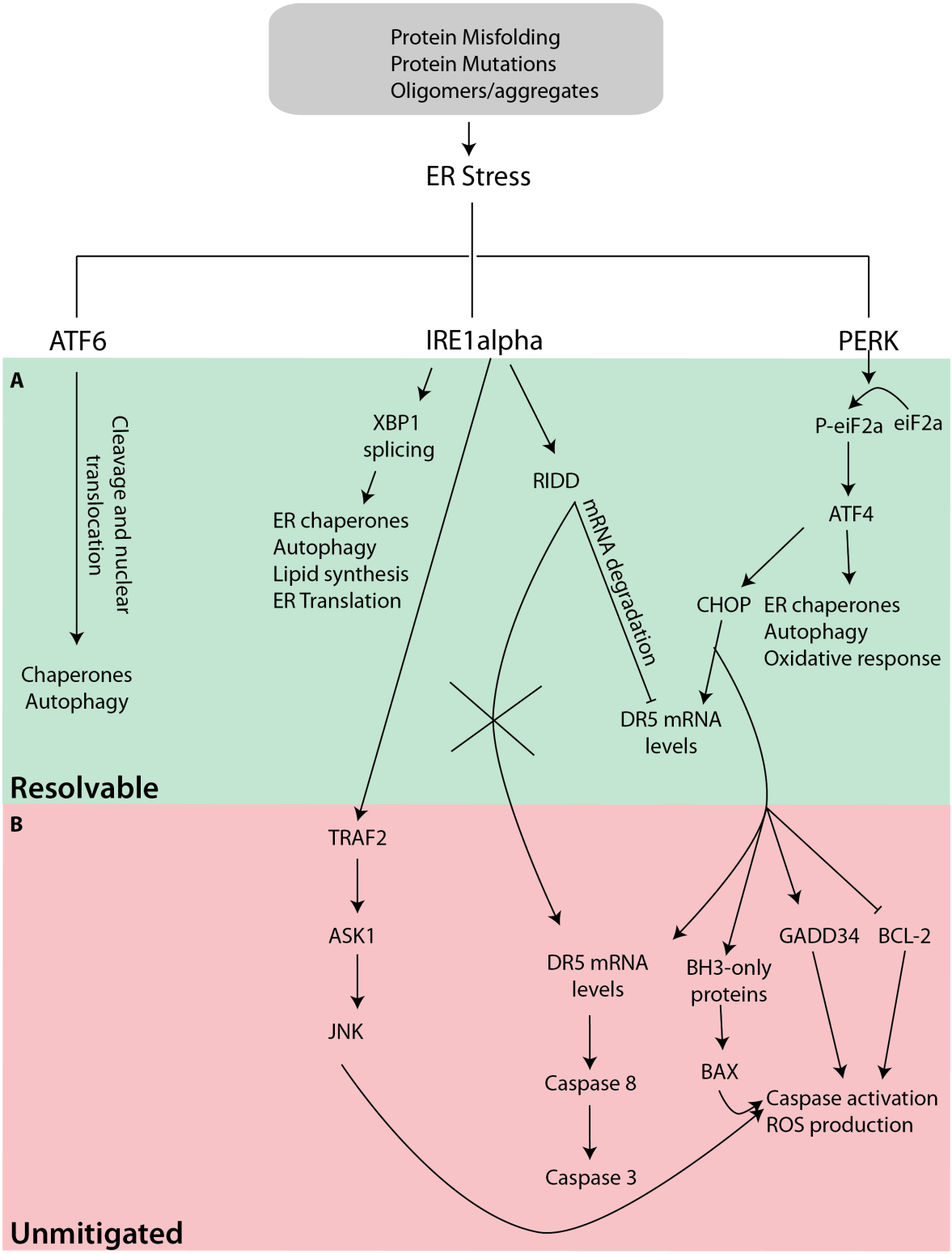


Figure 7. The two stages of ER stress. A) In resolvable ER Stress, the cell can respond with the activation of three pathways. The ATF6 can be cleaved in the cytosol and undergo nuclear translocation to activate genes related to chaperones and autophagy; IRE1alpha can promote the alternative splicing of XBP1 that will enhance the expression of genes associated to autophagy, lipid synthesis and ER chaperones; and IRE1alpha can also upregulate RIDD pathway that is responsible for mRNA degradation. Recent

data suggests that RIDD mechanism is crucial to maintain DR5 levels lower and avoid the activation of caspase pathways. On its turn, PERK can phosphorylate eiF2α that will act on ATF4 that will promote the expression of CHOP, a master player in ER stress. CHOP can increase the levels of DR5 mRNA levels and ATF4 can also, independently, increase the levels of proteins associated to autophagy, oxidative response and ER chaperones. **B)** Under unresolvable ER Stress conditions, several caspase pathways are activated. The better understood mechanisms is based on the increase of the DR5 mRNA levels due to the expression of CHOP that is not compensated by an increase in IRE1alpha and, subsequently, the RIDD pathway. The DR5 protein will then lead to the activation of the Caspase 8 pathway that culminates in caspase 3. Besides DR5, CHOP can also activate BAX and GADD34 and inhibit BCL-2 leading to apoptosis and ROS production. The IRE1alpha can, by itself, also promote apoptosis via the ASK/JNK pathway (286-289).

1.5.1. α-Syn, ATP13A2 and ER homeostasis

ER stress has been intimately linked to neurodegenerative disorders (290, 291). In the SN of PD patients a notorious increase in UPR markers was reported (292) with a similar activation of this cellular response being found in neurotoxin models of PD, including 6-hydroxydopamine (6-OHDA) and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyrine (293-296).

α-Syn oligomers accumulate preferentially in the ER (297), and the protein appears to be an important intermediate in ER stress upon manganese-induced toxicity, via PERK pathway (298). In a PD mouse model, neurons displaying α-Syn pathology exhibit abnormalities in the ER morphology, accompanied by upregulation of ER chaperones (299, 300). Overexpression of WT and mutant (A53T) α-Syn promotes an impairment of the ER to Golgi trafficking leading to ER stress, ERAD impairment, Golgi fragmentation and ultimately cell death (139, 301, 302). Also the α-Syn A53T mutant was associated to combined ER stress and mitochondria dysfunction (303), although the mechanisms underlying the ER stress component remain controversial (299).

Conversely, knowledge regarding the interaction between ATP13A2 and this organelle is less extended. Since it is a transmembrane protein, its processing requires a passage via the ER before being targeted to the lysosome. In some familial mutations, a lost of cellular localization and accumulation in the ER was

reported and increased susceptibility to ER stress-promoting compounds (189) (Fig. 8E).

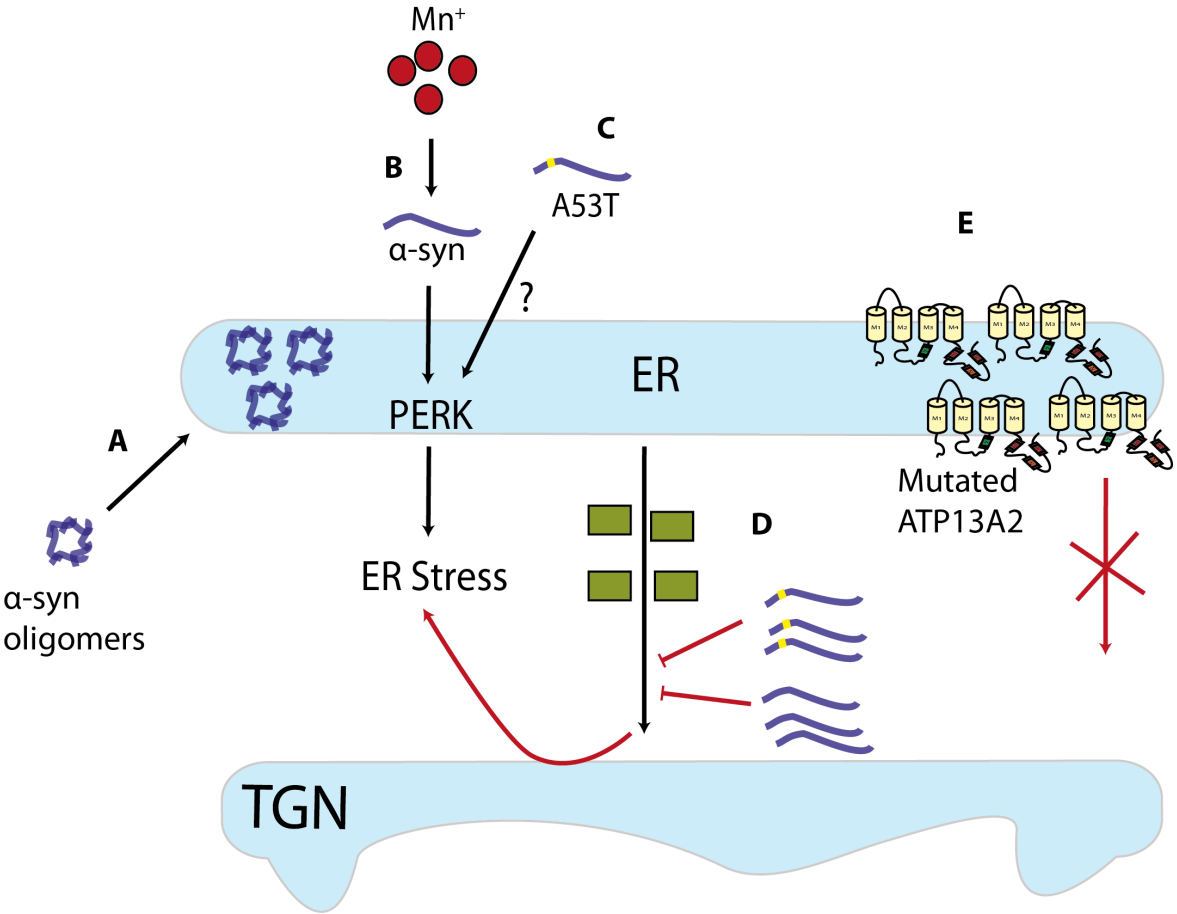


Figure 8. The effect of α-Syn and ATP13A2 on the ER. A) α-Syn oligomers have been found accumulated preferably in the ER. **B)** It has been described that α-Syn mediates Mn^{+} ER Stress and **C)** A53T α-Syn enhances cytotoxicity by promoting ER stress via PERK although the mechanism has not been fully validated. **D)** Increased levels of WT and A53T α-Syn in yeast and mammalian cells can block ER-to-Golgi trafficking promoting ER Stress. **E)** Some mutations in ATP13A2 disable the capability of the protein to be targeted to the lysosome and accumulate in the ER.

2. Aims of the study

Several genetic alterations have been associated with familial forms of PD. It has been suggested that the molecular imbalance underlying the disease onset can be common among all genes, since alterations in them can promote the appearance of identical pathological hallmarks in patients. Therefore, it becomes essential do understand how PD-associated proteins intersect in the intracellular ecosystem and how alterations in one protein can affect the homeostasis of others.

The objective of this thesis was to analyse the cellular interplay between two PD-related proteins, ATP13A2 and α-Syn, and the effect of a selected mutation in the homeostasis of this interaction. For this purpose, we started by studying how a mutation in ATP13A2, Dup22, could affect α-Syn and in particular the latter protein's propensity to oligomerize and aggregate. To achieve this goal we took advantage of pre-established cellular models that mimic both α-Syn oligomerization and aggregation. Additionally, using native α-Syn we confirmed the effect of ATP13A2 Dup22 on the α-Syn intracellular stability.

Simultaneously, to understand if the interaction between the two proteins altered cellular homeostasis, we investigated several pathways and organelles that have been previously associated to PD. In detail, we analysed the endolysosomal pathway, the ER homeostasis and mitochondria stability. Ultimately, we studied if the interplay between the two proteins had an effect on the overall intracellular toxicity and if specific pro-apoptotic pathways were activated.

In total, this study may provide new insights on the molecular mechanisms underlying the progression of PD and to better understand how two distinct disease-associated proteins can interact and influence cellular homeostasis.

3. Materials and Methods

3.1. Plasmid constructs

A total of 8 plasmids were used in the project as detailed in Table 2.

Table 2. Plasmid constructs

Protein	Vector	Reference
GFP	pcDNA 3.1+	NA
ATP13A2 WT GFP	pcDNA 3.1+	(189)
ATP13A2 Dup22 GFP	pcDNA 3.1+	(189)
α-Syn	pcDNA 3.1+	NA
SynT	pcDNA 3.1+	(304)
Synphilin-1	pcDNA 3.1+	(304)

3.2. Bacterial transformation and plasmid DNA purification

For transformation, the plasmid's DNA was incubated with DH5 alpha competent bacteria on ice for 30 minutes, followed by a heat shock at 42ºC for 45 seconds and 2 minutes on ice. Afterwards the DNA was incubated in SOC-medium for 1 hour at 37ºC. For large-scale purification with Nucleobond Xtra Midi (Macherey & Nagel, Germany), a 10 ml pre-inoculum in LB was prepared in the morning with the final LB volume of 100 ml being added overnight.

3.3. Cell Culture and Transfections

Human H4 cells were maintained at 37°C and 5% CO_2 in DMEM medium (PAN, Germany) supplemented with 10% Fetal Calf Serum and 1% penicillin-streptomycin. Cells were seeded in different well-plate formats, one day prior to transfection and kept up to 48 hours after transfection.

Transfections were performed with Fugene 6 (Roche, Germany), Metafectene (Biontex, Germany) or Calcium Phosphate. Fugene 6 and Metafectene transfections were done according to manufactures' instructions.

For Calcium Phosphate transfections, 3-5 hours prior to transfection fresh cell medium was added to the cells. For transfection, DNA was diluted in 1xHBS buffer (25mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), 140mM NaCl, 5mM KCl, 0.75 $Na_2HPO4*2H_20$, 6mM Dextrose, pH 7.1). After properly mixed, 2.5M CaCl2 was added dropwise and vigorously mixed. Followed 20 minutes of incubation, the mixture is added dropwise to the cells and the plate or dish gently rocked. The next morning, the medium was changed to avoid toxicity due to transfection procedure.

Experiments using the α-Syn Bimolecular Fluorescent complementation (BiFC) were perform in H4 cells stability expressing the two hemis of the system: VN-α-Syn and α-Syn-VC previously selected with G418 (Invivogen, USA)

3.4. Immunocytochemistry

For immunofluorescence microscopy, cells were fixed with 4% parafolmaldahyde 48 hours after transfection, sequentially treated with 0.5% Trinton X-100 and 1,5% normal goat serum or Bovine Serum albumin in Phosphate Buffered Saline (PBS). Primary antibodies were incubated in a 1:1000 solution overnight at 4ºC. Secondary Alexa Fluor antibodies were incubated for 2 hours at room temperature followed by Nuclei stained with Hoechst 33258 in a 1:5000 in PBS for 5 min. Images were captured, in a blind process, using a Leica DMI 6000B microscope (Germany) or Olympus IX81-ZDC (Germany) microscope and analyzed with Image J software (305). For counting the number of aggregates per cell, three different categories were chosen: cells with no aggregates, cells with less than 10 aggregates and cells with 10 or more aggregates.

NIAD-4 staining (Glixx Labs, USA), to identify the presence of amyloid-like structures, was performed by incubating cells with the dye at a final concentration of 10 μM for 30 min at room temperature followed by immunocytochemistry as previously described.

3.5. Live-cell imaging microscopy

For live imaging, cells were plated on µ-Dish 35 mm Ibidi dishes two days prior to transfection. Imaging was performed using an Olympus IX81-ZDC microscope under controlled conditions: 37ºC, 5% CO2 and 55-60% humidity. Recordings were performed up to 6 hours and started 6 hours after transfection.

For investigating mitochondrial morphology, live-cell imaging was performed using Mitotracker® (Invitrogen, USA). Cells were incubated in PBS with a final concentration of 100nM of Mitotracker for 20 minutes at 37ºC. Black and white images of mitochondria were obtained using a macro for imageJ first described in (306).

Living staining of lysosomes was performed using Lysotracker® (Invitrogen, USA). Cells were incubated in PBS for 30 minutes in a final concentration of 75 nM of the dye. Afterwards cells were analysed in the microscope without changing the media.

Live imaging of membranes was performed using an FM Dye. Technically this dye allows the distinction between extracellular and intracellular membranes based on incubation time. A short period incubation of 5 minutes stains extracellular membranes while a longer incubation of 30 minutes followed by washing in PBS allowed the visualization of intracellular membranes.

3.6. Immunoblotting analysis

For immunoblotting analysis, cells were incubated in lysis buffer (25mM Tris HCl pH 7.6, 150mM NaCl, 1%NP-40, 1% Sodium deoxycholate, 0.1% Sodium dodecyl sulfate) freshly supplemented with protease inhibitor cocktail (Roche, Germany) for 30 minutes on ice. After they were sonicated once for 10 seconds on ice and centrifuged at maximum speed (14000 rpm) for 10 minutes. The supernantant was collected and the pellet discarded.

Protein quantification was done using the Bradford assay (307) with absorbance measurements performed in the Infinite M200 PRO plate reader (Tecan Ltd, Switzerland) at 595 nm. Before loading onto the gel, between 30µg to 60µg of protein was mixed with 5x Laemmli buffer (250mM Tris pH 6.8, 10% Sodium dodecyl sulfate, 1.25% Bromophenol Blue, 5% β-Mercaptoethanol, 50% Glycerol).

Cell lysates were electrophoresed through 12% or 15% sodium dodecyl sulfate polyacrylamide (SDS-PAGE) gels under denaturing conditions and transferred to nitrocellulose (GE Healthcare) or PVDF (Milipore) membranes for 2 hours using a regular wet transfer procedure using a 25mM Tris, 192mM Glycin with 20% methanol buffer. Afterwards the membranes were blocked either with 5% skim milk or 5% Bovine Serum Albumin in tris-buffered saline for 1 hour and were then incubated with primary antibodies overnight at 4°C. HRP-conjugated secondary antibodies were applied for 2 hour at room temperature in a 1:10000 dilution in tris-buffer saline. Between blocking and antibodies incubations, membranes were washed 3 times for 10 minutes with tris buffer saline with 1% Tween 20.

Membrane visualization was performed on either an Alpha Imager (Alpha Innotech, USA) or Fusion Fx (Vilber Lourmat, France) with Immobilon Western Chemiluminescent HRP substrate (Merck Milipore, USA). Quantification of bands was performed using Image J software (305).

Table 3. Primary antibodies used.

Antibodies	Species	Company
α-Syn	Mouse	BD Bioscience (USA)
	Rabbit	Santa Cruz Biotech (USA)
p-α-Syn	Mouse	Wako (USA)
V5	Rabbit	Abcam (UK)
b-actin	Mouse	Sigma (USA)
CHOP	Mouse	Thermo Scientific (USA)
CatD	Goat	Santa Cruz Biotech (USA)
Atg5	Rabbit	Abcam (UK)
GM130	Mouse	BD Bioscience (USA)
Calnexin	Rabbit	Gene Tex (USA)

Table 4. Secondary antibodies used.

Antibodies	Recognizing	Company
Alexa Fluor 555 Donkey	Mouse	Invitrogen (USA)
	Rabbit	
Alexa Fluor 633 Donkey	Goat	
AMCA Goat	Mouse	Jackson Immunoresearch (USA)
	Rabbit	
ECL, HRP-Linked	Mouse	Amersham (UK)
	Rabbit	
ECL, HRP-Linked	Goat	Jackson Immunoresearch (USA)
Atto 647N	Mouse	Sigma (USA)

3.7. Proteinase K Resistance

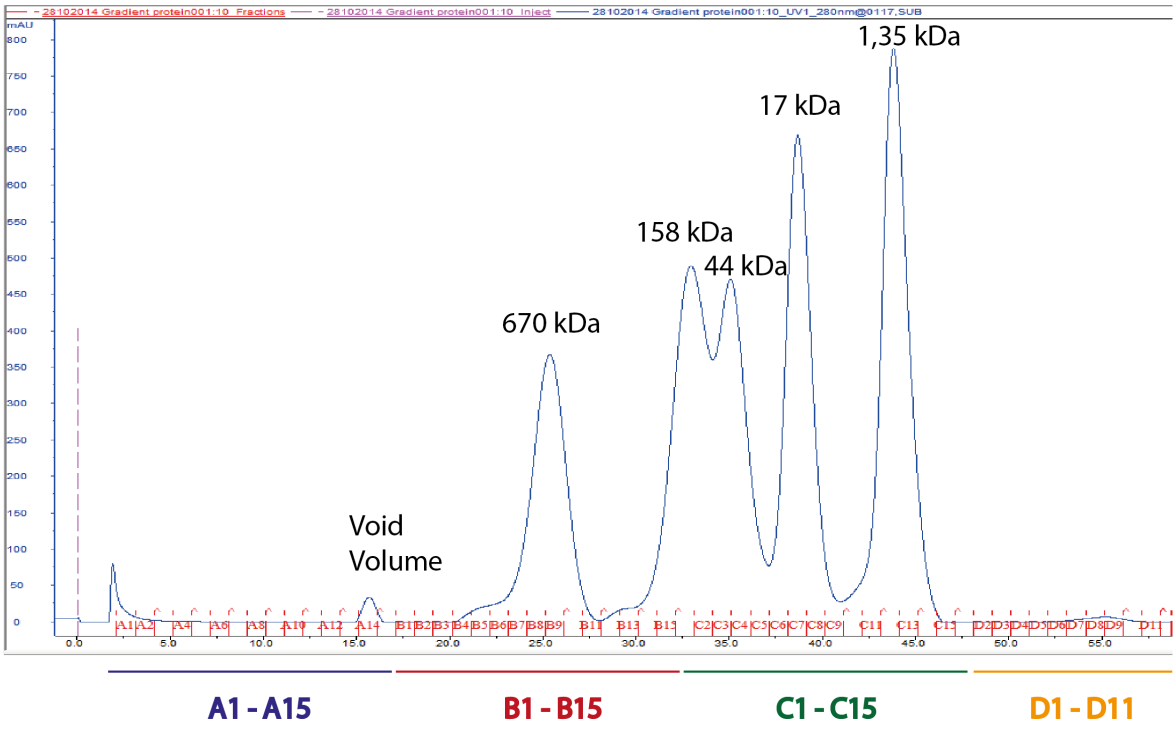
Cells were collected 48 hours after transfection in phosphate buffer (1X PBS with 1% TritonX-100). Lysates were sonicated for 10 seconds at 40-50% and afterwards incubated with 2,5ug/ml of freshly prepared Proteinase K for different time periods :0, 5 and 10 minutes. Finally the lysates were boiled at 95ºC for 5 minutes and loaded onto 12% SDS-PAGE gels, and immunoblots were performed as previously described.

3.8. Size Exclusion Chromatography and Dot blot

Cells were collected 48 hours after transfection in phosphate buffer (1X PBS with 0,5% TritonX-100) freshly supplemented with protease inhibitor cocktail (Roche, Germany) and centrifuged for 10 minutes at 10,000g. 2-3mg of total protein in a maximum volume of 500ul was filtered using a 0,45µm Spin-X centrifuge filter before loading onto a Superose 6 (Superose 6 10/300GL. GE Healthcare Life Science, Sweden) column and subsequent processed by Size Exclusion Chromatography by high-performance liquid chromatography (SEC-HPLC) (Äkta

Purifier 10, GE Healthcare Life Science, Sweden). The run was performed with a flow rate of 0,5ml/min for 1.2 Column Volumes and a total of 56 fractions with 500ul were collected. The theoretical molecular weight sizes for each fraction were assessed using a SEC standard (BioRad, USA). The standard is composed by 5 proteins of known molecular weights: Vitamin B12 (1,35 kDa), Myoglobin (17 kDa), Ovalbulmin (44 kDa), γ-globulin (158 kDa) and Thyroglobulin (670 kDa) (Fig. 9A). For the dot blot assay, these fractions were boiled at 95ºC for 10 minutes and centrifuged at 10,000g for 5 minutes. Afterwards they were loaded to a nitrocellulose membrane with the schematic exemplified in Fig. 9B.

A



B

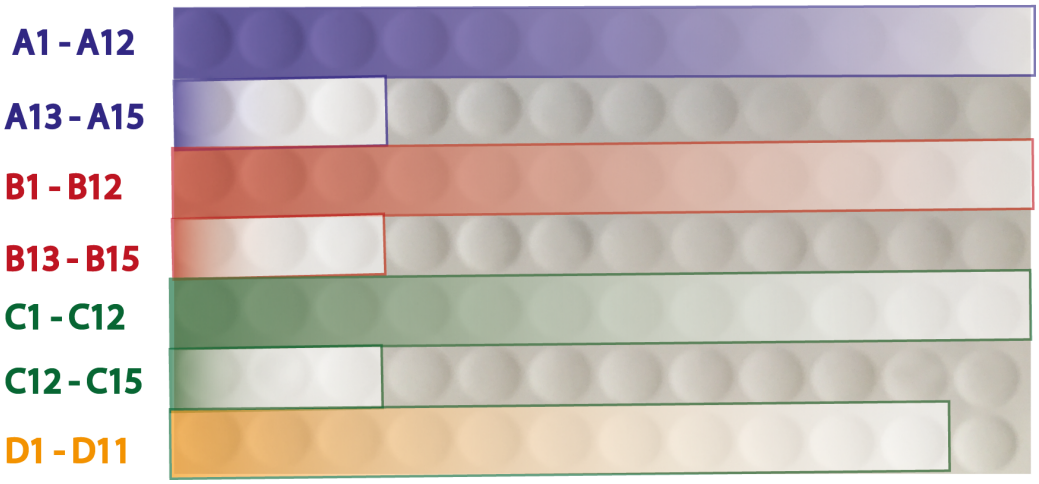


Figure 9. SEC-HPLC Gradient and Dotblot. A) A protein gradient was used as a standard to predict the approximate molecular weights of our samples. The run, with 1.2 column volumes of length, generated a total of 56 samples that were labelled as A1 to A15, B1 to B15, C1 to C15 and D1 to D11. **B)** Schematics of how SEC-HPLC samples were loaded onto the dotblot apparatus.

3.9. RNA extraction and cDNA synthesis

RNeasy Mini Kit (Qiagen, Germany) was used to extract and purify total RNA from H4 cells. RNA quality was determined spectrophotometrically, and RNA (300 ng) was reversed transcribed into cDNA, following genomic DNA removal, by using QuantiTect Reverse Transcription Kit (Qiagen, Germany) containing oligo-dT and random primers.

3.10. Real-time PCR analysis

Quantitative real-time polymerase chain reactions (qPCRs) were performed using a Mx300P cycler (Agilent Techonolgy, USA), and samples were prepared according to the Mesa Blue qPCR MasterMix Plus for SYBR Assay (Eurogentec, Belgium) protocol. For genes of interest, custom primers were designed and checked for target specificity using the bioinformatics Primer-Blast (308). The primers used can be seen in Table 5.

Table 5. Primers used for qPCR

Gene		Sequence
CHOP	Forward Primer	5'-AGTCTAAGGCACTGAGCGTA-3'
	Reserve Primer	5'-TTGAACACTCTCTCCTCAGGT-3'
DR5	Forward Primer	5'-TGCTCTGATCACCCAACAAG-3'
	Reserve Primer	5'-CTGAGATATGGTGTCCAGGTG-3'
ATP13A2	Forward Primer	5'-CAGTTTCATCCGTGAGGCT-3'
	Reserve Primer	5'-CACGACGTGATAGCCGATGA-3'
α-Syn	Forward Primer	5'-AGTGACAAATGTTGGAGGAG-3'
	Reserve Primer	5'-GCTTCAGGTTCGTAGTCTTG-3'
β-actin	Forward Primer	5'-GCGAGAAGATGACCCAGATC-3'
	Reserve Primer	5'-GCGAGAAGATGACCCAGATC-3'

β-actin was used as RNA loading control after confirming consistent expression across all samples. For each gene, dissociation curves were verified and negative controls, both non-reversed RNA and no-template, were included in each plate to ensure specificity of the amplification. Samples from three independent experiments were loaded in triplicates and amplified as follows: 5 minutes at 95ºC, 40 cycles of 30 seconds at 95ºC and 1.5 minutes at 60ºC. Relative quantification of targeted genes was calculated using the $2^{-\Delta\Delta Ct}$ method (309). Briefly, Ct values from genes of interest were obtained via MxPro software and subtracted to the corresponding Ct value of β-actin (ΔCt). Fold-change expressions were subsequently calculated by subtracting ΔCt control sample (GFP transfected samples) to ΔCt of experimental condition (ΔΔCt). ANOVA was used to analyze significant changes in gene expression.

3.11. Mitosox assay

Cells were plated in 96-well plates and the experiments performed 48 hours after transfection. Cells were washed with PBS an incubated in Mitosox Red (Invitrogen) diluted in PBS for 20 minutes at 37ºC. Afterwards cells were once again washed in PBS and the signal (Exc: 510nm, Emi: 580nm) was measured with the Infinite M2000 PRO (Tecan, Mainz, Germany). Three basal

measurements were performed with the cells being challenged with 5% H_2O_2 up to 30 minutes.

3.12. Cytotoxicity assay

Cytotoxicity was measured via release of Lactate dehydrogenase (LDH) into the culture medium with a commercial Cytotoxicity Detection Kit (LDH) (Roche, Germany). Basic LDH release was measured in non-transfected cells, the maximal LDH release was measured by cell lysis in 2% Triton X-100. Absorbance was measured with the Infinite M2000 PRO (Tecan, Mainz, Germany) plate reader at 490 nm. Experimental values were calculated in percentages of the maximal LDH release and normalized to GFP as a control.

4. Results

The findings described in this section are a partial representation of the following publications:

Guowei Yin*, Tomas Lopes da Fonseca*, Sibylle E. Eisbach, Ane Martín Anduaga, Carlo Breda, Maria L. Orcellet, Éva M. Szegő, Patricia Guerreiro, Diana Lazaro, Gerhard H. Braus, Claudio O. Fernandez, Christian Griesinger, Stefan Becker, Roger S. Goody, Aymelt Itzen, Flaviano Giorgini, Tiago F. Outeiro and Markus Zweckstetter (2014) alphα-Synuclein interacts with the switch region of Raba8a in a Ser129 phosphorylation-dependent manner. Neurobiol. Dis, 70C, 149 – 161. * Equal contribution

Tomás Lopes da Fonseca, Raquel Pinho and Tiago F. Outeiro – A familial mutation in ATP13A2 enhances alpha-synuclein aggregation and promotes cell death. *Accepted for publication* Human Molecular Genetics.

4.1. The ATP13A2 Dup22 mutant exhibits altered intracellular localization

ATP13A2 is a transmembrane protein that is found at the late endosomes and lysosomes (32). Considering that, throughout the project, we used ATP13A2 fused to GFP, we started by investigating if the cellular localization of the native protein was maintained. Live cell imaging using lysotracker showed that ATP13A2 WT fused with GFP is present in late endosomes and lysosomes, similarly to what has been described for non-tagged protein (Fig. 9A). The Dup22 mutation, that eliminates 6 of the 10 transmembrane domains, promoted a mislocalization of the protein as shown by loss of co-localization with lysotracker (Fig. 9B).
This data confirms that the familial mutation Dup22 promotes a loss of intracellular localization in ATP13A2.

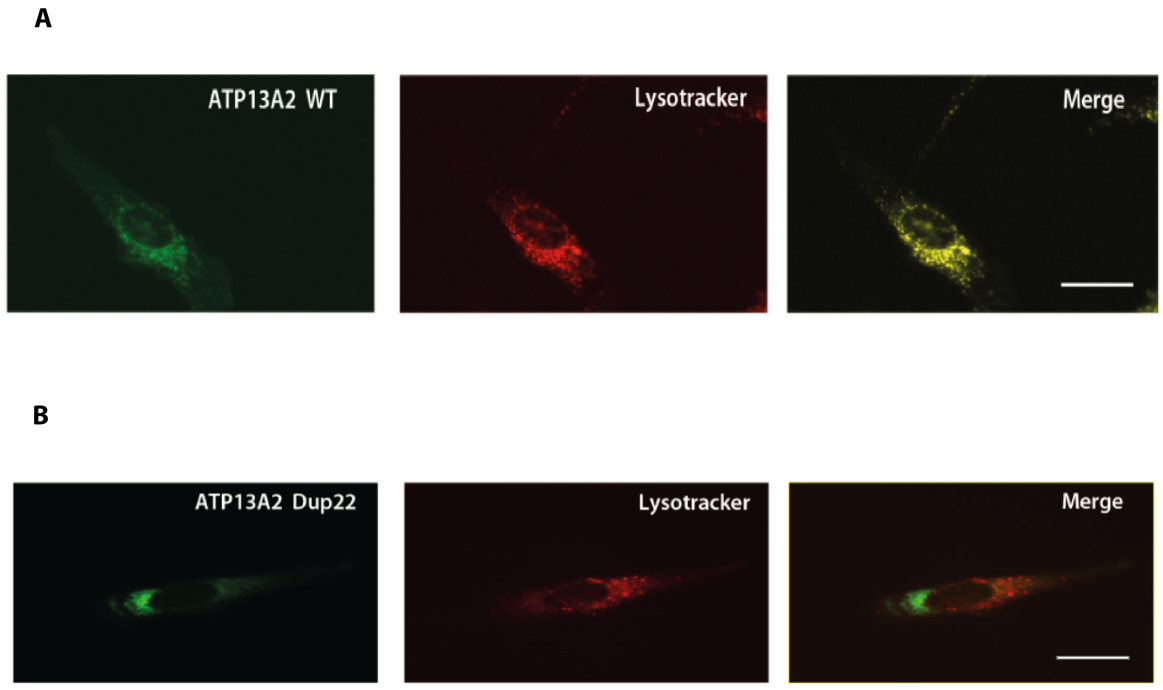


Figure 10. Dup22 mutation alters ATP13A2 cellular localization. A) ATP13A2 WT co-localizes with Lysotracker while **B)** its mutated version, ATP13A2 Dup22, loses its intracellular localization. Scale bar: 5 μm.

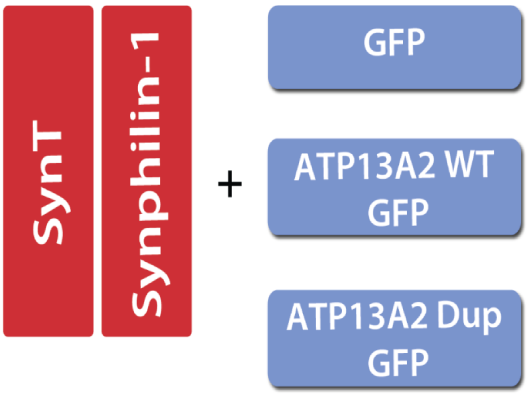
4.2. ATP13A2 Dup22 increases the propensity of α-Syn to aggregate in a cellular model

α-Syn aggregation and deposition inside brain cells is one of the hallmarks of synucleinopathies. Nevertheless, this is one of the most difficult to recapitulate in a petri disk and, particularly, in cell cultures. To better understand the effect of the ATP13A2 Dup22 mutation on α-Syn aggregation we used one of the few well-established cell-based models of α-Syn aggregation (304). Briefly, this model consists of the co-expression of SynT (α-Syn fused to an N-terminal fragment of GFP that renders α-Syn more prone to aggregation) and Synphilin-1 (Fig. 11A), an α-Syn-interacting protein that promotes α-Syn aggregation and is present in LBs in PD and other synucleinopathies. Co-expression of SynT and synphilin-1 results in the formation of intracellular inclusions (Fig. 11B) that share many of the features of LBs, such as Thioflavin S staining and co-staining with ubiquitin (304, 310).

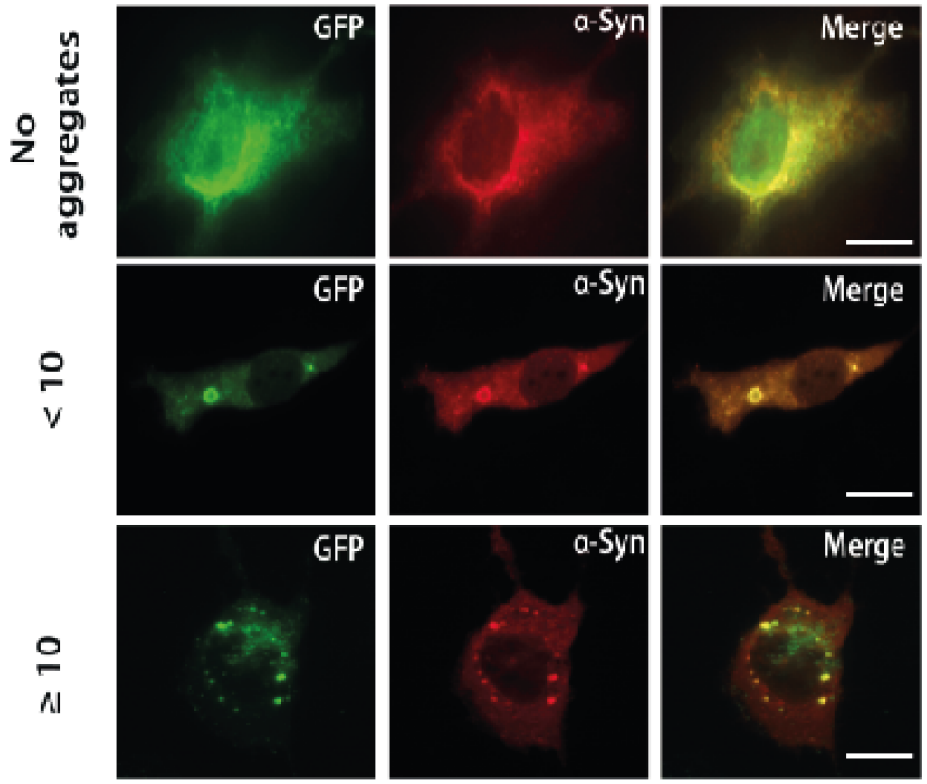
Using this model we observed that the ATP13A2 Dup22 mutation promoted two distinct effects: an increase in the percentage of cells with inclusions and an increase in the number of inclusions per cell (Fig. 11C). In contrast, the co-expression of ATP13A2 WT promoted no significant alterations on inclusion formation (Fig. 11C). Both ATP13A2 WT and Dup22 were present within the inclusions formed (Fig. 11D). Despite the alteration on inclusion formation, no alterations in the protein levels of ATP13A2 or α-Syn were observed (Fig. 11E), suggesting a mutual effect between the two proteins that did not influence the way cells processed the inclusions.

All together, these results indicate that ATP13A2 Dup22 mutation can drastically increase α-Syn aggregation propensity. Interestingly, this effect is independent of α-Syn protein levels.

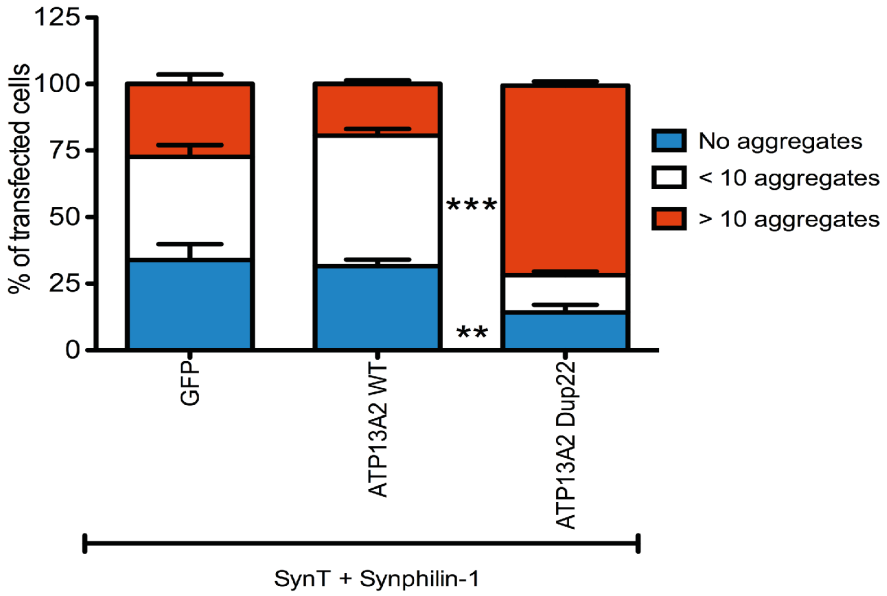
A


SynT
Synphilin-1
+
GFP
ATP13A2 WT GFP
ATP13A2 Dup GFP

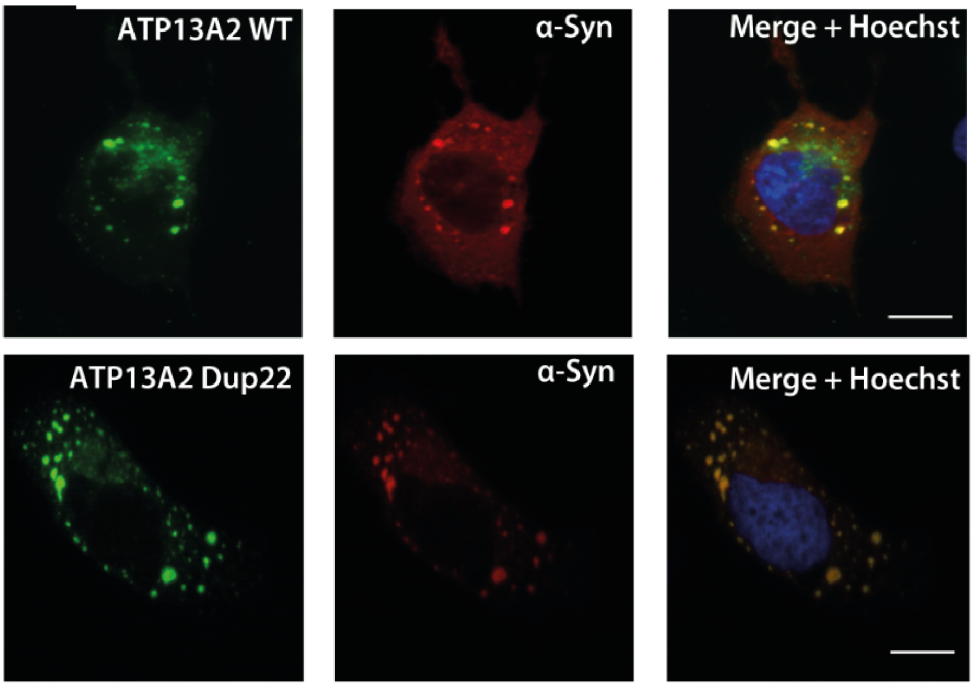
B


No aggregates
< 10
≥ 10
GFP
α-Syn
Merge

C


125
100
75
50
25
0
% of transfected cells
***
**
No aggregates
< 10 aggregates
> 10 aggregates
GFP
ATP13A2 WT
ATP13A2 Dup22
SynT + Synphilin-1

D


ATP13A2 WT
α-Syn
Merge + Hoechst
ATP13A2 Dup22
α-Syn
Merge + Hoechst

E

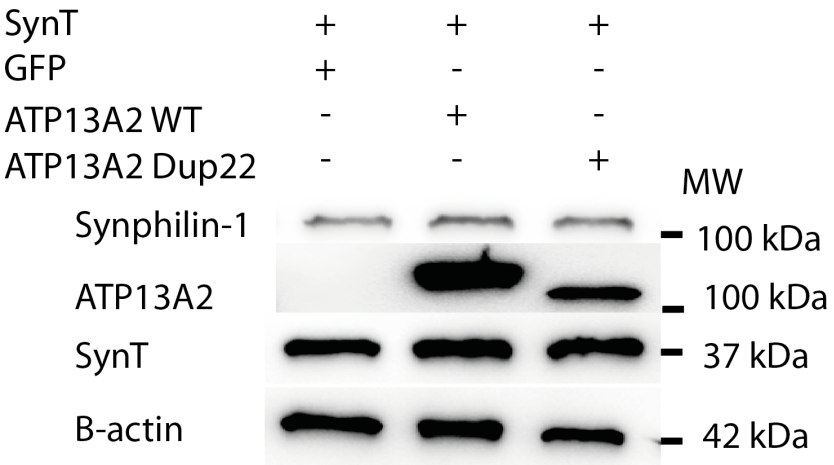

SynT + + +
GFP + - -
ATP13A2 WT - + -
ATP13A2 Dup22 - - +
MW
Synphilin-1
100 kDa
ATP13A2
100 kDa
SynT
37 kDa
B-actin
42 kDa

Figure 11. Effect of ATP13A2 on α-Syn aggregation. A) Triple transfections were executed to analyse the effect of ATP13A2 on α-Syn aggregation. In detail, SynT and Synphilin-1 were co-expressed with GFP, ATP13A2 WT GFP or ATP13A2 Dup22 GFP. **B)** Representative images for each category of number of aggregates: no aggregates, less than 10 aggregates, 10 or more aggregates. **C)** Expression of ATP13A2 Dup22 promoted an increased number of cells with aggregates and amount of aggregates per cell. **D)** ATP13A2 WT and Dup22 mutation co-localized with these proteinaceous intracellular inclusions (upper and lower painel, respectively). **E)** Immunoblot analysis revealed no difference in protein levels. All experiments are representative of N=3 independent experiments. Data are mean ± S.D. ANOVA with post-hoc Turkey's tests. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$. Scale bar: 5 μm.

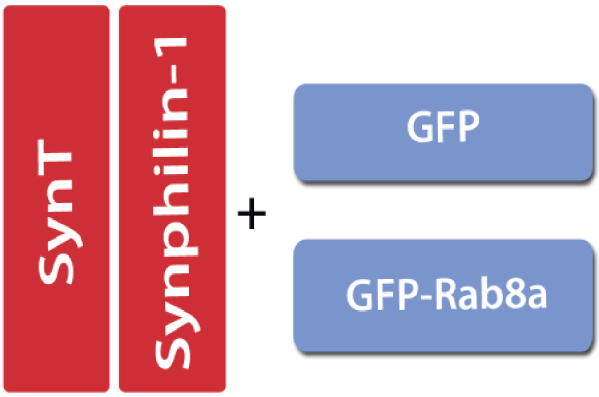
As mentioned above, ATP13A2 is a transmembrane protein belonging to the Endolysosomal machinery (32). This pathway has gained attention in the PD field as a possible mechanism underlying the intracellular pathology of the disease (221). Given the notorious effect of ATP13A2 on α-Syn aggregation, we then investigated the impact of another important player in this pathway, Rab8a, on α-Syn aggregation (237).

Rab8a is a Rab GTPase member of the endolysosomal pathway at the Golgi level, and has been extensively associated with α-Syn (236).

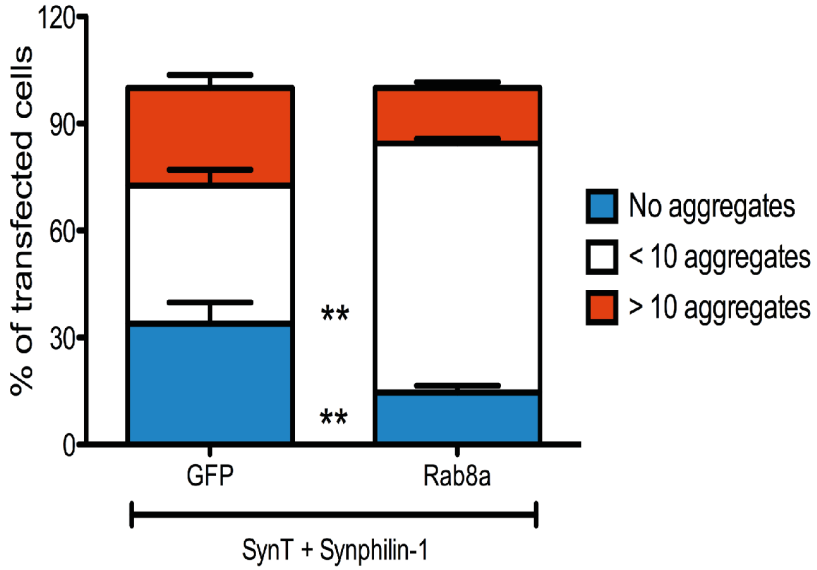
Taking advantage of the cellular model of α-Syn aggregation described above (Fig. 12A), we observed that Rab8a could also influence inclusion formation (Fig. 12B) without altering the levels of α-Syn (Fig.12C). *In vitro* studies further corroborate the effect of Rab8a on α-Syn aggregation (Fig. 12D). Interestingly, using immunoprecipitation assays, we concluded that α-Syn and Rab8a could directly interact *in vivo* including in the synaptic terminals (Fig. 12E). In Nuclear Magnetic Resonance (NMR) experiments we observed that the C-terminus of α-Syn is essential for this interaction and that this interplay was independent of Rab8a activity (Fig. 12F and G).

These data further confirms that the endolysosomal pathway plays an important role on α-Syn homeostasis. Furthermore the direct interaction between α-Syn and Rab8a at the synaptic level might indicate that α-Syn could be a functional piece of this intracellular mechanism.

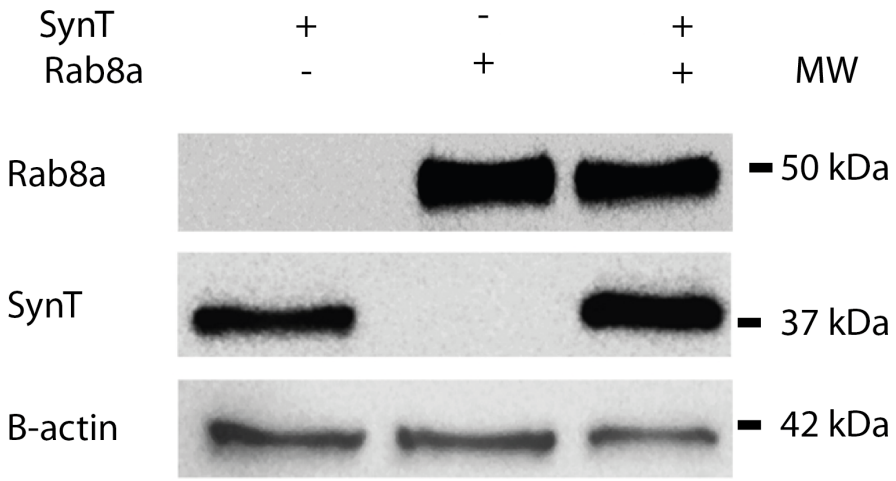
A


SynT
Synphilin-1
+
GFP
GFP-Rab8a

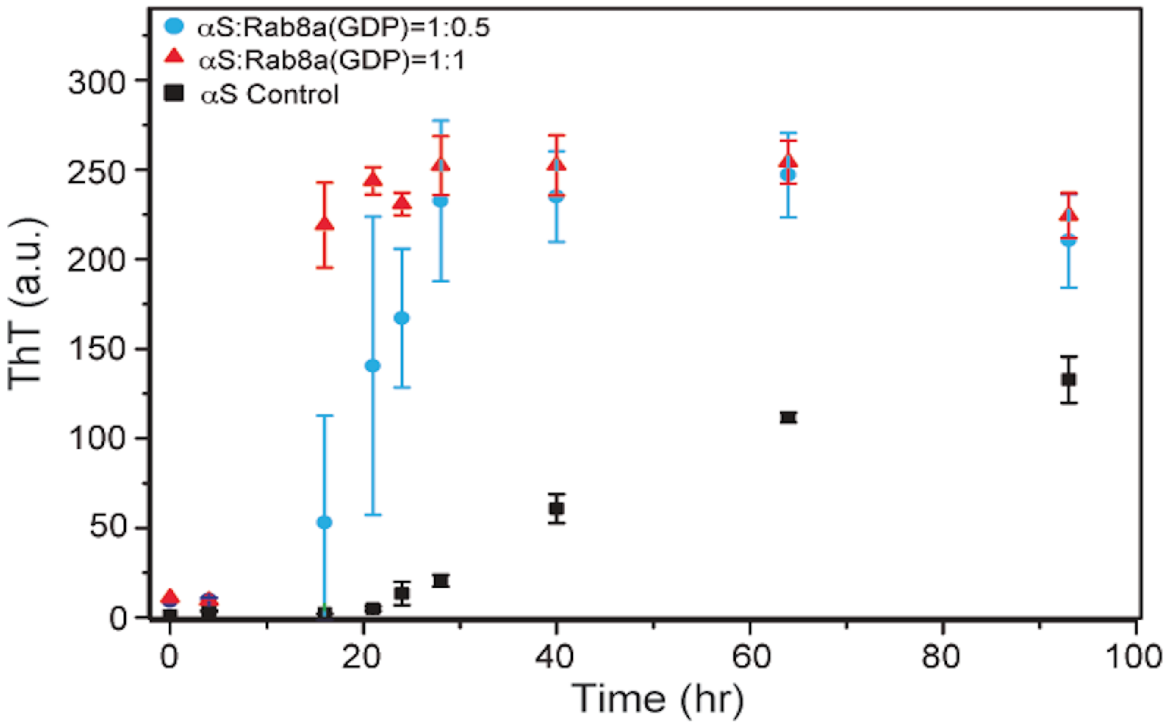
B


120
90
60
30
0
% of transfected cells
**
**
GFP
Rab8a
SynT + Synphilin-1
No aggregates
< 10 aggregates
> 10 aggregates

C


SynT + - +
Rab8a - + +
MW
Rab8a
50 kDa
SynT
37 kDa
B-actin
42 kDa

D


αS:Rab8a(GDP)=1:0.5
αS:Rab8a(GDP)=1:1
αS Control
ThT (a.u.)
0
50
100
150
200
250
300
0
20
40
60
80
100
Time (hr)

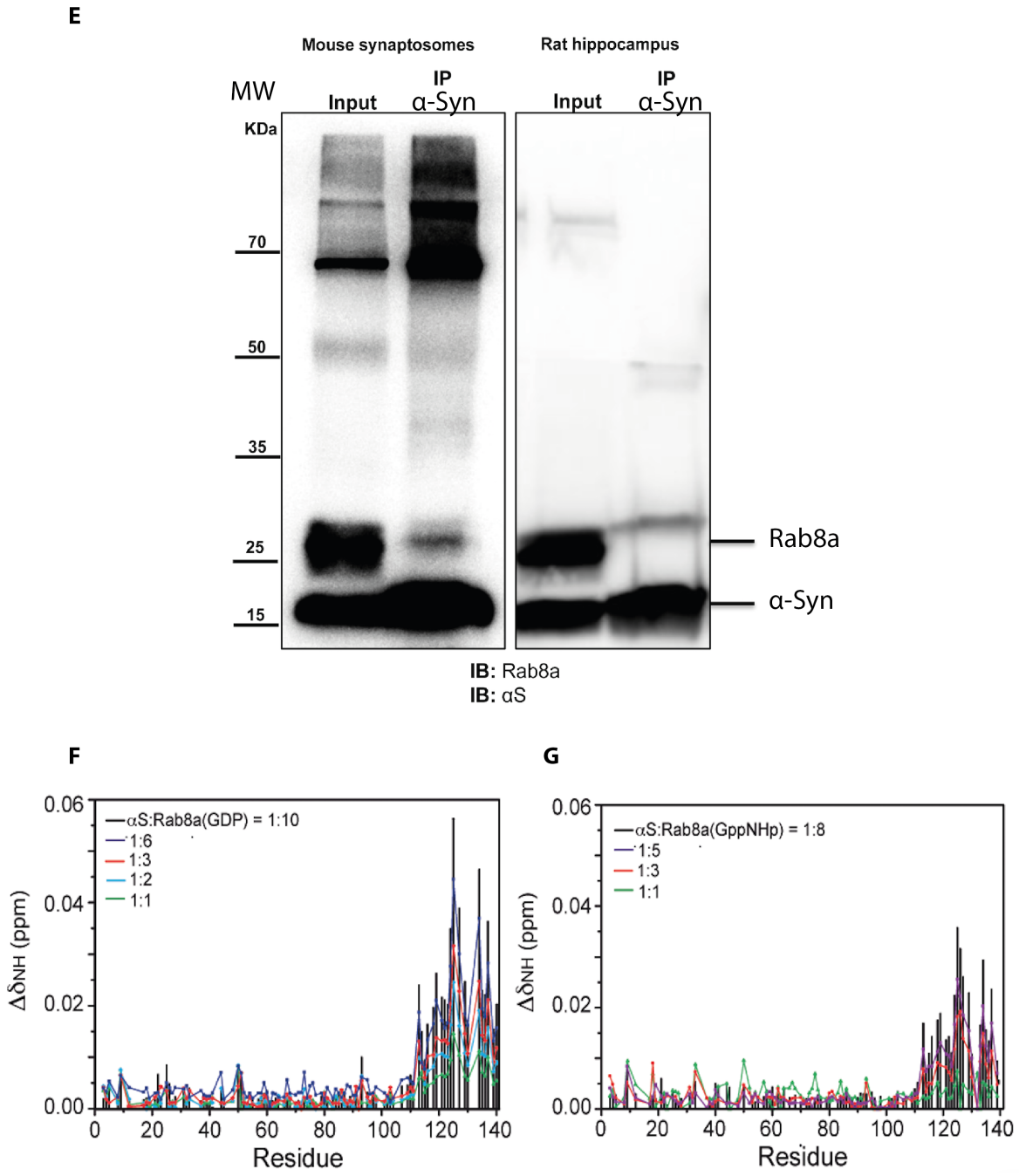


Figure 12. Rab8a modulates α-Syn aggregation in human cells. (A) Triple transfections were perfromed to analyse the effect of Rab8a on α-Syn aggregation. In detail, SynT and Synphilin-1 were co-expressed with GFP or Rab8a GFP. **(B)** Raba8a increases the total number of cells with aggregates when compared to the control, **(C)** without altering α-Syn total protein levels. **D)** In vitro studies, using recombinant protein, reveal that Rab8a can accelerate α-Syn aggregation and increase the amount of aggregated protein. **E)** α-Syn was immunoprecitated (IP) from rat hippocampus or mouse cortical synaptosomes and αS and Rab8a were detected (IB). **F)** Rab8a binds to the C-terminus of α-Syn. Averaged NMR chemical shift perturbation of 1H/15N resonances of α-

Syn in the presence of Rab8a (GDP) and **G)** Rab8a (GppNHp) for molar ratios up to 1:10 and 1:8 respectively of α-Syn/Rab8a. All experiments are representative of N=3 independent experiments. Data are mean ± S.D. ANOVA with post-hoc Turkey's test. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

The results described in Figure 12D, 12E, 12F and 12G were generated by colleagues from the labs of Prof. Dr. Tiago Outeiro's and Prof. Dr. Markus Zweckstetter.

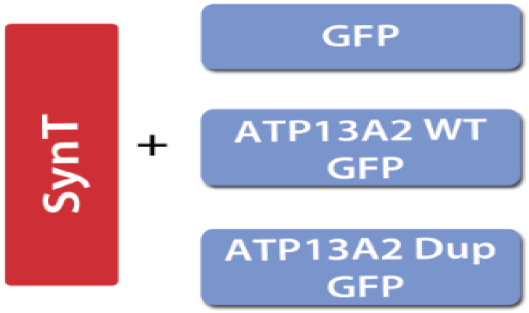
4.3. ATP13A2 Dup22 promotes the aggregation of SynT

The previous model relies on the co-expression of SynT and Synphilin-1. The latter is a well-known interactor of α-Syn that can influence its cellular homeostasis (311). For this reason we performed identical experiments to those described above, but in the absence of Synphilin-1 (Fig. 13A). In these experimental conditions we also observed the formation of intracellular aggregates and, similarly to the previous model, ATP13A2 Dup22 promoted the formation of more inclusions per cell, and increased the percentage of cells with inclusions (Fig. 13B and C). In this paradigm, without the presence of Synphilin-1, we observed that ATP13A2 WT also lead to an increase in the formation of inclusions (Fig. 13C), without altering the protein levels (Fig. 13D). The inclusions formed in this model, particularly upon the co-expression of ATP13A2 Dup22 and SynT, stained positive for phosphorylated α-Syn at residue serine 129 (p-α-Syn) and NIAD-4, a dye that binds to amyloid structures (312), two known markers of LBs (Fig. 13E and F).

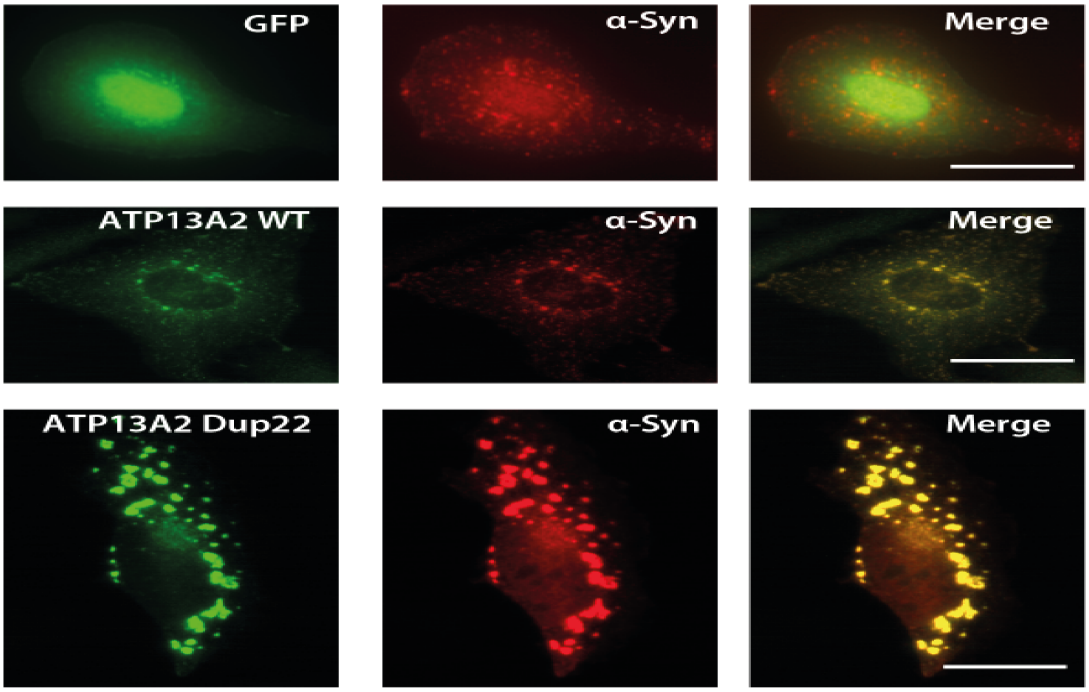
To further characterize the biochemical nature of the inclusions, we performed a proteinase K (PK) digestion assays. At the time points tested (0 minutes, 5 minutes and 10 minutes), we observed that co-expression of both ATP13A2 Dup22 and SynT resulted in a higher SynT resistance to PK treatment that was already noticeable after 10 minutes (Fig. 13G and H).

To conclude, we prove that ATP13A2 Dup22 can enhance SynT propensity to aggregate. Additionally, we showed that these aggregates are positive for p-α-Syn and amyloid structures and that, at a biochemical level, ATP13A2 Dup22 can also increase SynT resistance to PK treatment.

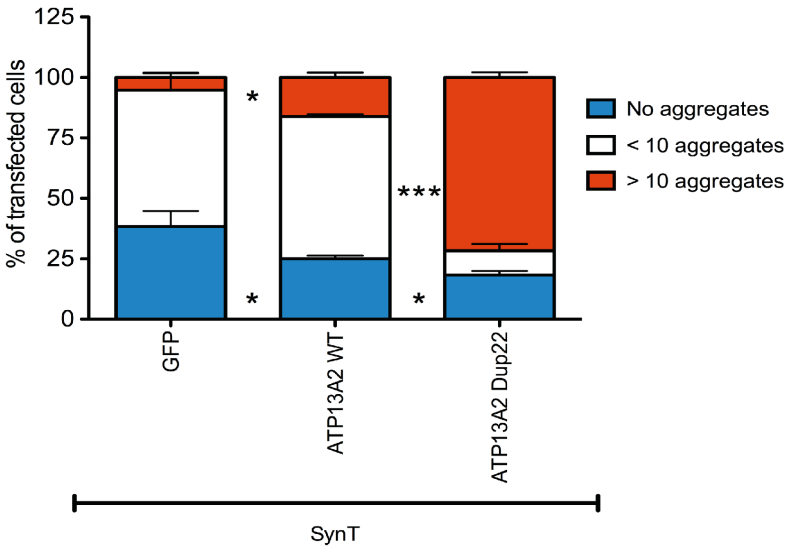
A


SynT
+
GFP
ATP13A2 WT GFP
ATP13A2 Dup GFP

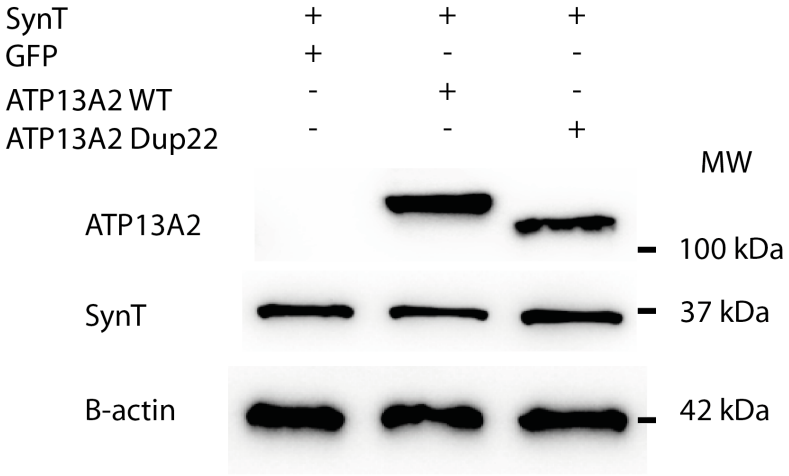
B


GFP
α-Syn
Merge
ATP13A2 WT
α-Syn
Merge
ATP13A2 Dup22
α-Syn
Merge

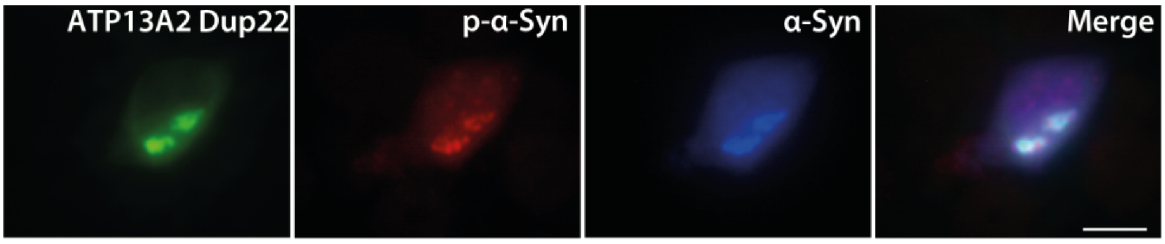
C


125
100
75
50
25
0
% of transfected cells
*
*
***
*
No aggregates
< 10 aggregates
> 10 aggregates
GFP
ATP13A2 WT
ATP13A2 Dup22
SynT

D


SynT + + +
GFP + - -
ATP13A2 WT - + -
ATP13A2 Dup22 - - +
MW
ATP13A2
100 kDa
SynT
37 kDa
B-actin
42 kDa

E


ATP13A2 Dup22
p-α-Syn
α-Syn
Merge

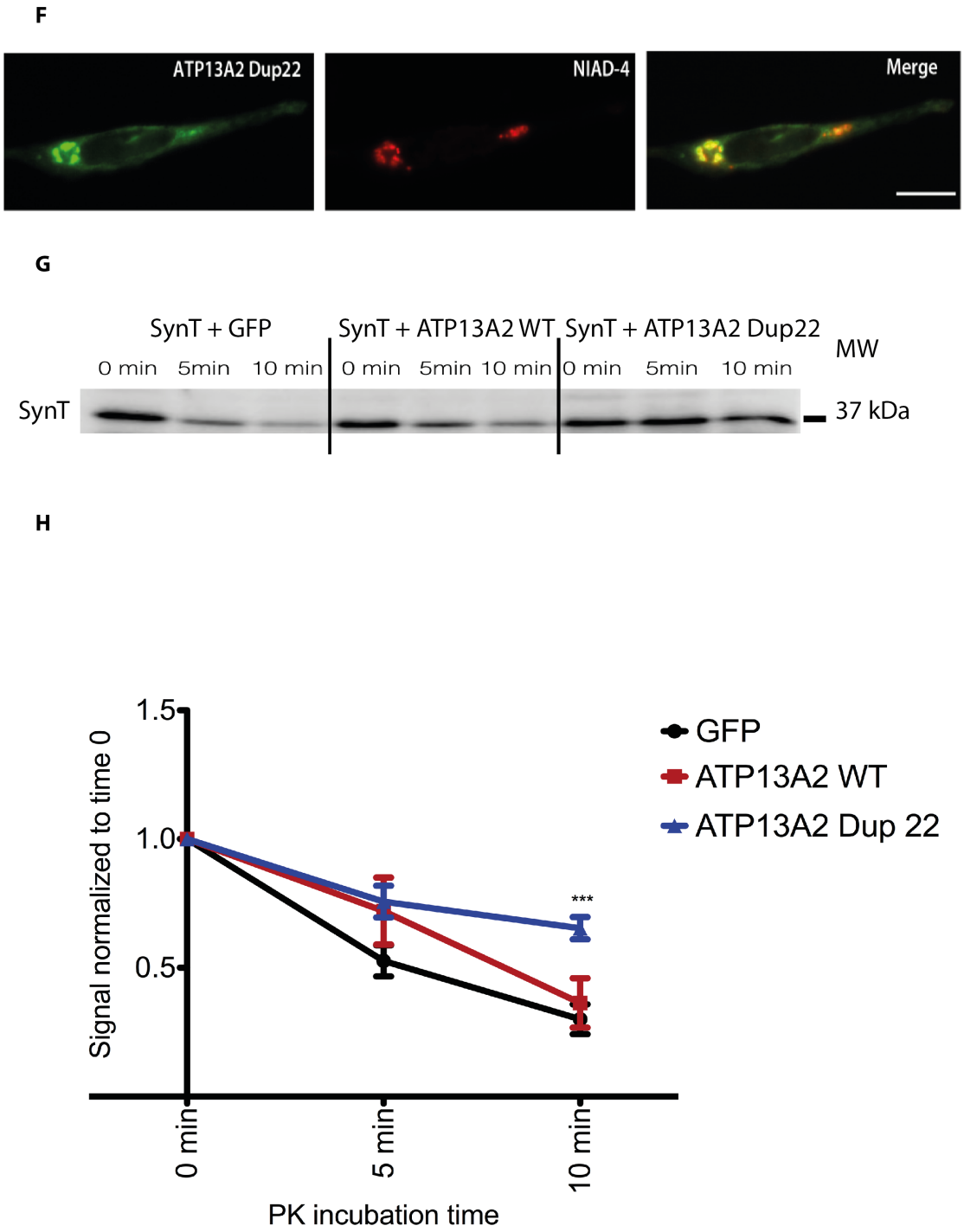


Figure 13. Effects of ATP13A2 on SynT aggregation. A) Double transfections were executed to analyse the effect of ATP13A2 on SynT. In detail, SynT was co-expressed with GFP, ATP13A2 WT GFP or ATP13A2 Dup22 GFP. . **B)** Expression of both WT and ATP13A2 Dup22 promoted the formation of SynT intracellular inclusions (middle and lower panel), even without the expression of Synphilin-1. **C)** Expression of ATP13A2 Dup22 promoted an increase in number of cells with aggregates and the amount of aggregates per cell. In this transfection paradigm, also ATP13A2 WT promoted a similar effect. **D)** Despite the increase in the amount of aggregates, no alterations in protein

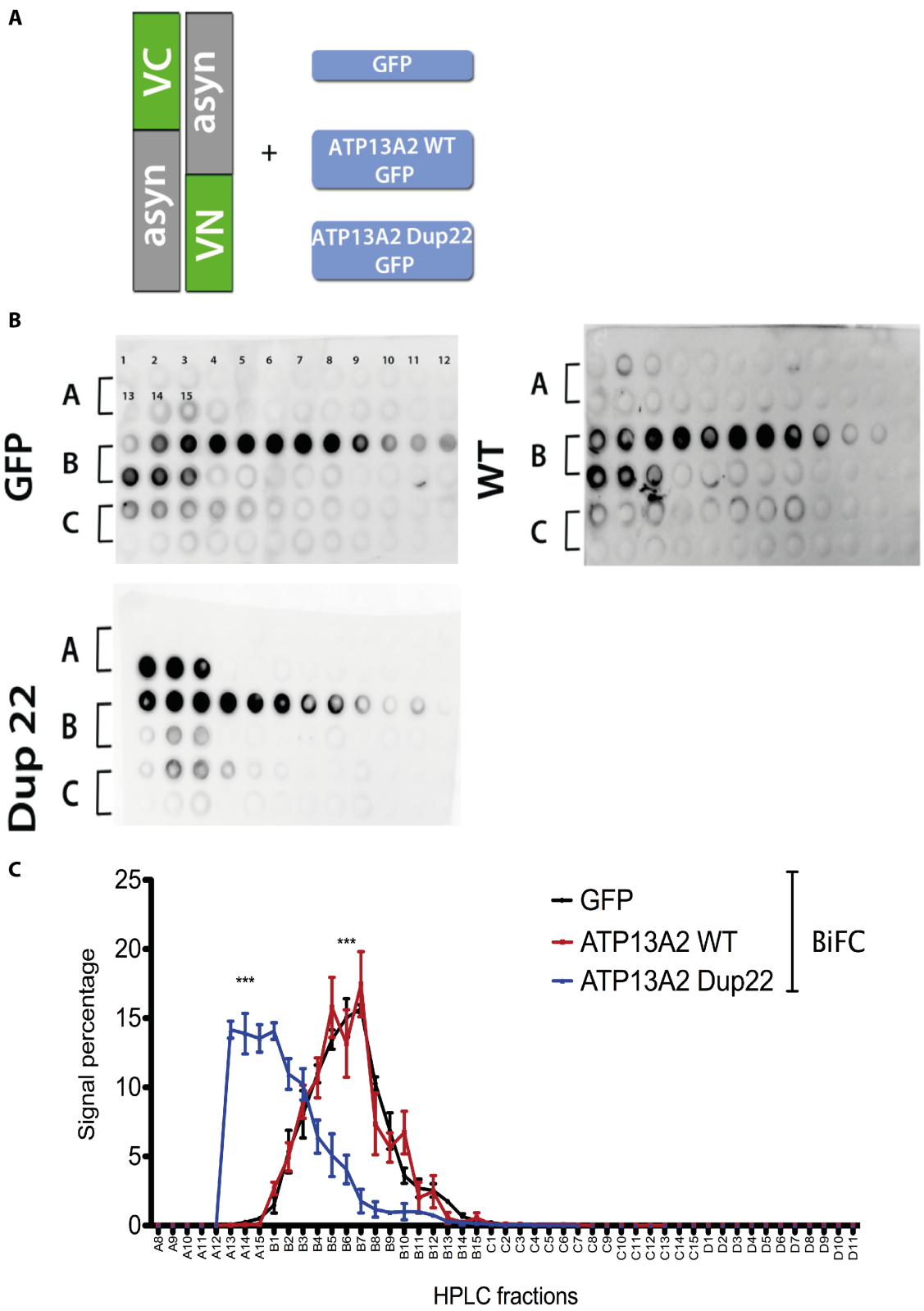
levels were detected by immunoblot. **E)** These aggregates stained positive for p-α-Syn and for **F)** NIAD-4, a marker for amyloid structures. **G)** Immunoblotting analysis for SynT reveals a higher resistance of the protein to PK treatment when co-expressed with ATP13A2 Dup22. **H**) Graphic representation of the effect of PK treatment. All experiments are representative of at least 3 independent experiments. Data are mean ± S.D. ANOVA with post-hoc Turkey's test. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$. Scale bar: 5 μm.

4.4. ATP13A2 Dup22 promotes the formation of higher molecular weight species in a cell model of α-Syn oligomerization

The precise origin of α-Syn toxicity is still unclear. Nevertheless, as previously stated, two diverging hypothesis suggest that either (i) large insoluble protein aggregates or (ii) intermediate oligomeric species, as the main culprits in synucleinopathies. To understand if the effect of the ATP13A2 Dup22 was exclusive to the latter stages of α-Syn aggregation, we used another cell model to study the impact of ATP13A2 on α-Syn oligomerization, based on the BiFC assay. BiFC was first generated in 2002 and later optimize for the study of α-Syn oligomerization (146, 313). Briefly, the assay is based on the capability of the reconstitution of a split fluorophore upon interaction of two proteins (Fig. 14A).

The majority of the studies using this assay use microscopy-based approaches in order to analyse changes in protein dimerization/oligomerization. This approach takes in consideration the assembly or disassembly of dimers, but does not provide direct evidence for the occurrence of changes in higher molecular species that were observed in the first report (146). To tackle this, we used SEC-HPLC followed by Dotblot analysis, and observed that the expression of ATP13A2 Dup22 promoted an increase in the molecular weight α-Syn oligomeric species (Fig. 14B and C). The expression of ATP13A2 WT promoted no alteration in the molecular weight pattern of α-Syn oligomers (Fig. 14B and C) and no detectable alteration in total protein levels (Fig. 14D). Importantly, we also found that the increase in the size of the oligomeric species leads to increased intracellular toxicity (Fig. 14E).

These data indicates that besides SynT aggregation, ATP13A2 Dup22 can also influence the initial steps of α-Syn oligomerization. Importantly, the formation of α-Syn higher molecular weight species promoted by ATP13A2 Dup22 is associated to an increase in intracellular toxicity.


A
VC
asyn
asyn
VN
+
GFP
ATP13A2 WT GFP
ATP13A2 Dup22 GFP
B
GFP
Dup 22
WT
A
B
C
1 2 3 4 5 6 7 8 9 10 11 12
13 14 15
C
Signal percentage
HPLC fractions
GFP
ATP13A2 WT
ATP13A2 Dup22
BiFC
***
***

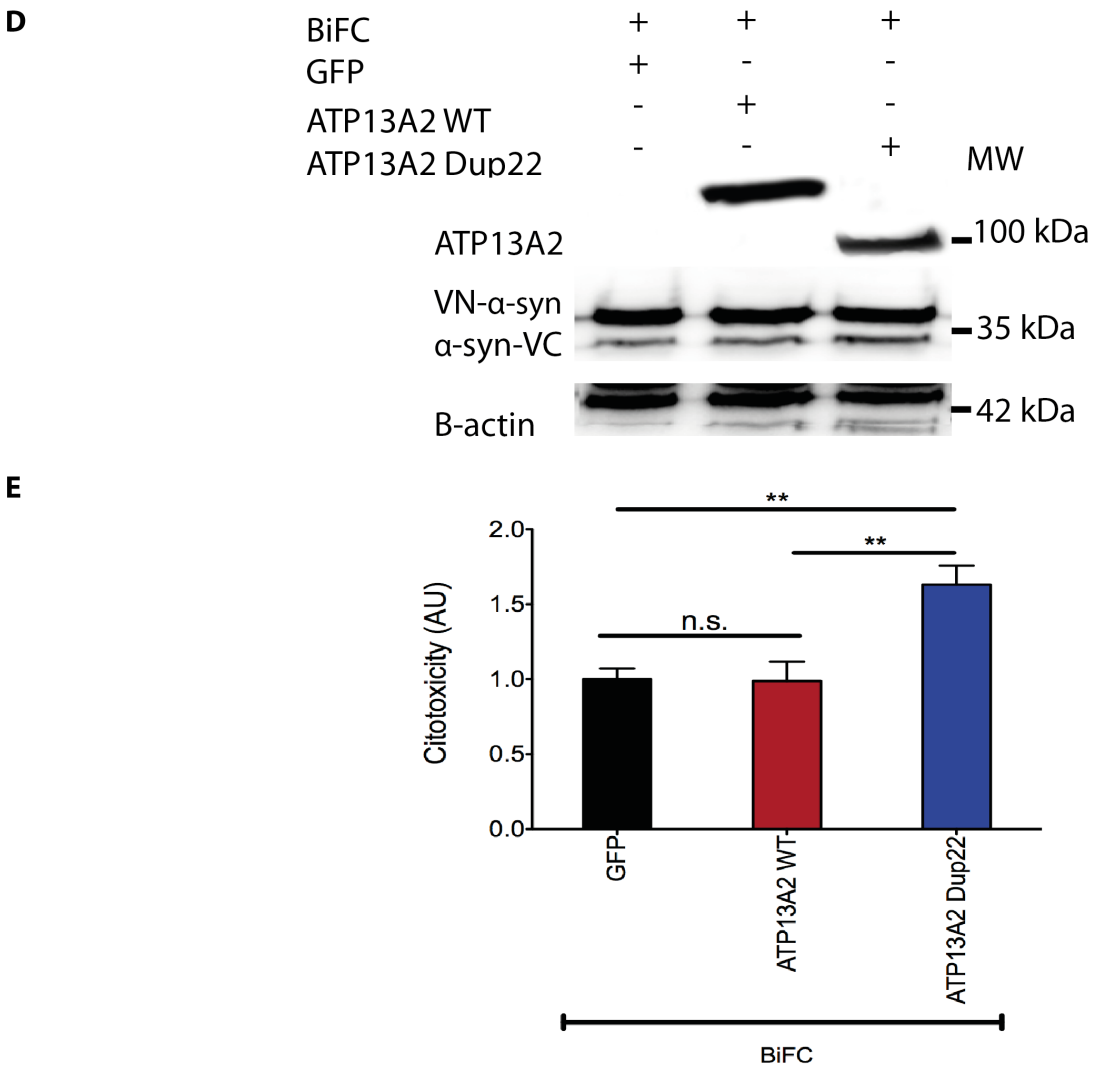
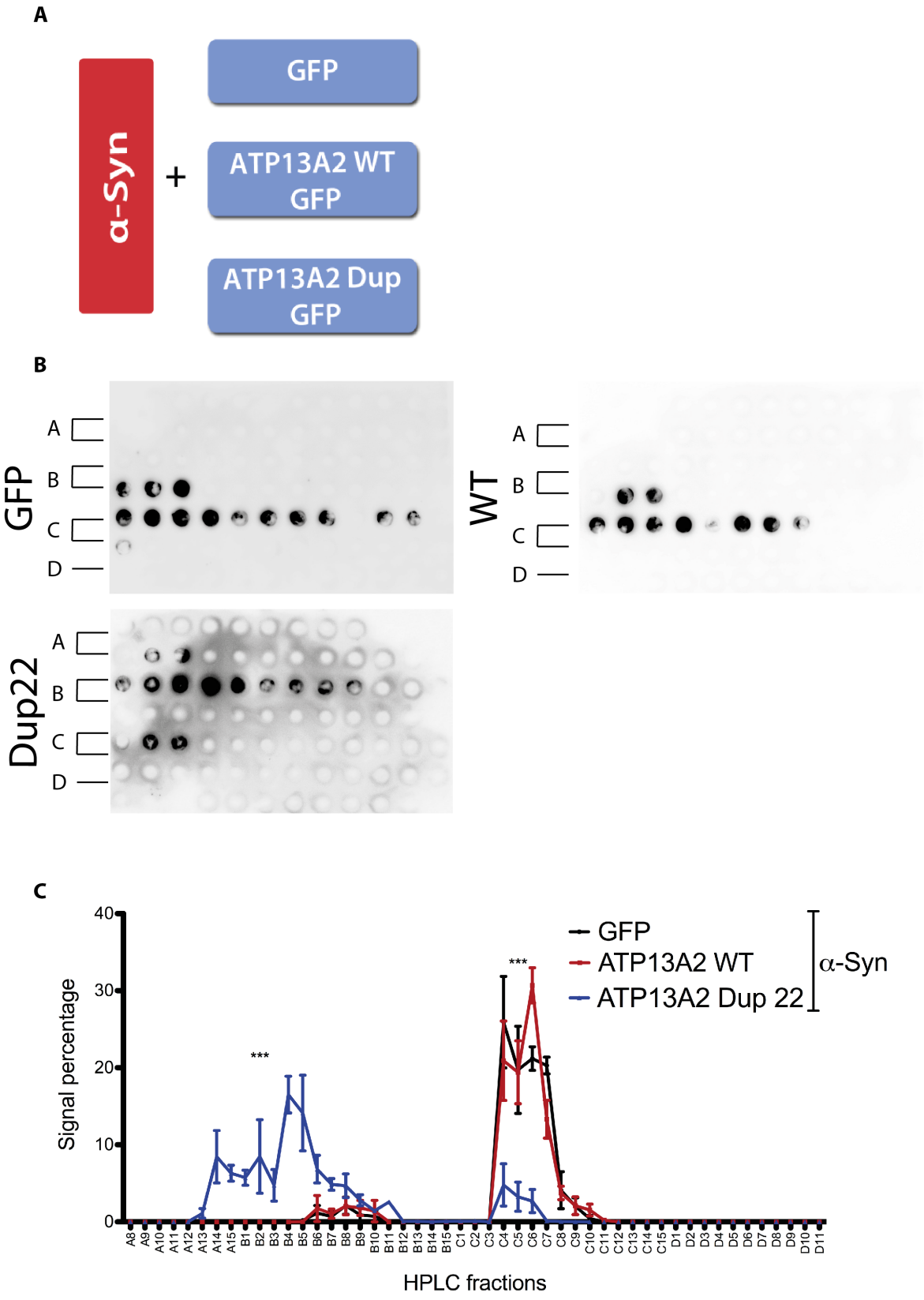


Figure 14. ATP13A2 Dup22 promotes the formation of high molecular weight α-Syn species. A) Triple transfections were executed to analyse the effect of ATP13A2 on α-Syn oligomerization. In detail, the two BiFC constructs (α-Syn-VC and VN-α-Syn) were co-expressed with GFP, ATP13A2 WT GFP or ATP13A2 Dup22 GFP. **B)** ATP13A2 Dup22 promoted the formation of high molecular weight α-Syn species, which appeared in early fractions of the HPLC, while no effect was observed with ATP13A2 WT. **C)** Graphic representation of α-Syn distribution throughout HPLC fractions, in all three experimental conditions. Total signal of all dots were calculated and individual dots were normalized to that sum. **D)** Immunoblot was performed with the same samples used for SEC-HPLC, and no differences in protein levels were detected. **E)** The formation of higher molecular weight species lead to the increase of cellular toxicity, measured by LDH release. All experiments are representative of 3 independent experiments. Data are mean ± S.D. ANOVA with post-hoc Turkey's. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

4.5. ATP13A2 Dup22 promotes the formation high molecular weight species of untagged α-Syn

The strong effect promoted by the ATP13A2 Dup22 mutation in the aggregation and oligomerization models prompted us to study the effect of this mutation on untagged α-Syn (Fig. 15A). Using SEC-HPLC, we observed that ATP13A2 Dup22 could enhance the formation of α-Syn oligomeric species (Fig. 15B and C) without altering the amount of protein (Fig. 15D). For this set of experiments we performed an additional control that consisted in the application of recombinant α-Syn in the SEC-HPLC (Fig. 15E). Since α-Syn is not a globular protein, the time taken by the protein to pass through the column is shorter, resulting in its appearance in earlier fractions. Monomeric α-Syn is mainly present in fractions C3 to C8. According to the protein gradient (Fig. 9A), these fractions correspond to proteins with molecular weights ranging from 44kDa to 17kDa.

The formation of higher molecular weight species by ATP13A2 Dup22 is also observed with untagged α-Syn. Combined, this date suggests that ATP13A2 plays a crucial role in all steps of α-Syn oligomerization and aggregation.


A
α-Syn
+
GFP
ATP13A2 WT GFP
ATP13A2 Dup GFP
B
GFP
WT
Dup22
A
B
C
D
C
Signal percentage
HPLC fractions
GFP
ATP13A2 WT
ATP13A2 Dup 22
α-Syn
***
***

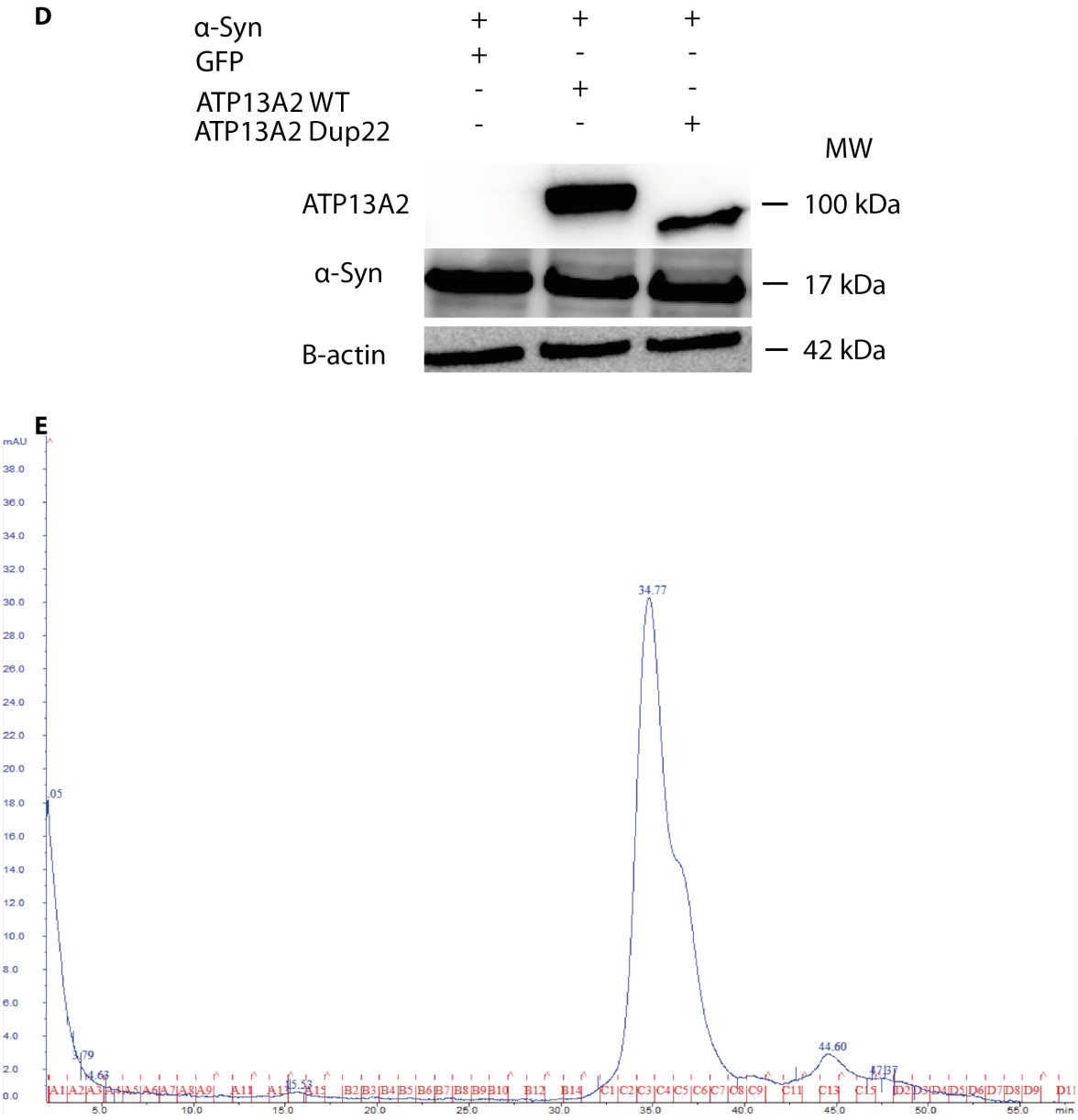


Figure 15. ATP13A2 Dup22 enhances the formation of higher molecular weight α-Syn species, when co-expressed with untagged α-Syn. A) Double transfections were executed to analyse the effect of ATP13A2 on α-Syn. In detail, α-Syn was co-expressed with GFP, ATP13A2 WT GFP or ATP13A2 Dup22 GFP. **B)** ATP13A2 Dup22 enhances the formation of higher α-Syn molecular species that appeared in early fractions of the SEC-HPLC. Cells expressing both ATP13A2 WT or GFP and α-Syn exhibited similar size exclusion pattern. **C)** Graphic representation α-Syn size fractioning, from all three experimental conditions, confirming a shift in the molecular size of α-Syn in the presence of the mutated ATP13A2. **D)** Before SEC-HPLC, all samples were analysed by immunoblot that show no difference in protein levels. **E)** Recombinant untagged α-Syn was used as a control. All experiments are representative of 3 independent experiments.

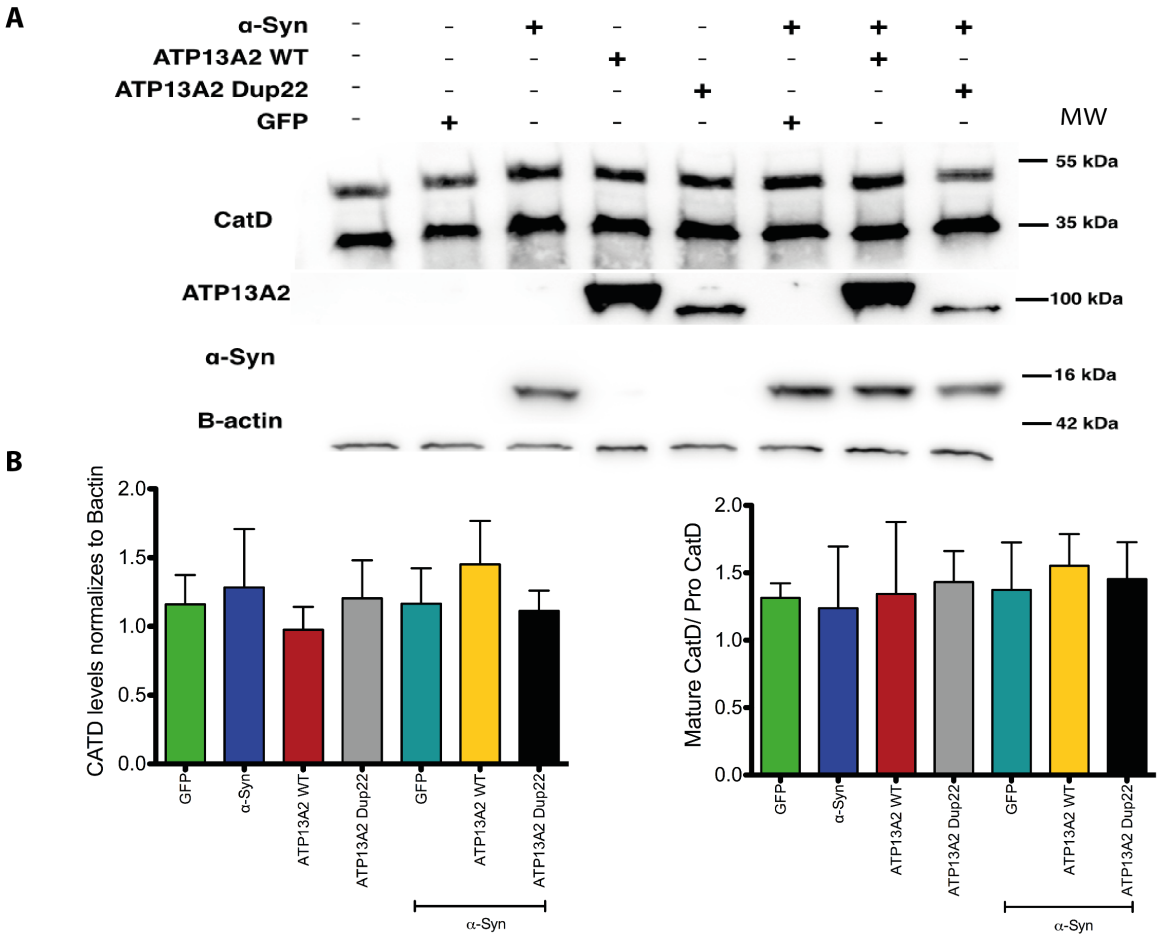
Data are mean ± S.D. ANOVA with post-hoc Turkey's test. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

4.6. The formation of higher molecular weight α-Syn species is independent of CatD maturation

Recently, a number of reports suggested that ATP13A2 is necessary for proper CatD maturation (163, 176, 264). As previously stated CatD is the most important protease when it comes to α-Syn degradation (250, 256). For this reason, we tested if the effects observed with untagged α-Syn (Fig. 15) and with the aggregation-prone form SynT (Fig. 13) were due to a decrease in total of CatD or to impairment in the maturation of the protease.

We observed that, contrary to other reports, the formation of high molecular weight α-Syn species was independent of both levels and maturation of CatD, when co-expressing untagged α-Syn together with ATP13A2 WT or Dup22 (Fig. 16A and B). An identical result was obtained when co-expressing SynT, suggesting that alterations in CatD are not involved in increased resistance to PK treatment (Fig. 16C and D).

All together, this data suggests that α-Syn oligomerization an aggregation promoted by ATP13A2 Dup22 is independent of the Endolysosomal pathway function and the maturation of its players.


A
α-Syn
ATP13A2 WT
ATP13A2 Dup22
GFP
MW
55 kDa
35 kDa
CatD
ATP13A2
100 kDa
α-Syn
16 kDa
B-actin
42 kDa
B
CATD levels normalizes to Bactin
2.0
1.5
1.0
0.5
0.0
GFP
α-Syn
ATP13A2 WT
ATP13A2 Dup22
GFP
ATP13A2 WT
ATP13A2 Dup22
α-Syn
Mature CatD/ Pro CatD
2.0
1.5
1.0
0.5
0.0
GFP
α-Syn
ATP13A2 WT
ATP13A2 Dup22
GFP
ATP13A2 WT
ATP13A2 Dup22
α-Syn

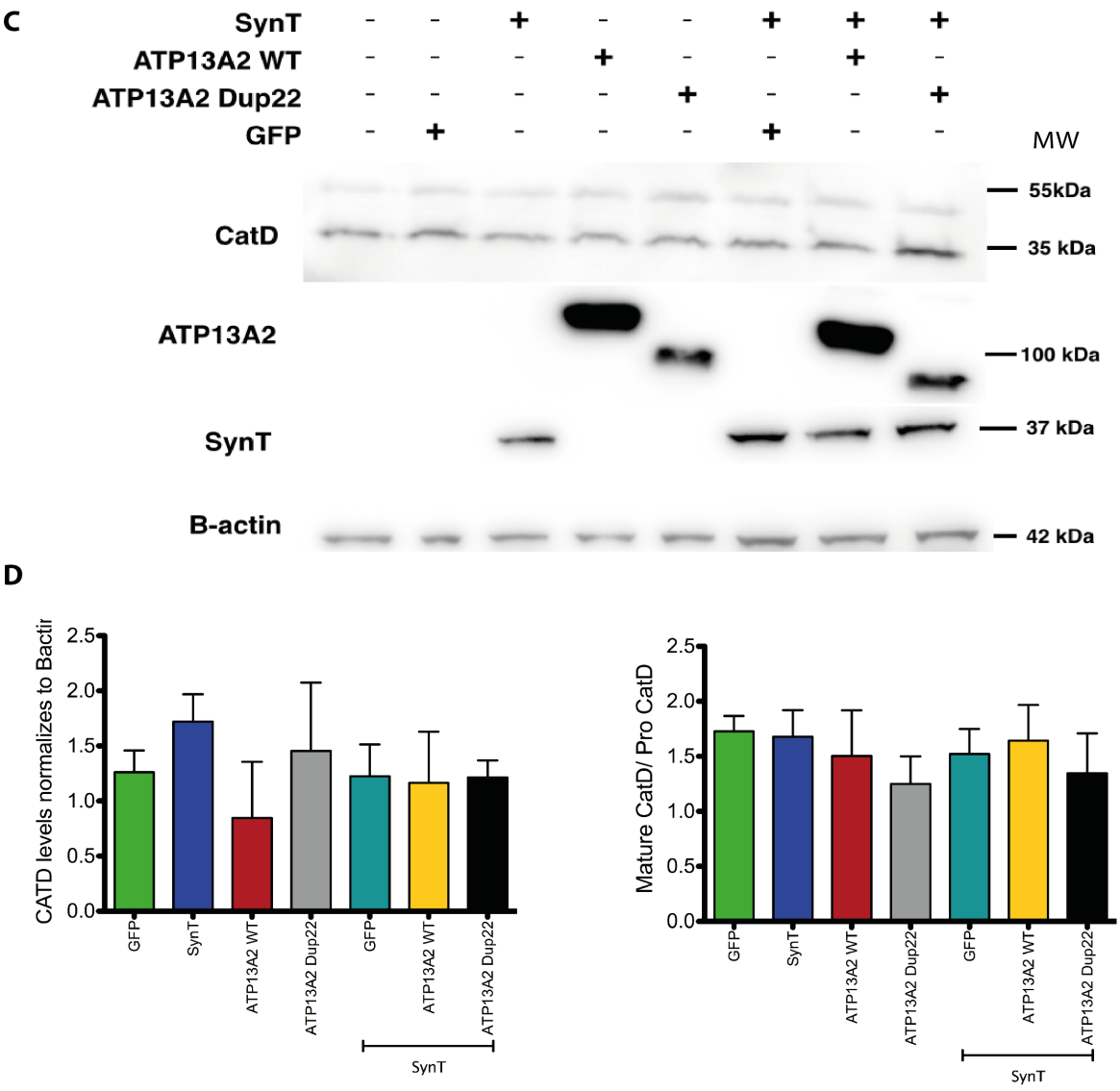
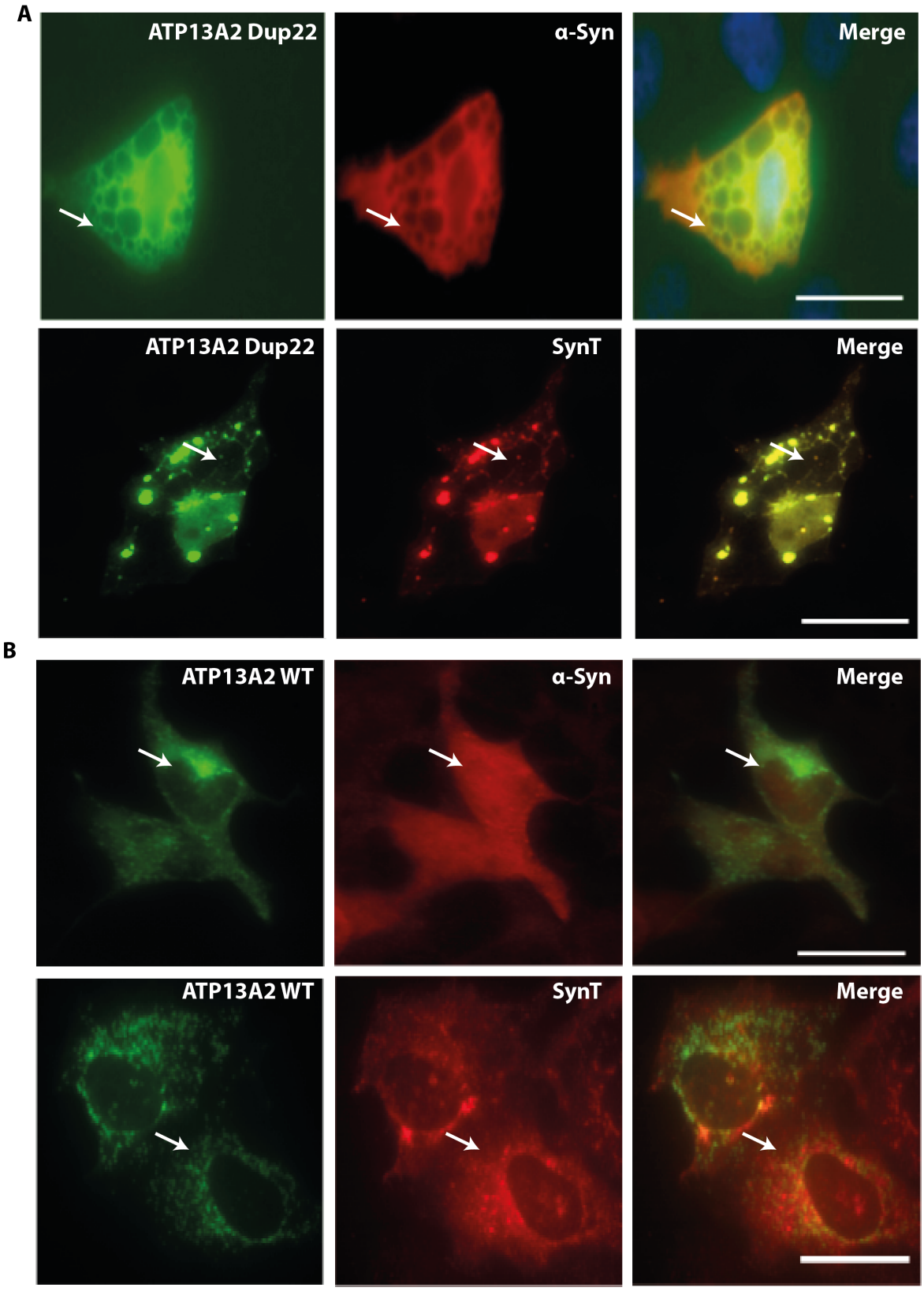


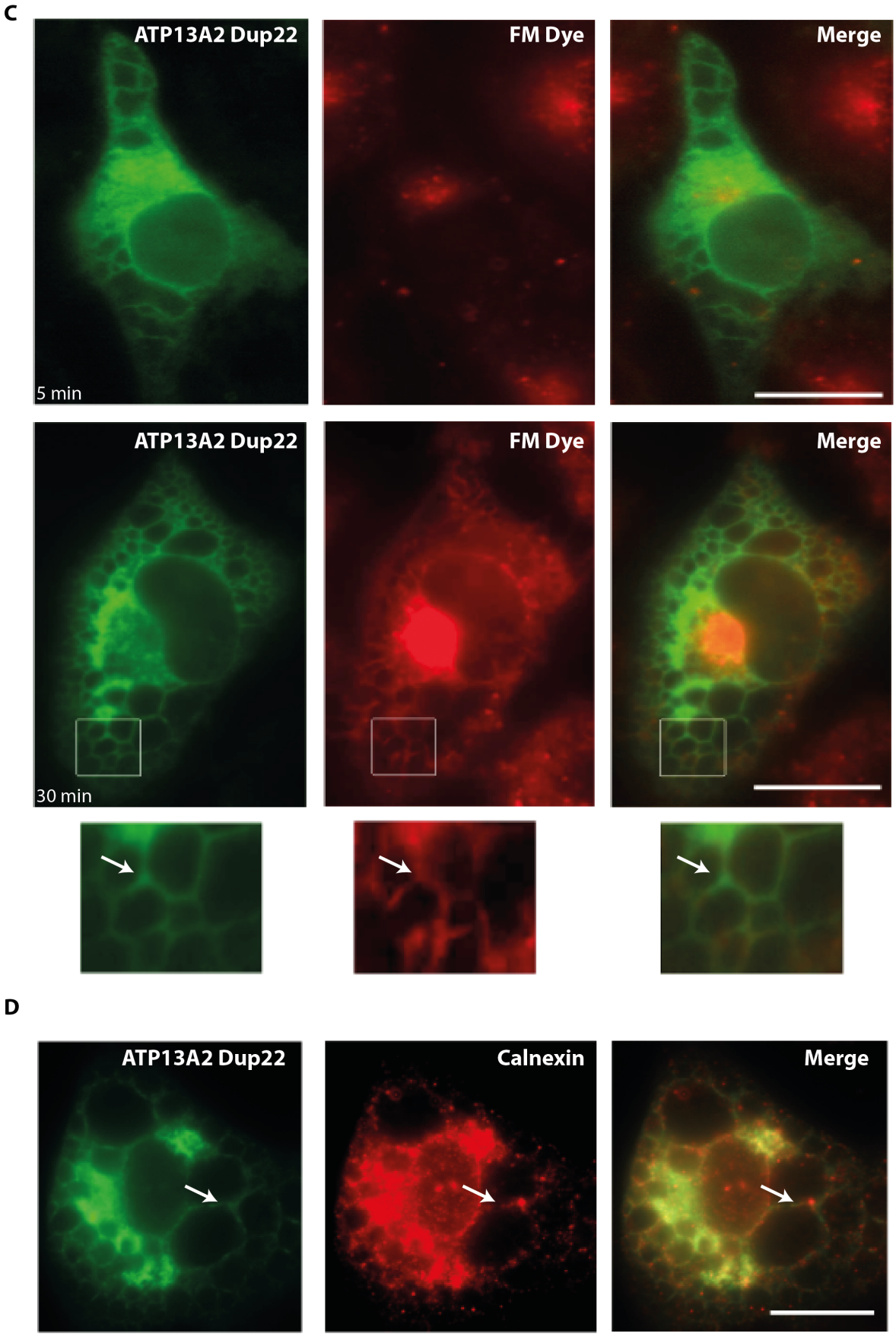
Figure 16. ATP13A2 Dup22 effect on α-Syn is independent of CatD maturation. Levels of CatD were assessed in the presence of either α-Syn (A and B) or SynT (C and D). **A)** Immunoblot analysis for CatD revealed an identical protein levels in all tested conditions, with no significant differences on the **B)** total levels of CatD and levels of this proteases maturation (left and right graph, respectively). **C)** Identical results were obtained when SynT was co-expressed, instead of untagged α-Syn, with no differences observed in **D)** the total levels of CatD and in the ratio between mature and pre CatD (left and right graph, respectively). All experiments are representative of 3 independent experiments. Data are mean ± S.D. ANOVA with post-hoc Turkey's test. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

4.7. ATP13A2 Dup22 and α-Syn co-localize in abnormal ER structures

To understand the impact of the interaction between ATP13A2 and α-Syn in the overall cellular homeostasis, we took a deeper look at different organelles. We first noticed that both α-Syn and SynT co-localize with ATP13A2 Dup22 in an abnormal, reticular structure (Fig. 17A upper and lower panels, respectively). Importantly, this was not observed upon the expression of both forms of α-Syn with ATP13A2 WT, or when ATP13A2 Dup22 was expressed alone (Fig. 17B upper and lower panels, respectively and 10B). Taking advantage of an FM Dye that enables the distinction between plasma membrane and intracellular membranes, depending on the incubation time, we concluded that the abnormal structure was not composed of membranes derived from the plasma membrane, but, instead, was composed of intracellular membranes (Fig. 17C upper and lower panels, respectively). Lastly, we used immunocytochemistry to investigate the composition of the membrane structures. We found that those structures stained positive for an ER marker, Calnexin, and negative for the Golgi Network, as assessed with an antibody against GM130 (Fig. 17D and E). Interestingly, ER staining in cells co-expressing ATP13A2 WT and α-Syn exhibited a normal ER staining pattern, and no difference was detected in the Golgi Network (Fig. 17F and G).

Combined, this data proves that ATP13A2 Dup22 and α-Syn can co-localize in abnormal membrane structures that are positive for the ER marker Calnexin. Importantly this network stained negative for other intracellular membrane structures.


A
ATP13A2 Dup22
α-Syn
Merge
ATP13A2 Dup22
SynT
Merge
B
ATP13A2 WT
α-Syn
Merge
ATP13A2 WT
SynT
Merge


C
ATP13A2 Dup22
FM Dye
Merge
5 min
ATP13A2 Dup22
FM Dye
Merge
30 min
D
ATP13A2 Dup22
Calnexin
Merge

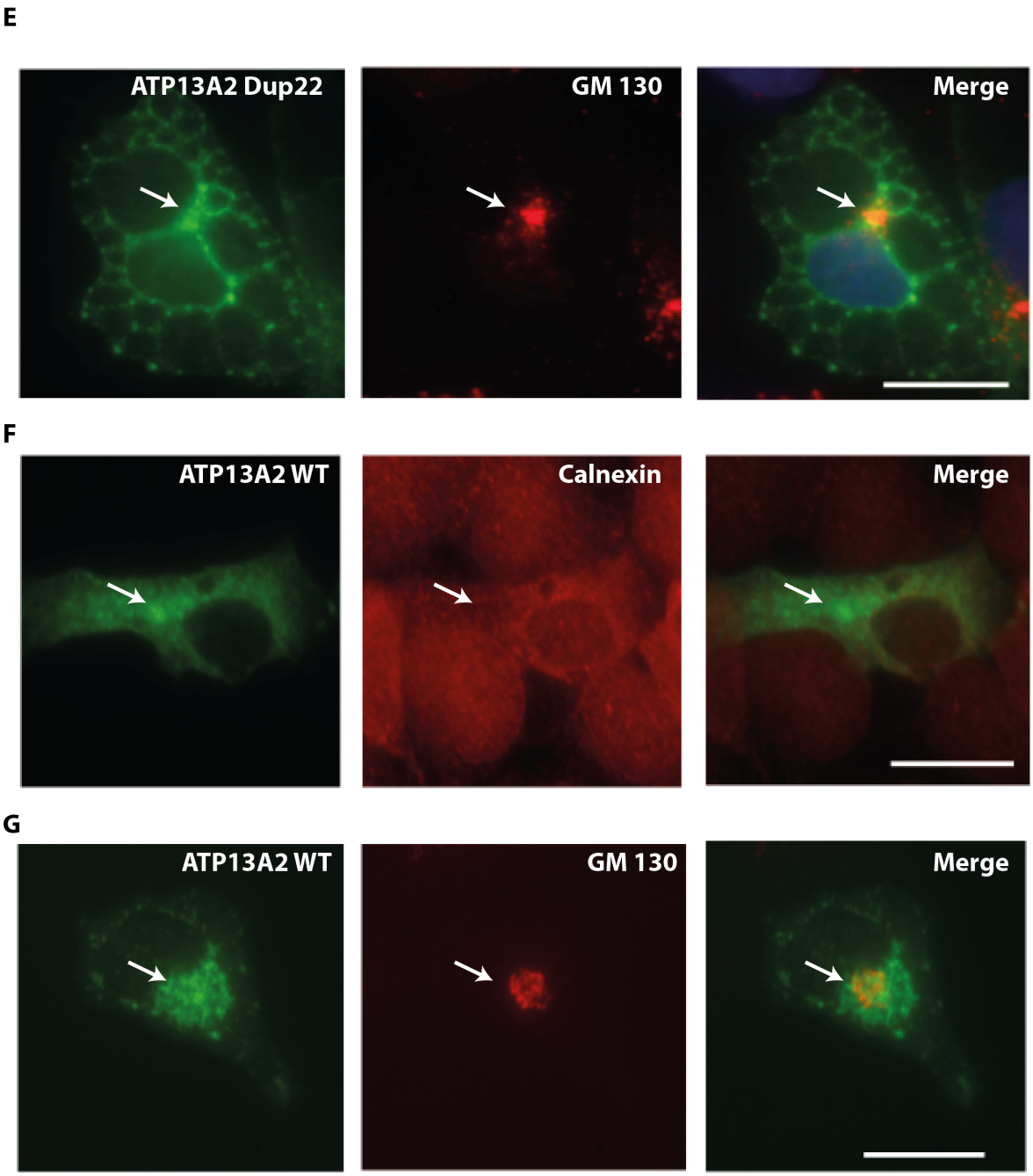
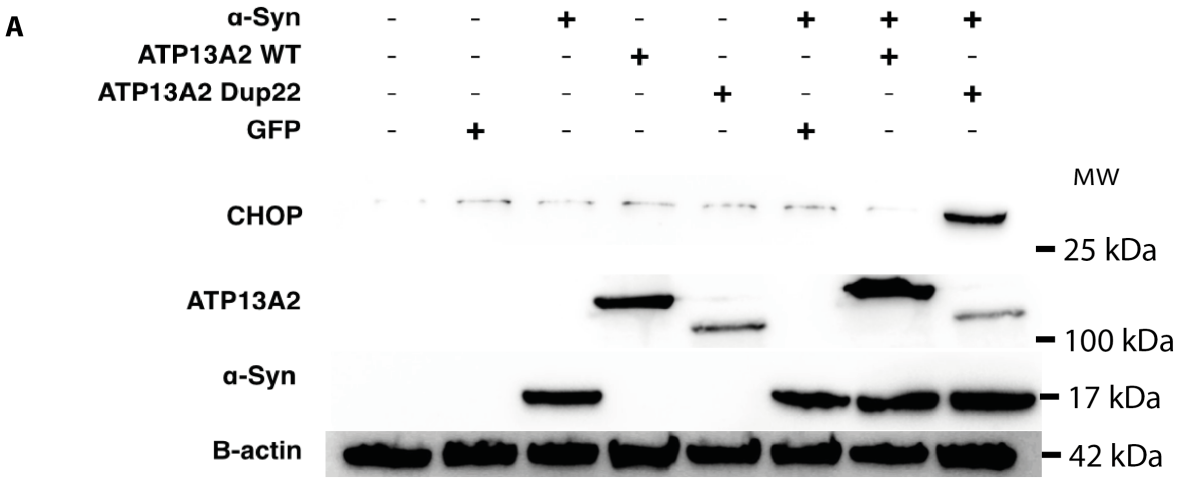


Figure 17. ATP13A2 Dup22 and α-Syn co-localize with reticular, altered ER. A) ATP13A2 Dup22, together with α-Syn (upper panel) or SynT (lower panel), are found in abnormal cellular structures. **B)** These structures were absent in the presence of ATP13A2 WT either with α-Syn (upper panel) or SynT (lower panel). **C)** Microscopic visualization with a FM dye showing that those structures are not constituted by plasma membrane (upper panel) but intracellular membranes instead (lower panel). **D)** The structures stained positive for the ER marker Calnexin and **E)** negative for GM130, a Golgi marker. **F)** Normal ER was observed when co-expressing ATP13A2 WT with α-Syn and **G)** no difference was detected in the Golgi network. Scale bar: 5 μm.

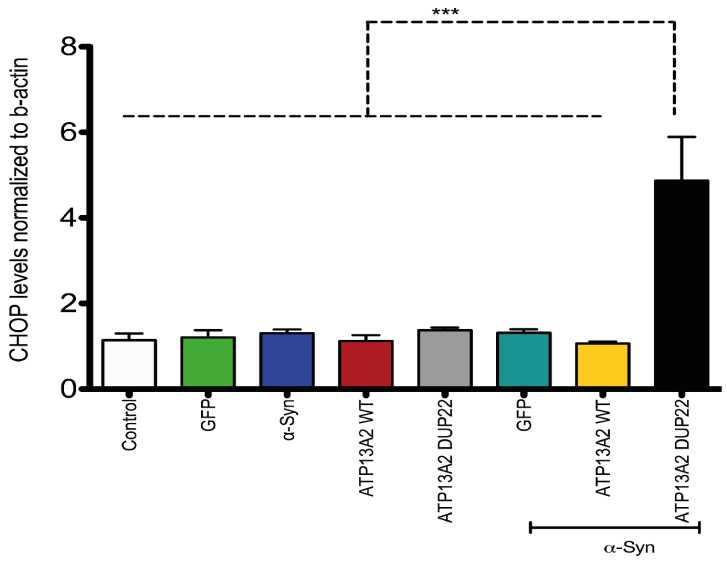
4.8. Co-expression of ATP13A2 Dup22 and α-Syn promotes ER Stress

The results described above suggested that the interplay between ATP13A2 Dup22 and α-Syn could have a direct impact on ER homeostasis. The two proteins have been separately associated with the ER compartment (32, 299). Thus, we further investigated if the altered ER structures were associated with alterations in the homeostasis of this organelle. When analysing ER stress markers, we observed a notorious activation of the PERK pathway, measured by increased CHOP levels. The co-expression of ATP13A2 Dup22 and α-Syn promoted an increase in CHOP, both at the protein (4.87 ± 1.76) (Fig. 18A and B) as well as at the mRNA levels (12.62 ± 0.75) (Fig. 18C). Importantly, this pathway was not found activated when expressing each protein separately (Fig. 18A, B and C). A similar result was obtained when using SynT, suggesting that this effect can occur independently of the formation of inclusions (Fig. 18D, E and F).

These results suggest that just the co-expression of α-Syn and ATP13A2 Dup22 increases ER Stress. Importantly this effect appears to be independent of α-Syn aggregation since both untagged α-Syn and SynT promote an increase in CHOP levels.

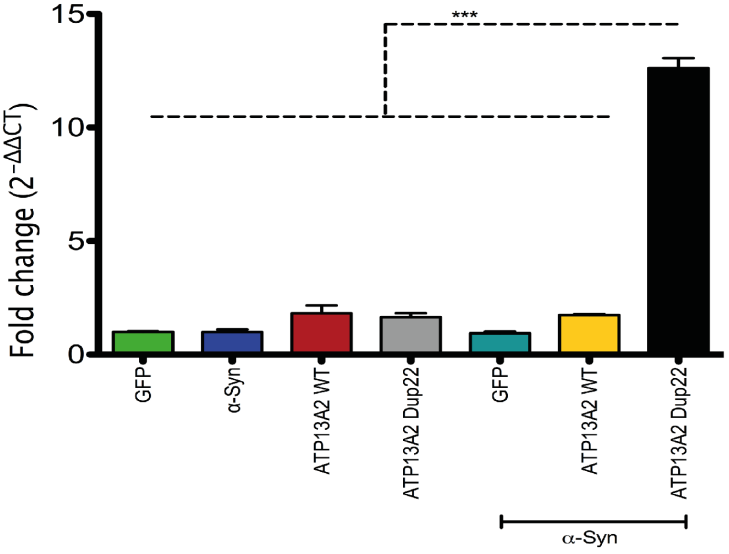


B

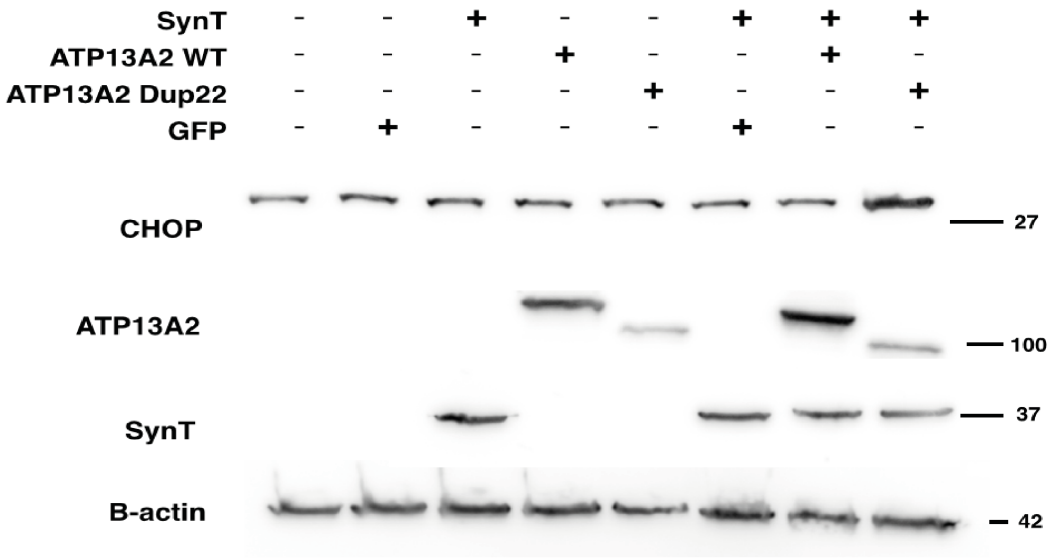


C

mRNA levels - CHOP



D



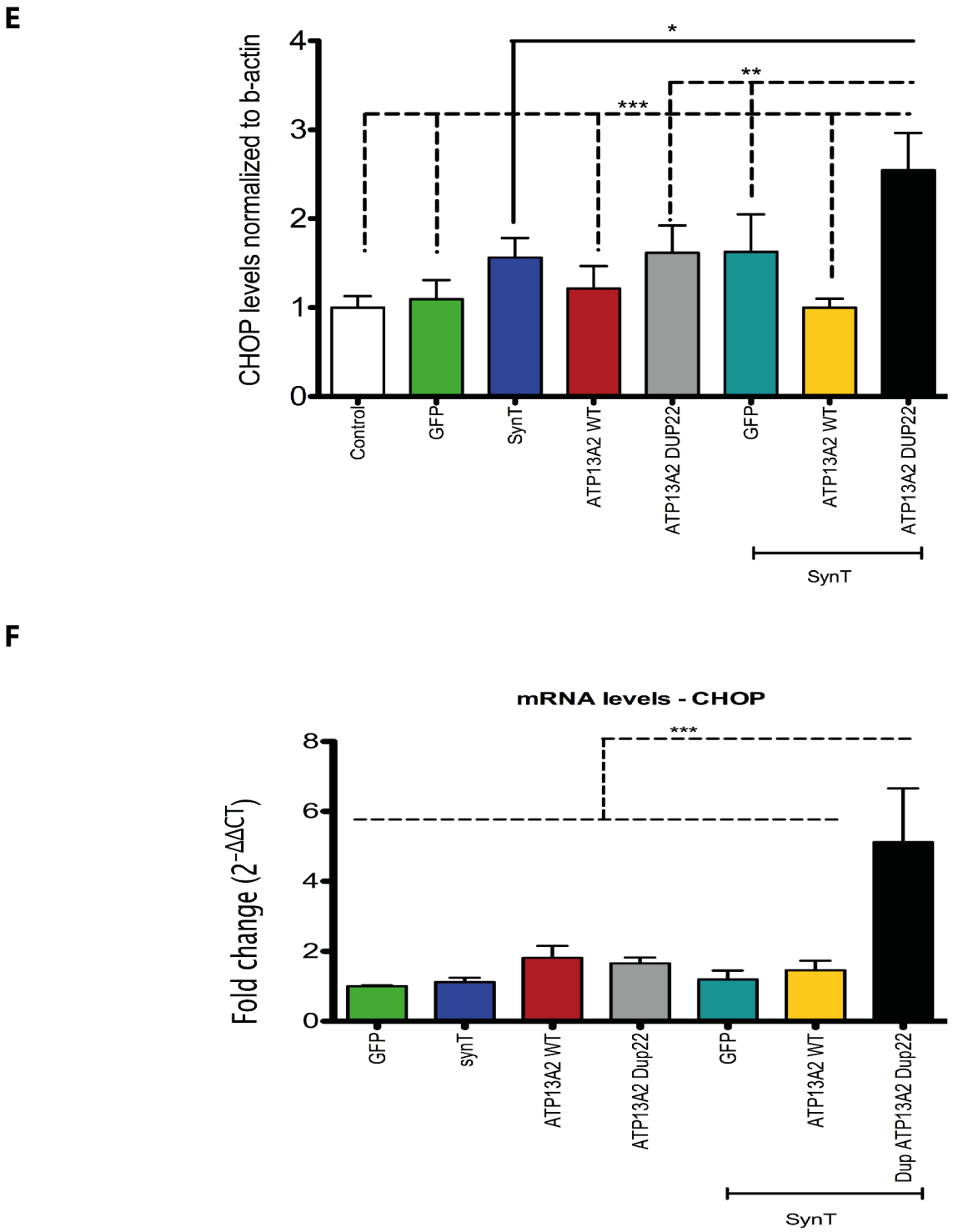


Figure 18. The interplay between ATP13A2 Dup22 and α-Syn induces ER stress. A) Immunoblot analysis revealed an increase in CHOP levels upon the co-expression of α-Syn and ATP13A2 Dup22. **B)** Quantification of immunosignal for CHOP, showing statistically significant differences between α-Syn and ATP13A2 Dup22 group compared to all other conditions. **C)** An increase in CHOP mRNA levels was confirmed by RT-PCR. **D)** The co-expression of SynT with ATP13A2 Dup22 generated an identical increase in CHOP levels that, upon quantification **E)**, was statistically different from all other

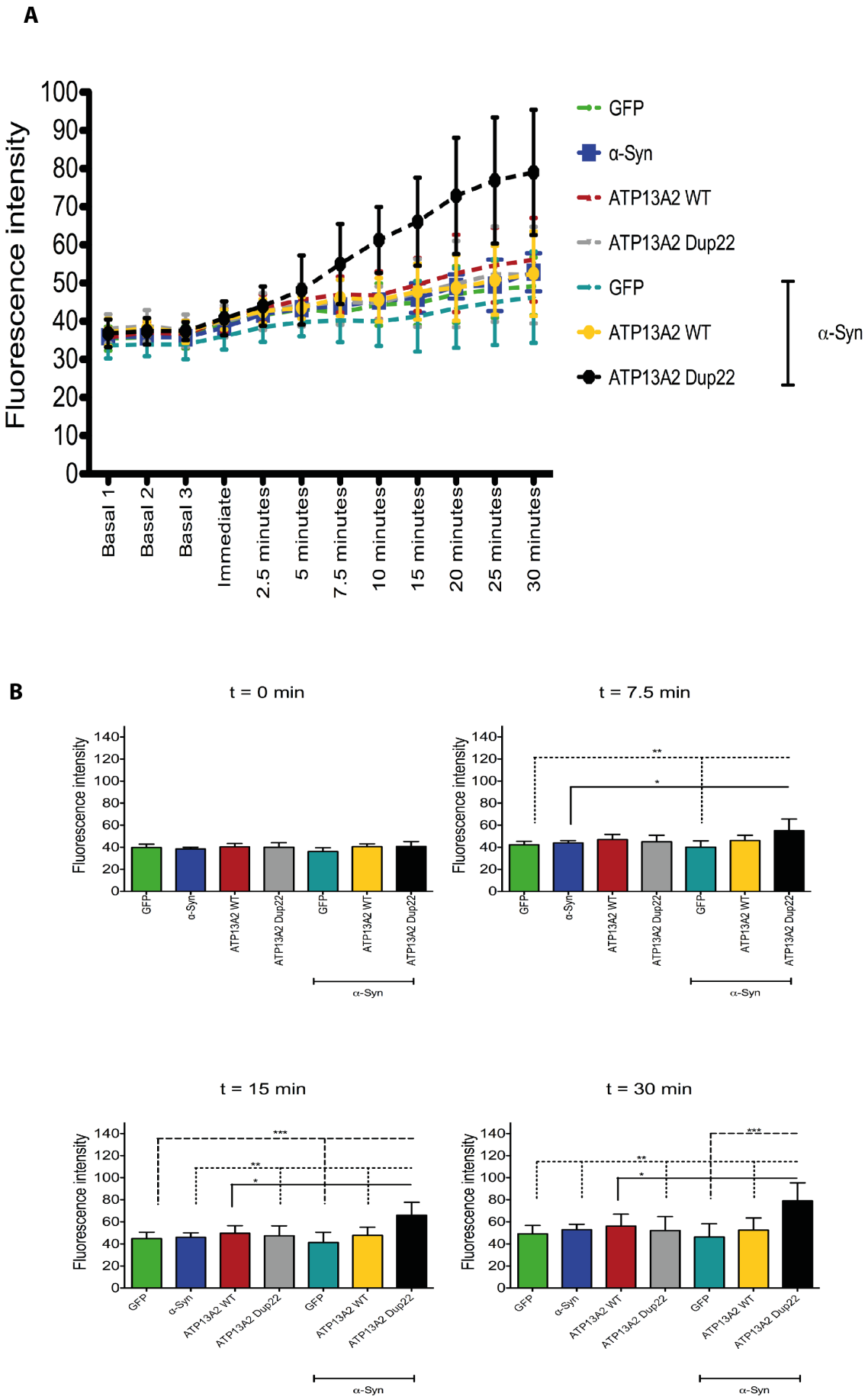
conditions. **F)** By RT-PRC we observed an increase in CHOP mRNA levels. All experiments are representative of 3 independent experiments. Data are mean ± S.D. ANOVA with post-hoc Turkey's test. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

4.9. ER Stress is accompanied by mitochondrial impairment

Mitochondria have a deep connection with neurodegeneration (314) including PD (315). Independently, both α-Syn and ATP13A2 has been associated with mitochondrial homeostasis and function (105, 156) and it is known that ER and mitochondria can physically interact (316). Therefore, we next tested if the interaction between the two proteins could alter the homeostasis of mitochondria.

We measured the production of Reactive Oxygen Species (ROS) using Mitosox, a probe that once inside the mitochondria is oxidized by superoxide. We observed that, under basal conditions, the co-expression of α-Syn or SynT with ATP13A2 Dup 22 did not alter ROS production (Fig. 19A, B, C and D). Nevertheless, the co-expression of ATP13A2 Dup22 and α-Syn increased the susceptibility of cells to oxidative stress, leading to the generation of higher levels of ROS upon challenge with H_2O_2 (Fig. 19A, B, C and D). After obtaining these results, we assessed mitochondrial morphology. Using Mitotracker and a specific imageJ plugin that allows a better observation of the mitochondria structure by generating optimized black and white figures, we observed a clear shift in these organelles morphology between three distinct intracellular paradigms. Cells not transfected presented elongated mitochondria (Fig. 19E1), transfected cells with no alterations in the ER structure, that showed mainly elongated but already some rounded mitochondria (Fig. 19E2), and transfected cells with abnormal ER structure, where the majority of the mitochondria exhibited a round shape (Fig. 19E3).

Together, these results show that the interplay between ATP13A2 Dup22 and α-Syn can promote an increase in ROS production when under oxidative stress. Interestingly we could observe an alteration in mitochondria shape when the altered ER structure is detected.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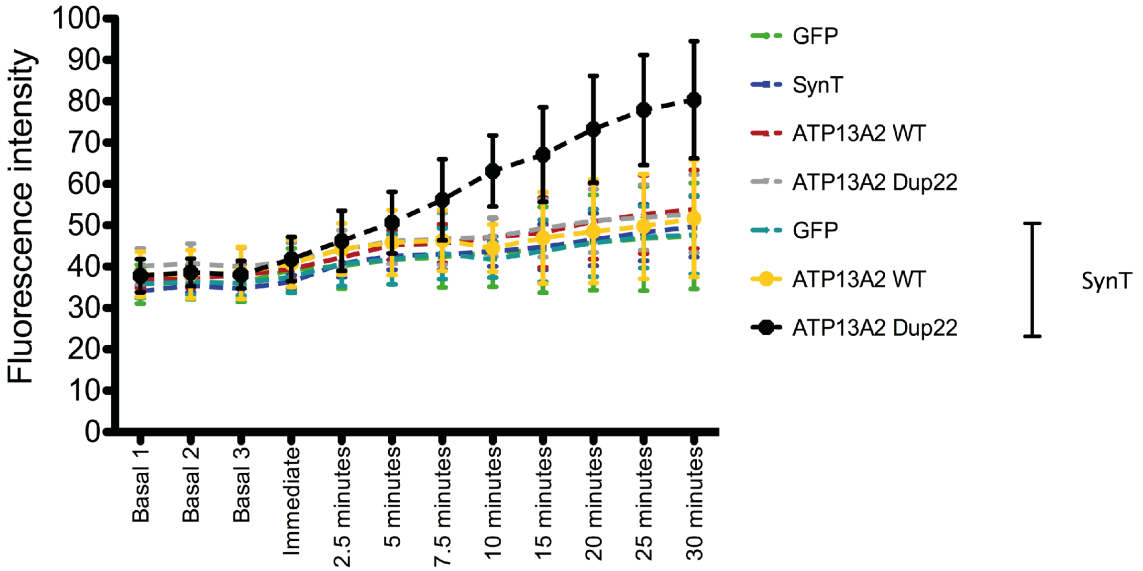
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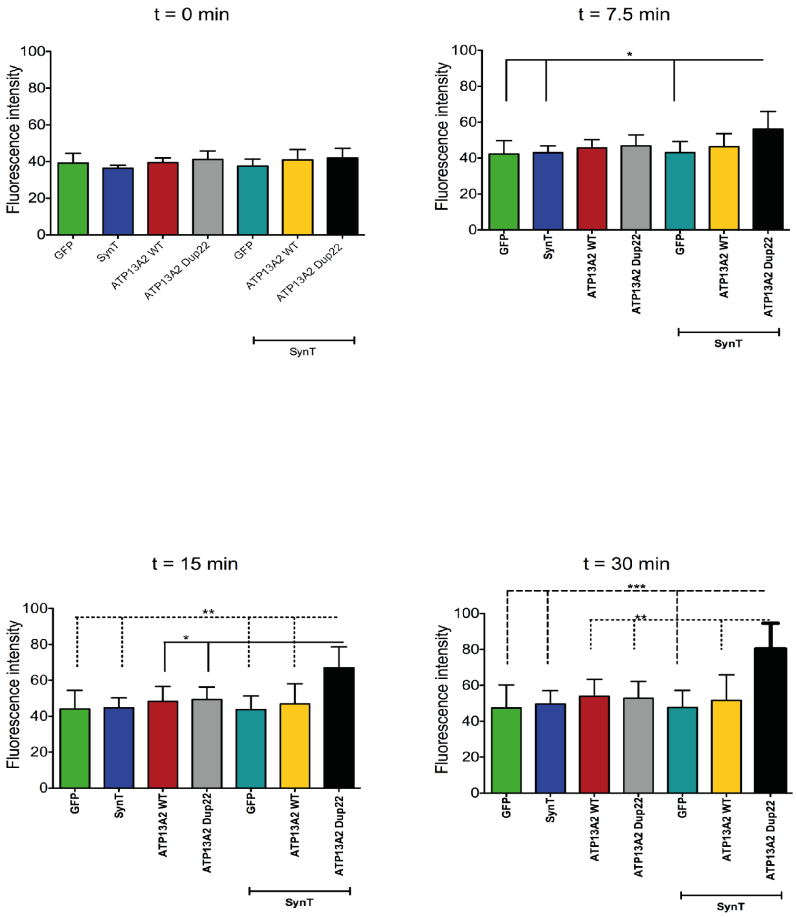
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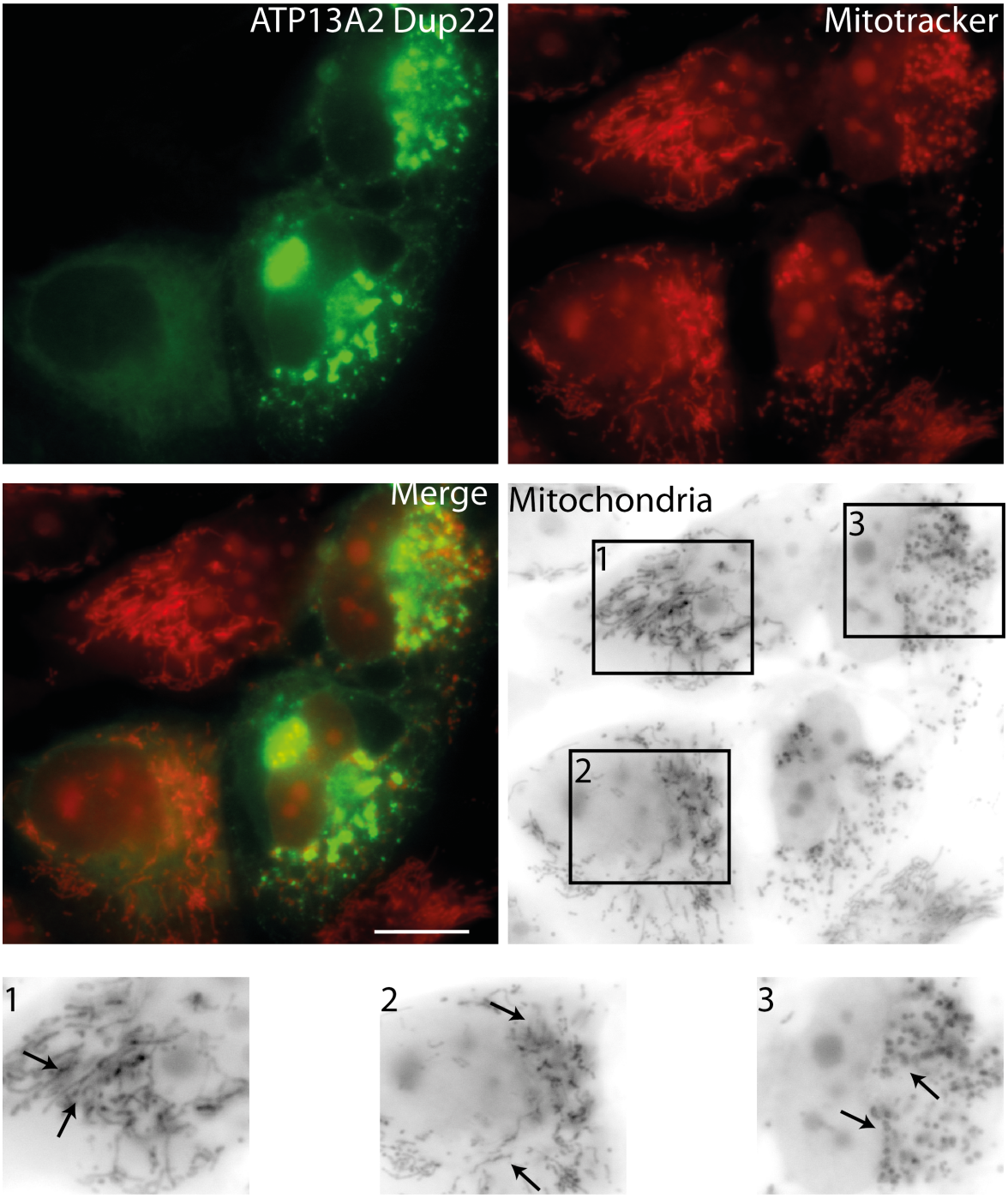
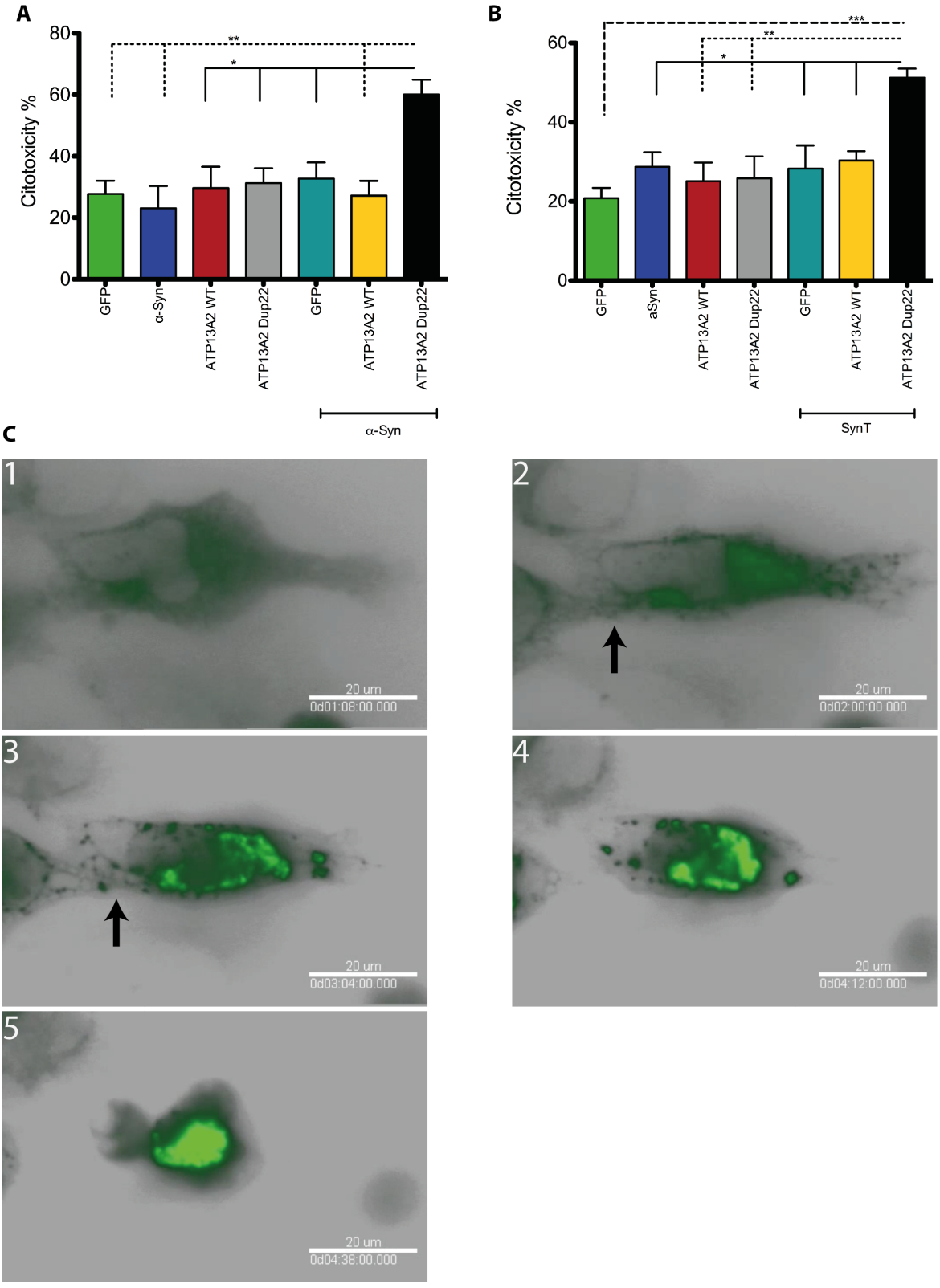


Figure 19. Co-expression of ATP13A2 Dup22 and α-Syn alter mitochondrial homeostasis. A) The expression of ATP13A2 Dup22 together with α-Syn increases the generation of ROS under oxidative stress. **B)** Statistical analysis show no differences under basal conditions no differences, while 7.5 minutes after challenging the cells with H_2O_2. **C)** The same effect is observed with the co-expression of SynT with **D**) the first significant difference being obtained at the same time point. **E)** Mitochondria morphology was observed with Mitotracker. Black and white optimized images were obtained using an ImageJ plugin. Zoomed images of cells co-expressing ATP13A2 Dup22 and α-Syn show

that **1)** untransfected cells present elongated mitochondria, **2)** cells transfect but without abnormal ER structure show mainly elongated mitochondria but already some rounded-shape ones and, finally **3)** cells with the abnormal ER structure show the majority of cells with rounded mitochondria. All experiments are representative of N=3 independent experiments. Data are mean ± S.D. ANOVA with post-hoc Turkey's test. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$. Scale bar: 5 μm

4.10. Coexpression of ATP13A2 Dup22 and α-Syn increases cytotoxicity

ER stress and mitochondrial impairment are intrinsically implicated in the overall cell homeostasis (317, 318). Considering our previous results, we asked whether those cellular alterations were associated with increased intracellular toxicity. By measuring the levels of LDH in the supernatant of these cells - a common measure of the membrane integrity and, hence, of cellular toxicity - we observed that the co-expression of ATP13A2 Dup22 together with α-Syn or SynT increased the cytotoxicity (Fig. 20A and B). Expressing the proteins separately did not produce the same effect, suggesting that the interaction between the two proteins is necessary to promote toxicity. Using live-cell imaging, we observed that the formation of abnormal ER structures precedes cell death, hinting that this organelle might be directly associated to the cell fate (Fig. 20C). A recent study concluded that increased CHOP levels could lead to unmitigated ER Stress that culminates in increase DR5 mRNA levels and, ultimately, in cell death (286). For this reason we analysed DR5 mRNA levels and concluded that the co-expression of ATP13A2 Dup22 with both α-Syn and SynT lead to a significant increase of DR5 mRNA levels (4.81 ± 0.55 and 3.48 ± 1.2, respectively) (Fig. 20D and E).
Lastly, we observed that the interaction between ATP13A2 Dup22 and α-Syn increases intracellular toxicity. Furthermore, we observed an increase in the mRNA levels of DR5, which suggests an activation of apoptotic pathways.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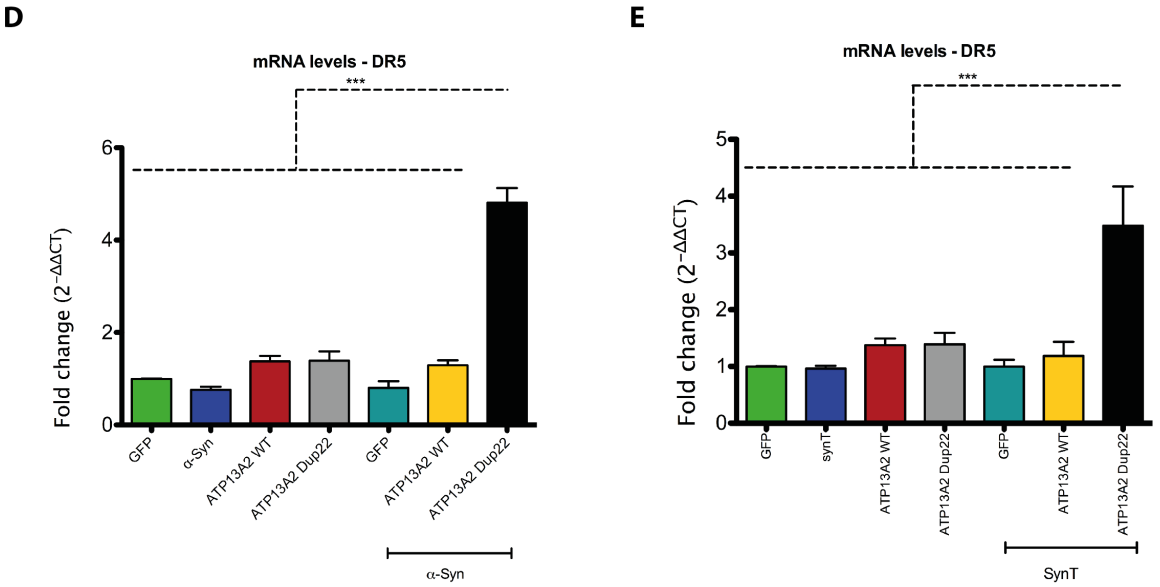

Figure 20. The interaction between ATP13A2 Dup22 and α-Syn increases cellular toxicity. A) The co-expression of α-Syn with ATP13A2 promoted an increase in intracellular toxicity, measured by LDH release. **B)** An identical effect was observed with SynT. **C)** Live-cell imaging performed at different time-points showed that the formation of abnormal ER structures anticipated cell death. Importantly, increased DR5 mRNA levels were observed with when ATP13A2 Dup22 was expressed together with either **D)** α-Syn and **E)** SynT. All experiments are representative of N=3 independent experiments. Data are mean ± S.D. ANOVA with post-hoc Turkey's tests. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$. Scale bar: 20 µm.

5. Discussion

PD is a complex age-associated neurodegenerative disorder primarily known for its typical motor symptoms. However, despite intensive research, the molecular background and triggers of the disease remain mostly unknown (319). α-Syn aggregation in LBs and Lewy neurites is a classical histopathological feature shared between sporadic and familiar forms of PD, and deep efforts have been made to understand the molecular basis of the aggregation process (320). Deciphering which molecules or proteins can affect α-Syn aggregation might ultimately lead to the development of novel therapeutics for synucleinopathies.

The cellular imbalance preceding α-Syn oligomerization and aggregation is elusive but impairment of the degradation pathways has been repetitively associated to this process (238, 321). Both proteasome and lysosome are involved in α-Syn degradation but only the latter organelle can effectively handle molecular species bigger than dimers (196, 239). ATP13A2 was the first lysosome-related protein to be found mutated in PD patients (Table 1), conferring extra notoriety to this organelle in the neurodegeneration context. This late endosome and lysosome transmembrane protein has been, since the beginning, associated to α-Syn homeostasis although the mechanisms underlying this effect are controversial (166, 193). An initial study using ATP13A2 KO mice described the formation of neuronal α-Syn aggregates (161). A recent work concluded that the accumulation of α-Syn is independent of the protein itself, since not all aggregates stained positive for α-Syn but were positive for ubiquitin (176). This new data suggests that the presence of α-Syn in these proteinaceous inclusions occurs after their formation and that α-Syn does not play an important role in their formation. Despite studies performed in conditions lacking ATP13A2 no work has, so far, addressed the effect of ATP13A2 familial mutations on its interaction with α-Syn.

The present study aimed to understand the effect of a familial mutation in ATP13A2, Dup22, on α-Syn and how the interaction between the two proteins can affect intracellular balance. Taking advantage of a well-described cellular model to study α-Syn aggregation we concluded that ATP13A2 Dup22 could enhance this process by increasing both the number of inclusions per cell, and the number of

cells with these inclusions. Importantly, we have previously reported that these intracellular inclusions exhibit one of the main characteristics of LBs present in PD patients, amyloid structures (312). To further understand the importance of the endolysosomal pathway on α-Syn aggregation we studied another protein that has been recently linked to protein clearance and secretion via this mechanism: Rab8a (237). We were able to show that this TGN Rab GTPase can also influence α-Syn aggregation by enhancing, both *in vitro* and *in vivo* this cellular phenomena (312). Importantly, the effect of Rab8a is due to its direct interact with α-Syn, a process that requires the C-terminus of the latter. Together, this data strengths the hypothesis that the endolysosomal pathway plays an important role on α-Syn homeostasis. Furthermore the direct interaction between the two proteins suggests that α-Syn might have a functional role in this pathway. Reinforcing this theory is the fact that VAMP2, a known interactor of α-Syn, has also been connected to the endolysosomal machinery (116, 119).

To further understand the effect of the Dup22 mutation of ATP13A2 on α-Syn aggregation we removed Synphilin-1 from the previously described model. Synphilin-1 is known to interact with α-Syn, and this protein could mask any direct effect promoted by ATP13A2 on α-Syn aggregation. For this reason we co-expressed SynT with both ATP13A2 WT and Dup22, in the absence of Synphilin-1. Here, we observed that ATP13A2 Dup22 could enhance the amount of cells with aggregates and, particularly, with large intracellular inclusions. ATP13A2 WT also increased the amount of intracellular inclusions, when compared to GFP, although to a much lesser extend when compared to ATP13A2 Dup22. Biochemically, ATP13A2 Dup22 promotes an increase in the resistance of SynT to PK treatment, a characteristic that was not observed when SynT was co-expressed with ATP13A2 WT. Increased α-Syn resistance to PK treatment has been described in LBs and in α-Syn transgenic mice (322, 323). Furthermore, these inclusions presented similar characteristics to LBs found in PD patients, such as amyloid structures and S129 p-α-Syn (62, 63). Considering that both ATP13A2 WT and Dup22 can enhance SynT aggregation but just the latter increases SynT resistance to PK treatment suggests that the aggregates in the two conditions might have different conformations. On the other hand, the higher resistance to PK might be just the result of the significant increase in number of aggregates promoted by ATP13A2 Dup22.

Extensive research in PD, and more particularly on α-Syn, has so far failed to reach a consensus regarding which protein species induce intracellular toxicity and neurodegeneration (146, 147, 149, 150). Recent work shown that both oligomers and fibrils can promote a toxic effect and are partners in pathology development (151). Here we show that ATP13A2 Dup22 is able to promote the formation of higher molecular weight α-Syn species in a cellular oligomerization model. This effect was accompanied by an increase of intracellular toxicity. The observed effect promoted by ATP13A2 Dup22 in those different cellular models suggested that this mutation could modulate the overall homeostasis of α-Syn. To validate this hypothesis we performed experiments with untagged α-Syn and concluded that ATP13A2 Dup22 could also promote the formation of high molecular weight α-Syn species. This data proves that ATP13A2 Dup22 can influence α-Syn aggregation in all stages of the process either by promoting the formation of higher molecular weight species in untagged α-Syn and in the oligomerization model or by enhancing aggregate formation with SynT.

The function of ATP13A2 is still unknown but physiological levels of the protein seem to be necessary for the proper maturation of CatD (163, 176, 264). CatD is one of the two aspartic proteases known to be present in cells (324). Once α-Syn is targeted for degradation via the lysosome, CatD is the main protease responsible for its clearance (250, 256). Furthermore, mutations in both ATP13A2 and CatD are known to cause NCL (161, 186, 265, 325). Surprisingly, our immunoblot analysis showed no alterations in CatD maturation. We speculate that this result is due to the presence of endogenous levels of ATP13A2, which may be able to assure the proper maturation process of CatD. Since Dup22 is a loss of function mutation it is expectable that the generate protein would not interfere with the proper function of the endolysosomal machinery when endogenous ATP13A2 is expressed. Our results suggest that the observed increase in oligomerization and aggregation promoted by ATP13A2 Dup22 is, at least partially, independent of the function of the endolysosomal pathway and from CatD maturation. Furthermore, and considering that different α-Syn species are degraded by different mechanisms one could speculate that the effect promoted by ATP13A2 Dup22 in all α-Syn species might be independent of any degradation mechanism.

For a protein to exert its normal function it requires a critical process of folding to ensure that the proper conformation is achieved. The misfolding and aggregation of α-Syn has been, for many years, one of the main dogmas surrounding the study of PD pathology and PD-associated proteins (326). The folding machinery relies heavily on the ER and its chaperones and, therefore, this organelle has been extensively studied in PD context (290, 327). Additionally, the accumulation of misfolded proteins triggers an ER response, known as ER-stress, that consists in the UPR signalling cascade (289). This machinery can undergo two opposite responses, according to the degree of stress, that either culminate in cell survival or apoptosis (328). Despite prompting different fates, the molecular organizers are identical rendering hard to identify the triggers that can activate a pro-survival or a cell-death response. Here we show that α-Syn and ATP13A2 Dup22 can co-localize in abnormal intracellular membrane structures that stained positive for Calnexin, a common ER marker. The ER specificity of these structures was confirmed using a marker for the Golgi network, GM130, which showed no co-localization with the reticular structure. Moreover, we observed a normal ER distribution when co-expressing α-Syn with ATP13A2 WT, and no difference on Golgi network in all the conditions.

The presence of ATP13A2 Dup22 at the ER membrane has been previously described (189). This mutation in ATP13A2 eliminates several transmembrane domains and promotes a mislocalization of the protein. Since transmembrane proteins are processed via the ER, before being targeted to their last cellular destination, ATP13A2 Dup22 appears to accumulate in this organelle. This mutation, along with others in ATP13A2, can sensitize cells to ER Stress (189). More interesting, though, is the presence of α-Syn in the ER upon its co-expression with ATP13A2 Dup22. α-Syn typically presents an ubiquitous intracellular expression, but it has been described that toxic oligomeric α-Syn species can preferentially accumulate in the ER (297). This accumulation was reported in both α-Syn transgenic mice and in brain samples obtained from PD patients (297, 299). Nevertheless is still unknown if these oligomers are formed in the ER or if they are generate somewhere in the cell and then accumulate in this organelle. In the mice models, the presence of these species in the ER lead to chronic ER Stress and the activation of pro-apoptotic caspase cascades (299). A similar ER-mediated toxicity was observed in cell culture upon the overexpression

of mutant α-Syn A53T, together with mitochondrial impairment (303). Interestingly, as we described previously, ATP13A2 Dup22 can enhance the formation of α-Syn higher molecular weight species. One can speculate that these species are the ones accumulating with ATP13A2 Dup22 in the abnormal ER structures but further studies are required.

The molecular mechanisms underlying ER Stress are commonly divided in three, according to their initiator at the membrane level. The PERK and IRE1alpha pathways are activated in both resolvable and unmitigated ER Stress while activation of ATF 6 cascade has only been described in the first type of response. From the trio, α-Syn has been particularly linked to the PERK pathway, since its intracellular presence is necessary for manganese-induced toxicity (298). As previously mentioned, PERK phosphorylates eiF2α that promotes the expression of CHOP, a major regulator of ER Stress (329). In our study, we prove that the accumulation of both ATP13A2 Dup22 and α-Syn at the ER leads to the activation of the PERK pathway that was measured by CHOP mRNA and protein levels. The fact that ATP13A2 Dup22 presence in the ER is not enough, *per si*, to increase CHOP level indicates that α-Syn accumulation at the ER is necessary to activate the PERK pathway. Additionally we observed that CHOP mRNA and protein levels with untagged α-Syn were higher when compared to SynT. This suggests that the intermediate oligomeric species that originate from the co-expression of ATP13A2 Dup22 and α-Syn could be the main responsible for the observed ER Stress.

In addition to the ER, other cellular organelles such as mitochondria, are thought to play a role in PD pathogenesis. The connection between mitochondria impairment and PD has been strengthen by both genetic and environmental causes (330). Mitochondria are the main cellular energy producers and also responsible for nearly 90% of the intracellular ROS production (331). ROS are oxygen species formed by the partial reduction of oxygen that can impact several cellular pathways such as iron homeostasis, cell proliferation and survival, and DNA damage (332). In normal conditions, ROS are important members of intracellular signalling but an imbalance on its production can lead to their accumulation and deleterious action. Some speculate that this imbalance can be easily reached in dopaminergic neurons due to tyrosine hydroxylase (333) since it

has been shown that tyrosine hydroxylase is an additional mechanism for intracellular ROS production (334).

Some studies report that α-Syn can be found in both outer and inner mitochondria membranes (335, 336). It has been suggested that α-Syn contains, in its N-terminus, a hidden mitochondrial localization sequence, and that its presence in this organelle can decrease the complex I activity (336). Moreover, mitochondrial α-Syn may impact the organelle movement, fusion and fission but the mechanisms underlying this effect are still arguable (337, 338). ATP13A2, on its turn, has been extensively associated with mitochondria, either by influencing the degradation of the organelle or due to its interaction with metals, particularly with Zn^{2+} and Mn^{+} (156, 159, 160, 170).

Due to the intimate cross talk between ER and the mitochondria, and the role of both α-Syn and ATP13A2 on mitochondrial homeostasis, we investigated whether the interplay between these two proteins could promote mitochondria dysfunction. Here we show that the interaction between ATP13A2 Dup22 and α-Syn sensitizes cells to oxidative stress by increasing the production of ROS, more specifically superoxide, upon H_2O_2 challenge. Interestingly, no alterations were observed in basal levels, compared to all other conditions, suggesting that this effect might not be primordial in the cascade of intracellular events promoted by the interplay between these proteins. Furthermore, we concluded that both untagged α-Syn and SynT had an identical effect, with both significantly increasing ROS production. This indicates that, in terms of superoxide production, the existence intracellular aggregates do not represent an additional factor underlying ROS production. Mitochondrial morphology is crucial for cell physiology, and it has been shown that changes in mitochondria shape are associated with neurodegeneration and can lead to increased ROS production (339). By incubating our cells with Mitotracker we observed that cells displaying ER-reticulated network also exhibit loss of normal mitochondria shape. Our results indicate that the interplay between ATP13A2 Dup22 and α-Syn lead to changes in mitochondria morphology, from typical elongated to round, may be responsible for increased ROS production.

Ultimately, we wanted to understand if the observed cellular effects would promote an overall alteration of the intracellular toxicity. We verified that the interaction between ATP13A2 Dup22 and α-Syn or SynT lead to increase LDH release, an indicative of higher intracellular toxicity. This result confirms that the presence of

both proteins, ATP13A2 Dup22 and α-Syn, at the abnormal ER structures is necessary for triggering the cellular stress that culminates in cell death. Taking advantage of time-lapse imaging we observed that the formation of the abnormal ER structures preceded cell death. A recent study has described that the balance between the activation of a pro survival or apoptotic ER stress response has on DR5 mRNA levels an important tipping point (286). Briefly, PERK and IRE1alpha pathways can compensate each other in order to provide a pro-survival response, by maintaining DR5 mRNA levels low. Nevertheless, upon unmitigated and chronic ER stress situations, CHOP promotes the upregulation of DR5 mRNA levels that cannot be attenuated by the RIDD mechanism of the IRE1alpha pathway. We observed that the co-expression of ATP13A2 Dup22 with α-Syn or SynT promoted a significant increase in DR5 mRNA levels. The expression of the proteins separately did not lead to this effect suggesting that, once again, the presence of both proteins at the ER is necessary to increase the DR5 mRNA levels and activate pro-apoptotic responses.

These results highlight the role of endolysosomal proteins in α-Syn homeostasis, as both Raba8a and ATP13A2 are able to modulate the aggregation propensity of α-Syn. We show that a PD-associated mutation in ATP13A2 (Dup22) can promote the formation of high molecular weight α-Syn species and that the interaction between these two proteins can initiate a cascade of events that culminate in cell death. In detail, ATP13A2 Dup22 and α-Syn co-localize in abnormal ER structures and induce ER Stress that is accompanied by alteration in mitochondria morphology and homeostasis. Ultimately, we conclude that these deleterious effects culminate in increased intracellular toxicity via increased DR5 mRNA levels and the activation of pro-apoptotic mechanisms.

6. Conclusions and Outlook

α-Syn intracellular dynamics is one of the most studied topics in PD and synucleinopathies. The complex and far-reaching network that encompasses this protein has dazzled researchers for near a quarter of a century and many questions are still unsolved.

In this study, we explored the effect that endolysosomal proteins can have on α-Syn intracellular homeostasis. We started by showing that an important protein of this pathway, Raba8a, could influence α-Syn aggregation *in vivo* and *in vitro*. We further focused our attention on another player of the pathway, ATP13A2, and a mutated version, ATP13A2 Dup22, consisting on a 22 base pairs duplication in the ATP13A2 gene sequence. This mutation has been described in an early-onset PD case and generates a shorter protein, due to the lost of several transmembrane domains, that presents an erroneous intracellular localization at the ER.

Taking advantage of an α-Syn version that is prone to aggregate (SynT), we observed that ATP13A2 could enhance its aggregation and that ATP13A2 Dup22 increased the resistance of SynT to PK digestion. Furthermore, using SEC-HPLC we show that the presence of ATP13A2 Dup22 enhanced the formation of higher molecular weight α-Syn species, both a pre-establish oligomerization model and when co-expressed with the untagged protein. Interestingly, the effect promoted by ATP13A2 Dup22 was independent of CatD maturation, a process in which ATP13A2 is known to play an important role. We speculate that endogenous ATP13A2 is sufficient to guarantee the normal maturation and CatD, which suggests that alternative mechanisms may underlie the effect of ATP13A2 Dup22 on α-Syn. In future studies it would be interesting to knockdown endogenous ATP13A2 to better understand the overall impact of the Dup22 mutation on α-Syn oligomerization and aggregation.

Besides an impact on aggregation and oligomerization, we prove that the interplay between α-Syn and ATP13A2 Dup22 has a deleterious effect in cellular homeostasis. We show that both proteins can co-localize in abnormal ER structures leading to a significant increase in ER Stress, particularly the PERK pathway, an effect that is only observed upon the co-expression of both proteins. Changes in the ER pattern are accompanied by alterations in mitochondria

homeostasis. We show that ATP13A2 Dup22 and α-Syn sensitize cells to oxidative stress by enhancing the production of ROS upon challenge with H_2O_2. This effect was observed both in the presence and absence of inclusions suggesting that, in this paradigm, these species may not the main cause behind mitochondrial functional impairment and altered morphology. In further experiments, in would be worth investigating other ER stress pathways and to understand if the mitochondria impairment is caused by the overall intracellular imbalance or by the direct interaction with the ER (316). Additionally, it would be interesting to understand which α-Syn species are accumulating in the ER by taking advantage of α-Syn antibodies that can recognize different species of the protein.

Finally, we show that the interaction between ATP13A2 Dup22 and α-Syn promotes the upregulation of DR5 mRNA levels, and increase of cellular toxicity. Moreover, we discovered that the formation of the abnormal ER structures precedes cell death. Additional work is necessary to further understand the mechanisms underlying cell death and which caspase pathways lead to the apoptotic-like phenotype.

Altogether, our study provides new hints on how endolysosomal pathway can alter α-Syn homeostasis. We show that two proteins crucial in different stages of this degradation and secretion pathway, ATP13A2 and Rab8a, can alter α-Syn aggregation. Additionally, we bring a new insight on how the interaction between two PD-associated proteins, ATP13A2 and α-Syn, can lead to cellular death. In particular we show that the interplay between the familial mutation Dup22 in ATP13A2 and α-Syn can promote unmitigated ER stress, mitochondria impairment and the activation of apoptotic pathways. This knowledge provides a better understanding of the molecular interconnections underlying PD pathology and unveils new possible targets for a therapeutic approach.

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