Universidade de Aveiro Departamento de Ciências da Saúde
2013

Emanuel Ferreira Fernandes

O papel do complexo Sinfilina-1A/PPP1 na formação dos corpos de Lewy

The role of Synphilin-1A/PPP1 complex in Lewy bodies formation

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Tese apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biomedicina Molecular, realizada sob a orientação científica da Doutora Odete Abreu Beirão da Cruz e Silva, Professora Auxiliar do Departamento de Biologia da Universidade de Aveiro.

Dedico este trabalho científico à minha família e aos meus amigos, que sempre me deram a coragem e inspiração necessárias a sua realização. Dedico-o ainda a todos aqueles que queiram usufruir da informação aqui contida ou que, simplesmente, apreciem esta obra e se sintam inspirados por ela.

o júri

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À minha família, porque os últimos são sempre os primeiros. E vocês sabem sempre o que eu penso… mesmo antes de o ter pensado. Este trabalho é para vocês…

palavras-chave Doença de Parkinson, Corpos de Lewy, Sinfilina-1A, Fosfoproteína Fosfatase 1

resumo

Uma das características principais da doença de Parkinson é o aparecimento de inclusões citoplasmáticas, chamadas corpos de Lewy, maioritariamente nos neurónios dopaminérgicos remanescentes, no tronco cerebral dos pacientes afetados. Várias proteínas têm sido identificadas nos corpos de Lewy, mas o seu mecanismo de formação permanece por clarificar. Entre as várias proteínas já identificadas encontram-se a sinfilina-1, uma proteína interactora da α-sinucleina, e a sinfilina-1A, uma variante da sinfilina-1. Ambas têm sido consideradas elementos chave na doença de Parkinson, já que a sua sobreexpressão em células embrionárias 293 de rim humano, com ou sem a α-sinucleina, conduz à formação de inclusões citoplasmáticas parecidas com corpos de Lewy. Posto isto, têm sido envidados esforços no sentido de clarificar os mecanismos reguladores da agregação da sinfilina-1 e da sinfilina-1A, como forma de revelar novos aspetos da formação dos corpos de Lewy.

Embora tenham sido descritas cinases capazes de fosforilar a sinfilina-1, não há informações concretas sobre as fosfatases responsáveis pela sua desfosforilação. Este vazio começou a ser preenchido com a identificação da sinfilina-1A como uma nova proteína interactora da fosfoproteína fosfatase 1 em cérebro humano, através do sistema dois híbrido de levedura. Deste modo, no presente trabalho, procede-se ao estudo da função fisiológica do complexo sinfilina-1A/fosfoproteína fosfatase 1, demonstrando-se a capacidade da sinfilina-1A de recrutar de forma específica a fosfoproteína fosfatase 1 para corpos de inclusão, com recurso a imunofluorescência. Adicionalmente, as consequências do bloqueio desta interação são exploradas utilizando um mutante da sinfilina-1A incapaz de interagir com a fosfoproteína fosfatase 1, revelando um aumento das propriedades agregativas da sinfilina-1A. Finalmente, também é avaliada a capacidade de a sinfilina-1A selvagem e mutada produzirem agressomas, quando sobreexpressas e sem inibição do proteassoma, mas os resultados não são claros e não permitem a classificação das inclusões documentadas no presente trabalho como agressomas. Em conjunto, estes resultados sugerem que a sinfilina-1A tem a capacidade de afetar o endereçamento da fosfoproteína fosfatase 1 nas células, sendo a formação de corpos de inclusão dependente e, mais concretamente, controlada pela atividade da fosfoproteína fosfatase 1. Postula-se que um menor endereçamento da fosfoproteína fosfatase 1 para os corpos de inclusão conduza a estados hiperfosforilados que favorecem a agregação proteica.

keywords

Parkinson's disease, Lewy bodies, Synphilin-1A, Phosphoprotein Phosphatase 1

abstract

One of the major Parkinson's disease hallmarks is the development of cytoplasmic inclusions, termed Lewy bodies, mainly within surviving neurons in the brainstem of affected patients. Many proteins have been identified in the Lewy bodies, but their formation mechanism remains unclear. Among the proteins already identified in the Lewy bodies are synphilin-1, a α-synuclein-interacting protein, and synphilin-1A, a synphilin-1 splice variant. Synphilin-1 and synphilin-1A have been considered key elements in Parkinson's disease as their overexpression in human embryonic kidney 293 cells, with or without α-synuclein, leads to the formation of Lewy body-like cytoplasmic inclusions. Therefore, efforts have been made to clarify the regulatory mechanisms behind synphilin-1 and synphilin-1A aggregation as a means to uncover new aspects of Lewy bodies formation.

Although kinases able to phosphorylate synphilin-1 have been described, there are no specific data concerning the phosphatases responsible for its dephosphorylation. This gap was filled with the identification of synphilin-1A as a novel phosphoprotein phosphatase 1-interacting protein in human brain, through yeast two hybrid. Hence, in the present work, the physiological role of synphilin-1A/phosphoprotein phosphatase 1 complex is studied, being demonstrated the ability of synphilin-1A to specifically target phosphoprotein phosphatase 1 to inclusion bodies, using immunofluorescence experiments. Moreover, the consequences of disrupting this interaction are explored using a synphilin-1A mutant unable to interact with phosphoprotein phosphatase 1, revealing an enhancement of synphilin-1A aggregative properties. Also, the ability of wild type synphilin-1A and the mutant form to produce aggresomes upon overexpression and without proteasome inhibition is addressed but the results are unclear, does not allowing the classification of the inclusions documented in this work as aggresomes. All together, these results suggest that synphilin-1A is able to affect phosphoprotein phosphatase 1 targeting within cells, being inclusion bodies formation dependent and, most specifically, controlled by phosphoprotein phosphatase 1 activity. It is postulated that decreased phosphoprotein phosphatase 1 recruitment to inclusion bodies produces hyperphosphorylated states that favor protein aggregation.

Table of Contents

Abbreviations

α-syn	α-synuclein
53PB2	p53 binding protein 2
ALS	Autophagy-lysosomal system
ANK	Ankyrin
ANKRD42	Ankyrin repeat domain-containing protein 42
ATP	Adenosine-5'-triphosphate
Aβ	Amiloid β protein
BCA	Bicinchoninic acid
Bcl-2	B-cell lymphoma 2 (apoptosis regulator protein)
BM	Binding motif
BSA	Bovine serum albumin
CC	Coiled-coil
CKII	Casein kinase II
CNS	Central nervous system
CO_2	Carbon dioxide
DAT	Dopamine active transporter
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
E1	Ubiquitin-activating enzyme
E2	Ubiquitin-conjugating enzyme
E3	Ubiquitin-protein ligase
EGFP	Enhanced green fluorescent protein
ER	Endoplasmic reticulum
GDP	Guanosine-5'-diphosphate

GEF	Guanine nucleotide exchange factor
GFP	Green fluorescent protein
GPi	Internal globus pallidus
GSK3β	Glycogen synthase kinase 3 β
GTP	Guanosine-5'-triphosphate
HDAC	Histone deacetylase 6
HEK293	Human embryonic kidney 293
HSP	Heat shock protein
IF	Immunofluorescence
LBs	Lewy bodies
MAPB1	Microtubule-associated protein 1B
MBS	Protein phosphatase myosin-binding subunit
MPTP	1-methyl-4-phenyl-1,2,3,6 tetrahydropiridine
MT	Mutant
MTOC	Microtubule-organizing centre
MYPT1	Myosin phosphatase target subunit 1
$NaHCO_3$	Sodium Bicarbonate
PBS	Phosphate buffered saline
PD	Parkinson's disease
PIP	PPP1-interacting protein
PPP1	Phosphoprotein phosphatase 1
PPP2	Phosphoprotein phosphatase 2
PPP1c	Phosphoprotein phosphatase 1, catalytic subunit
PPP1CA	Phosphoprotein phosphatase 1, catalytic subunit, α isozyme
PPP1CC	Phosphoprotein phosphatase 1, catalytic subunit, γ isozyme
PPP1CC1	Phosphoprotein phosphatase 1, catalytic subunit, γ isozyme, isoform 1

PPP1CC2	Phosphoprotein phosphatase 1, catalytic subunit, γ isozyme, isoform 2
PSP	Protein serine/threonine phosphatase
PTP	Protein tyrosine phosphatase
Pu	Putamen
ROS	Reactive oxigen species
SARP	Several ankyrin repeated protein
SD	Standard deviation
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate-Polyacrilamide gel electrophoresis
SIAH	Seven in absentia homolog
SNpc	Substantia nigra pars compacta
Sph1	Synphilin-1
Sph1A	Synphilin-1A
TBST	Tris buffered saline-tween
Th	Thalamus
TIMAP	TGF-β1 inhibited, membrane associated protein
UCH-L1	Ubiquitin carboxyl-terminal hydrolase isozyme L1
UPS	Ubiquitin-proteasome system
WT	Wild type

1. Introduction

1.1 PARKINSON'S DISEASE OVERVIEW

1.1.1 Parkinson's Disease: Basics and Epidemiology

Parkinson's disease (PD) is a neurodegenerative motor disorder first described in 1817 by James Parkinson in *"An Essay on the Shaking Palsy"*[1]. According to epidemiological data, PD is the second most common neurodegenerative disorder after Alzheimer's disease, affecting about 0,3% of the whole population in industrialized countries[2, 3]. Moreover, the likelihood of developing PD increases with age: prevalence rises from 1% in individuals over 60 years of age to 4% in individuals over 80[3, 4]. Indeed, the mean age of onset is around 60 years, being the incidence between 8 and 18 per 100,000 person/years[3, 4]. Thence, population aging has led to an increasing concern on this pathology and to more investment in its understanding.

Two main types of PD are considered according to age of onset: (1) sporadic late-onset PD which accounts for 90% of the cases, with onset over the age of 50; and (2) rare early-onset PD which accounts for 5-10% of all cases, occurring before the age of 50[3-5].

1.1.2 Parkinson's Disease: Pathological and Clinical Hallmarks

In terms of pathological hallmarks, PD is mainly characterized by progressive degeneration and loss of dopaminergic neurons in the *substantia nigra pars compacta* (SNpc) while surviving neurons develop proteinaceous deposits in the cytoplasm (Lewy bodies - LBs) and within neuritis (Lewy neuritis)[6-8]. However, neuronal loss and LBs formation are also seen in other brain regions besides SNpc[8, 9]. Indeed, Braak and colleagues have found evidences that Lewy pathology in PD is frequently ordered and sequential, beginning in the olfactory region and/or in the dorsal motor nucleus of the vagus (stages I-II). Then, in the midstage of the disease, SNpc is affected together with other upper brainstem regions (stages III-IV), being the cerebral hemispheres involved only in latter stages (stages V-VI)[8, 9]. Dopaminergic neurons loss leads to dopamine deficiency in the striatum, producing a well characterized phenotype termed parkinsonism, which includes resting tremor, rigidity, bradykinesia and postural instability (Figure 1)[10, 11]. Furthermore, PD patients often suffer from non-motor symptoms such as autonomic dysfunction[12], depression and even dementia[2, 10, 11].

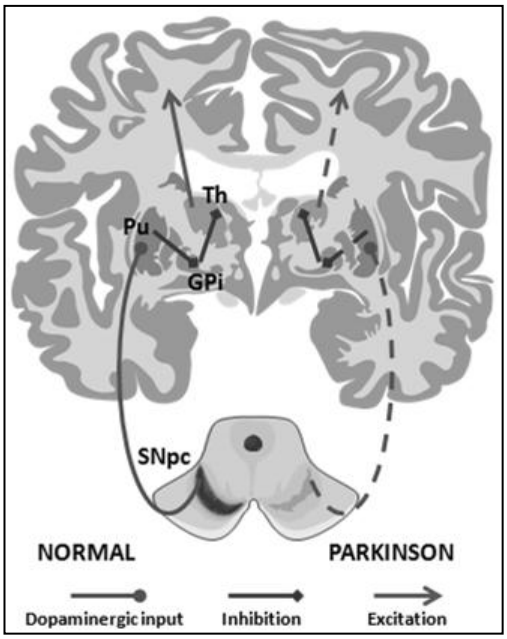

Figure 1 – Neuropathology of Parkinson's disease. In normal condition, dopaminergic neurons located in the *substantia nigra pars compacta* project to putamen. These projections modulate striatum activity. Upon activation, putamen inhibits internal globus pallidus, decreasing the inhibition exerted by internal globus pallidus in thalamus. Thalamic deinhibition allows the cortical excitation necessary for movement initiation. In PD patients, *substantia nigra pars compacta* looses pigmentation due to neuromelanin-rich, dopaminergic neurons loss, reducing putamen activation. This event reinforces internal globus pallidus inhibition on thalamus, causing difficulties in movement initiation[6]. (SNpc, *substantia nigra pars compacta;* Pu, putamen; GPi, internal globus pallidus; Th, thalamus. Dash arrows represent PD affected pathways. For simplification, only the direct pathway is represented.)

1.1.3 Parkinson's Disease: Etiology and Pathogenesis

Despite all knowledge concerning clinical and pathological hallmarks, PD etiology remains in debate. Currently, there are three main suggested causes for PD development: (1) environmental factors (such as exogenous toxins); (2) genetic factors; and (3) endogenous toxins[2, 6, 13, 14].

Numerous studies have presented evidence suggesting the role of exogenous toxins for PD development, particularly 1-methyl-4-phenyl-1,2,3,6 tetrahydropiridine (MPTP)[15], paraquat and rotenone[6, 16]. Therefore, these compounds are traditionally used to produce PD-animal models, being able to interfere with mitochondrial function and producing oxidative stress[6]. After a period in which the "environmental toxin hypothesis" was the main explanation for PD, a revolution has occurred in 1997 with the discovery that mutations in the gene for α-synuclein (α-syn) were able to produce familial forms of this pathology[17]. After that, many other mutated proteins associated to inherited rare forms of PD have been identified[18]. Besides exogenous toxins and genetics, a third hypothesis suggests that distortions in the normal metabolism of some substances, as dopamine, could produce endogenous harmful compounds able to take part in the degeneration process[14].

Whichever the etiological factor to initiate sporadic PD, there are two main explanations for its pathogenesis. The first one emphasizes protein misfolding and proteasome/lysosome dysfunction[6, 19-21], while the second one emphasizes mitochondrial dysfunction, oxidative stress and dopamine oxidation[6, 22, 23]. Obviously, they are not mutually exclusive, being the present aim in PD research to identify the sequence of these events and if they ultimately engage in a common mechanism for cell death (Figure 2).

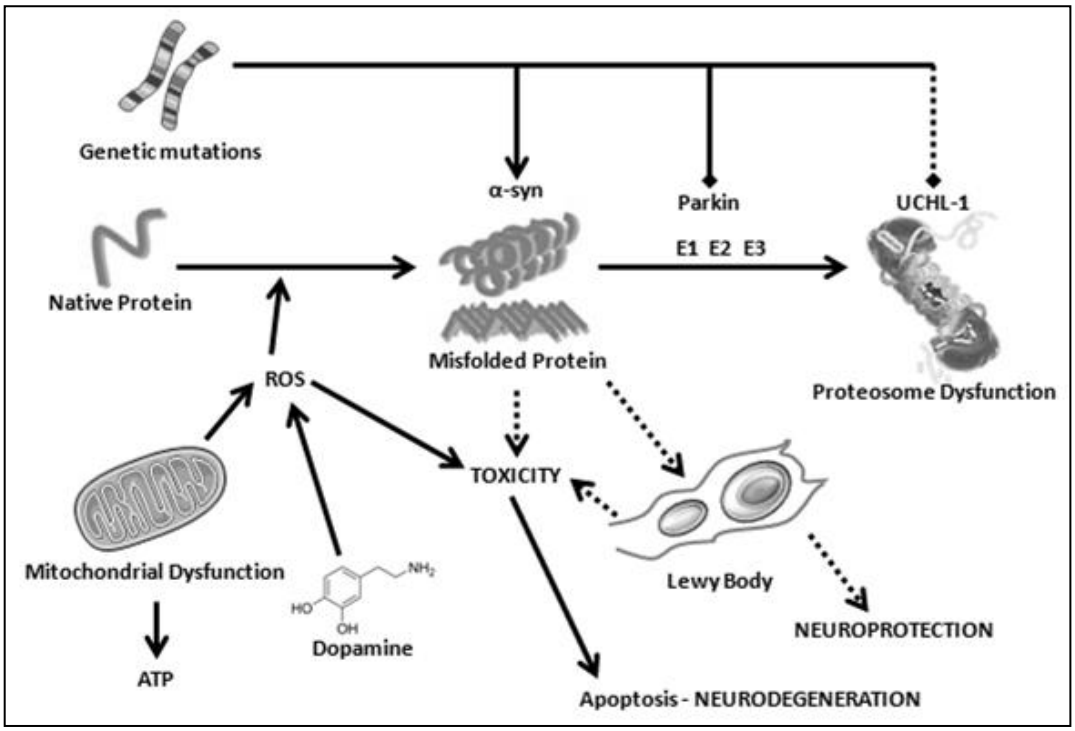

Figure 2 – Parkinson's disease pathogenesis. A growing body of evidence suggests that the accumulation of misfolded proteins is likely to be a key event in PD pathogenesis. Protein misfolding could be induced by genetic mutations producing aggregation-prone proteins (α-synuclein) or interfering with proteins involved in quality control and misfolded protein degradation (Parkin and UCH-L1). On the other hand, oxidative stress related with mitochondrial dysfunction and abnormal dopamine metabolism may also produce protein misfolding. It is unclear if misfolded proteins cause toxicity directly or via aggregates formation. The role of Lewy bodies remains to be clarified as some studies suggest a protective role while others suggest that Lewy bodies display toxicity. Other possible mechanisms of promoting cell death include ATP depletion and apoptosis[6]. (α-syn, α-synuclein; ROS, reactive oxygen species; UCH-L1, ubiquitin carboxyl-terminal hydrolase isozyme L1. Dash arrows represent the main events that need to be clarified.)

Despite the relevance of mitochondrial integrity, the hypothesis concerning protein misfolding and proteasome/lysosome dysfunction as explanation for PD pathogenesis will be emphasized in the present work. Indeed, neurons are cells incapable of full regeneration, being prone to suffer from protein accumulation due to their long life span. Furthermore, the production of abnormal proteins is prominent in the central nervous system (CNS) due to the high oxidative metabolism in neuronal cells[24]. Taken together, all these features determine that protein clearance is vital for proteostasis and neuron's survival.

1.2 PROTEOLYTIC STRESS AND AGGRESOMES FORMATION

Proper folding is vital for protein function. Nevertheless, the energy levels that separate native and non-native conformations are small enough to allow even native proteins to unfold under specific stress conditions[25, 26]. Thence, under stress conditions, some proteins suffer misfolding, being converted in toxic, aggregation-prone forms which must be properly cleared in order to avoid cellular toxicity[26].

1.2.1 Preventing Proteolytic Stress: Chaperones, UPS and ALS

Preventing the accumulation of aggregation-prone proteins is the most effective way to control protein aggregation which is achieved by folding facilitation through chaperones intervention and proteolytic degradation of abnormal proteins[26, 27]. Two are the main chaperone classes that prevent misfolded proteins accumulation: heat shock proteins (HSP) HSP60 and HSP70[28, 29]. However, misfolded proteins that could not be refolded are usually degraded by cytosolic ATP-dependent AAA+ proteases, such as the 26S proteasome[26].

The ubiquitin-proteasome system (UPS) is the major system in eukaryotic cells responsible for the disposal of abnormal and soluble proteins as well as short-lived regulatory proteins from the nucleus, cytosol and endoplasmic reticulum (ER). This system comprises three enzymes, i.e. the ubiquitin-activating enzyme (E1), the ubiquitin-conjugating enzyme (E2) and the ubiquitin-protein ligase (E3), in combination with the 26S proteasome[30]. The first three enzymes target proteins for proteasomal degradation by addition of ubiquitin moieties. On the other hand, the 26S proteasome possesses the degradation machinery, comprising a catalytic core (the 20S proteasome) and a regulatory cap (PA700) that regulates proteolysis. The catalytic core houses 3 proteases (chymotripsin-like, trypsin-like and peptidyl-glutamyl-peptide hydrolytic proteases) which degrade targeted proteins to small peptides and amino acids[24].

Despite the importance of UPS, cells possess another protein clearance system, the autophagy/lysosomal system (ALS). ALS is the major system responsible for disposal of insoluble bulk material such as protein aggregates and even unwanted organelles. This system requires the formation of spherical structures that contain acidic hydrolases able to breakdown macromolecules [26, 31].

Although there is cellular machinery responsible for protein clearance, when the generation of misfolded proteins exceeds the refolding or degradative capacity of the cell, protein aggregates accumulate. In this case, cells become under proteolytic stress, a feature clearly behind PD

pathogenesis, as mutations affecting UPS components, lysosomes and some aggregation-prone proteins produce familial cases of PD[32].

1.2.2 Dealing with proteolytic stress: aggresomes formation

When quality-control systems are overrun, protein aggregation takes place. However, recent data have suggested that even protein aggregation is a regulated process within cells[26].

Indeed, upon expression of some heterologous proteins or proteasome inhibition, mammalian cells produce specialized, non-permanent inclusion bodies in the cytoplasm termed aggresomes[33, 34]. These inclusions appear mainly as single spheres (1-3μm in diameter) or ribbons, being located to an indentation of the nuclear envelope at the microtubule-organizing centre (MTOC) and are surrounded by a cage-like shell made of vimentin[34, 35]. Aggresomes formation usually begins in the cell periphery through the assembly of small aggregates[34] which are transported to the final perinuclear site at the MTOC in a dynein-based manner[36]. Besides the dynein motor complex, histone deacetylase 6 (HDAC6), ataxin-3 and ubiquilin-1 are also important for recognition and transportation of cargo proteins into aggresomes[37-39]. Interestingly, some observations have suggested a potential role for Parkin, an E3 ubiquitin ligase, in substrates targeting into aggresome-autophagy pathway through K63-linked polyubiquitylation of misfolded proteins[37]. In light of that, in order to classify an inclusion as aggresome, two main criteria must be fulfilled: (1) the inclusion must co-localize with MTOC markers (such as γ-tubulin); and (2) its formation must be dependent on microtubules dynamics (being inhibited by drugs able to interfere with microtubules polymerization/depolymerization)[40, 41].

Curiously, aggresome formation is not a dead-end situation. Actually, heat shock proteins and components of the UPS are recruited to these inclusions to facilitate protein clearance[26]. Furthermore, aggresomes could be interesting in PD studies because it has been suggested that LBs, the typical PD inclusions, are a form of aggresome. Indeed, they have aggresomal appearance and contain centrosome/aggresome-specific proteins (as γ-tubulin) together with heat shock proteins and UPS elements commonly seen in aggresomes. Finally LBs reveal high levels of oxidized, phosphorylated and ubiquitylated proteins that normally do not accumulate in cells. It has been suggested that LBs might be cytoprotective under stress conditions, enhancing misfolded proteins clearance[24, 42, 43].

1.3 LEWY BODIES OVERVIEW

LBs are cytoplasmic rounded inclusions of 8-30 μm in diameter first described by Friedrich Lewy[44] after analysis of several brains from PD patients. Since their discovery, LBs were subjected to intensive study and other inclusion bodies were described for other neurodegenerative disorders besides PD. Nevertheless, after many years of study, the actual function of these inclusions is still highly controversial as some claim that inclusion bodies are part of the pathological process while others suggest a protective role against toxicity[45].

1.3.1 Lewy Bodies Structure and Classification

LBs may be classified according to their morphology and location in two main types: (1) brainstem (classic) LBs; and (2) cortical LBs[46]. The brainstem LBs usually occur in the brainstem nuclei and diencephalon, being spherical cytoplasmic inclusions characterized by hyaline eosinophilic cores, concentric lamellar bands, narrow pale halos and immunoreactivity for α-syn and ubiquitin (Figure 3A)[47]. By contrast, the cortical type is usually devoid of the halo (Figure 3B)[48].

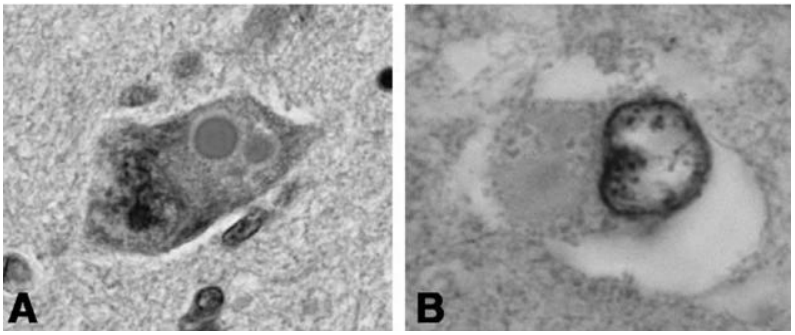

Figure 3 – Lewy bodies structure and classification. A) Classical Lewy body in a neuron in the dorsal raphe nuclei. B) Cortical Lewy body in a neuron in the medial temporal lobe. (*Adapted from*[49])

In terms of composition, LBs are a rather heterogeneous mixture of insoluble proteins and lipids, being α-syn derivatives their main components. However, apart from α-syn, a huge number of proteins have been identified so far, belonging to many different classes and families. Indeed, these proteins include: (1) UPS-related proteins (ubiquitin, ubiquitylated proteins, ubiquitin ligases, proteasome elements, parkin and DJ-1); (2) cytoskeletal proteins (neurofilaments, tubulins, microtubule associated proteins and gelsolin); (3) protein kinases and phosphatases; (4) chaperones (HSP70 and HSP90); (5) torsin A, 14-3-3; (6) inflammatory proteins as complements; (7) α-syn-binding proteins as synphilin-1and microtubule-associated protein 1B (MAP1B); (8) Alzheimer's disease related proteins (microtubule-associated protein tau and Aβ); (9) apoptotic proteins (Bcl-2

associated death proteins and extracellular regulated kinases); and (10) other proteins as cytochrome c, transglutaminase and glutathione peroxidase. Furthermore, these proteins may be ubiquitylated, phosphorylated, nitrated and/or oxidized[2, 50-52].

1.4 MOLECULAR BASIS OF PARKINSON'S DISEASE: ALPHA-SYNUCLEIN

Despite the complexity of PD, α-syn has been gathering great attention because there is evidence that it could be a major element in PD pathogenesis. Indeed, some familial forms of the disease are associated with mutations in the gene SNCA, in which α-syn is encoded[17, 53]. These mutations could be point mutations (A30P, A53T and E46K) or locus duplications and triplications, all of them causing parkinsonism with an autosomal dominant pattern of inheritance[2]. Moreover, α-syn can also be detected in LBs even in sporadic cases of PD[53]. Taking in account the relevance of α-syn, efforts have been made in order to uncover its physiological function and its role in LBs formation.

1.4.1 α-synuclein structure and function

α-syn is a small, insoluble, intrinsically unfolded protein of 140 amino acids encoded by the SNCA gene located at chromosome 4 (Figure 4)[54, 55]. In physiological conditions, α-syn is distributed to almost all subcellular compartments within neurons, but is enriched in the nucleus and presynaptic terminals[56, 57].

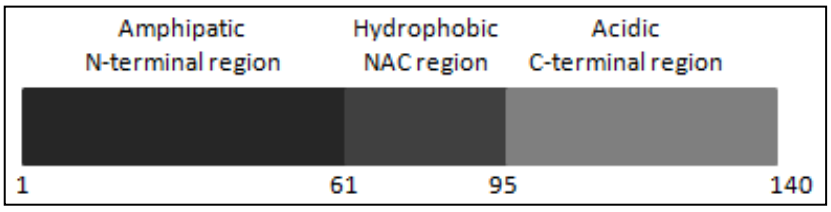

Figure 4 – α-synuclein structure. The amphipatic N-terminal region (dark gray) allows the association between α-synuclein and lipid layers. The hydrophobic NAC (non-Aβ component) region (medium gray) comprises the aggregative-prone domain of α-synuclein. The acidic C-terminal region (light gray) does not associate with membranes and seems to be important for chaperone-like functions of α-synuclein. Upon association with lipid micelles or membranes, α-synuclein acquires a secondary structure with two α-helixes. The point mutations A30P, A53T and E46K are located in the amphipatic N-terminal region. On the other hand, the acidic C-terminal region balances aggregative properties of NAC region, hindering α-synuclein spontaneous aggregation[58].

Although its function remains unclear, it has been suggested that α-syn has two major action sites within cells, namely presynaptic terminals[57] and plasma membrane[59]. At the presynaptic terminals, α-syn is thought to modulate synaptic vesicle function as it reversibly binds to brain vesicles and components of the vesicular trafficking machinery[57, 60, 61]. On the other hand, some data suggest an additional role in plasma membrane dynamics, probably protein trafficking. This is

consistent with some results which demonstrate α-syn involvement in the membrane localization of dopamine active transporter (DAT)[62]. Many other functions are suggested but they are beyond the scope of this introduction[63, 64].

1.4.2 α-synuclein in Parkinson's Disease

A growing body of evidence favors a critical, although in most cases, not necessarily a prime causal role of α-syn in PD pathogenesis. Indeed, families bearing SNCA mutations usually develop PD, being mutated α-syn the causal factor in these cases[2, 17]. Nevertheless, in sporadic patients in whom α-syn is not mutated, disease causes and mechanism seem to converge primarily around oxidative stress and impairment of protein catabolism. It is not clear if these causalities involve α-syn from the beginning or if defects in the handling of this protein just favor disease progression and neurons damage[65].

Findings suggesting a critical role of α-syn in PD include: (1) the presence of α-syn inclusions in the brain areas affected by PD; (2) the inheritance of PD in families bearing SNCA mutations; (3) the ability of α-syn to produce toxic oligomers, protofibrils and fibrils; (4) the formation of α-syn aggregates in chemically induced animal models of PD; and (5) neuronal dysfunction, synaptic terminals loss, neurons loss and damage to lysosomes upon α-syn overexpression in animal and cell models. Nevertheless, any of these studies present an unequivocal proof for a prime causal role of wild type α-syn in idiopathic PD[65].

Assuming that α-syn is involved in PD pathogenesis, it is important to evaluate which forms could be toxic. One interesting hypothesis postulates that post-translational modifications and molecular crowding of α-syn accelerate the formation of β-sheet-rich, spherical protofibrils. Latter, also chain-like and annular protofibrils are formed. Annular protofibrils are able to permeabilize membranes, allowing dopamine release within neurons which enhances oxidative stress. This hypothesis is corroborated by a cell-based study in which is proven that prefibrillar aggregates are able to produce toxicity in the absence of insoluble inclusions[65].

Besides soluble forms, α-syn is also able to form fibrillar and/or amorphous proteinase-resistant aggregates. These forms also display toxicity but perhaps only in latter stages of the disease process. Indeed, in early phases, they might act as cellular "sinks", trapping soluble oligomers. Obviously, these insoluble forms take part in LBs formation[45, 65].

1.5 MOLECULAR BASIS OF PARKINSON'S DISEASE: SYNPHILIN-1

In attempting to uncover α-syn function, a yeast two hybrid screen was performed aiming the identification of some α-syn-interacting proteins[53]. Interestingly, a novel protein, synphilin-1 (Sph1), has come into light and nowadays is intensively studied in order to clarify the mechanism of LBs formation. The importance of Sph1 for PD pathogenesis comes from its ability to interact with α-syn both *in vivo* and *in vitro*[53, 66]. Moreover, both proteins have nearly the same subcellular localization and both can be detected in LBs[67-69]. At the beginning no mutations or polymorphisms in Sph1 were associated with familial cases of PD[70-72]. Nevertheless, the R621C mutation was documented latter in two apparently unrelated German PD patients reinforcing the suggestion that Sph1 may actually play a role in PD[73]. Indeed, this mutation seems to represent a potential susceptibility factor for PD development.

1.5.1 Synphilin-1 structure and function

Sph1 is encoded by the SNCAIP gene, which is located in the chromosome 5 near markers WI-4673 and AFMB352XH5, and its open reading frame is contained within 10 exons[70]. As a protein, Sph1 is made of 919 amino acids and is composed by different structural domains: ankyrin-repeats, a coiled-coil domain and a putative ATP, GTP-binding domain (Figure 5)[53].

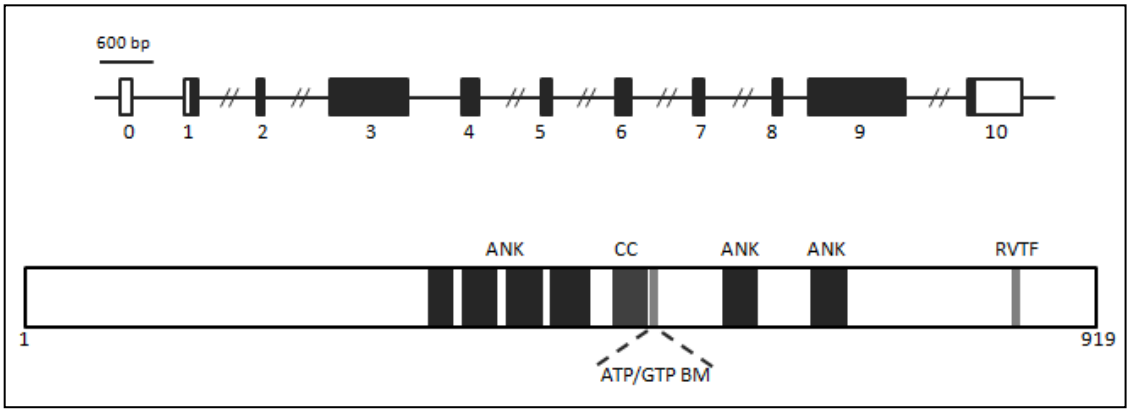

Figure 5 – SNCAIP gene and synphilin-1 structure. Sph1 coding region begins in the middle of exon 1 and ends within the very first few nucleotides of exon 10. Exons 4 through 7 contain the ankyrin-like repeats and a portion of the coiled-coil domain, while exon 8 contains the remainder of the coiled-coil domain and the ATP, GTP-binding domain. In this figure, the location of the RVTF (the PPP1 binding domain) motif is also specified at the C-terminus. Of note, Sph1 possesses many domains able to participate in protein-protein interactions, reinforcing its possible function as a linking protein[70, 74]. (ANK, ankyrin-like repeats; CC, coiled-coil domain; ATP/GTP BM, ATP, GTP-binding domain.)

In terms of tissue and subcellular localization, Sph1 shows a distribution similar to α-syn suggesting a relationship between these proteins. Indeed, Sph1 is widely distributed in brain and highly enriched in neurons, being found in the cell bodies of immature neurons and suffering

redistribution towards the presynaptic nerve terminals during development. Thence, Sph1 is particularly abundant in the neuropil and is located in the vicinity or associated to synaptic vesicles[70, 75]. Interestingly, a contrasting observation has come from a rat model in which Sph1 remains in the soma of *substantia nigra* neurons, contributing to the susceptibility of this area to LBs formation[75].

Although its actual function remains elusive, Sph1 is a synaptic vesicle-binding protein thought to anchor α-syn to vesicle membranes[75] and may affect dopamine release as well[76]. Recent data also suggest a possible contribution of Sph1 to UPS modulation[77].

1.5.2 Synphilin-1 isoforms: Synphilin-1A

There are more Sph1 isoforms within cells besides the most prominent 919 amino acids form[78]. However, synphilin-1A (Sph1A) is particularly important, consisting in an unusual Sph1 splice variant[74]. Sph1A is characterized by the absence of Sph1 exons 3 and 4, displaying an extra exon (9A) between exons 9 and 10. Curiously, although Sph1A has a start codon different from Sph1, the merge of exons 2 with 5 produces a frame shift leading to an identical reading frame for both proteins after exon 2. In summary, Sph1A only differs from Sph1 at the N-terminus and at the C- terminus (due to exon 9A) (Figure 6)[74, 79].

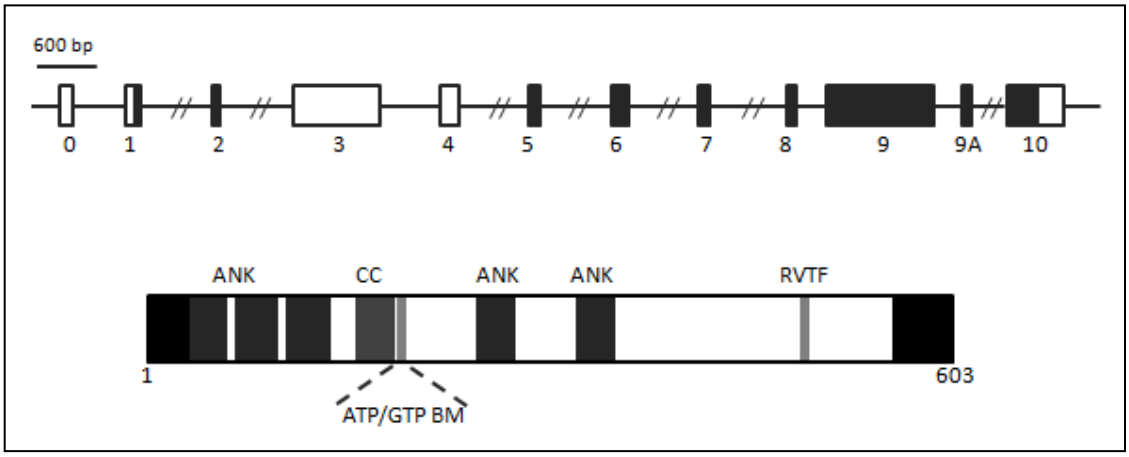

Figure 6 – Alternative splicing of SNCAIP gene and synphilin-1A structure. Sph1A coding region begins in a start codon different from Sph1 and finishes nearly in the middle of exon 10. Thence, Sph1A differs from Sph1 at the N-terminus and at the C-terminus. Furthermore, Sph1A lacks exons 3 and 4 and displays an extra exon, 9A. Despite the alternative splicing, Sph1A is essentially equal to Sph1 in terms of structural domains, also containing the RVTF motif (the PPP1 binding domain). Nevertheless, lacks the first and part of the second ankyrin-like repeats. It is suggested that Sph1A aggregation properties are in part due to its shorter N-terminus, allowing ankyrin-like repeats to be more exposed[74]. (ANK, ankyrin-like repeats; CC, coiled-coil domain; ATP/GTP BM, ATP, GTP-binding domain.)

Sph1A is an aggregation-prone isoform, being able to spontaneous aggregation in cell models even in the absence of proteasomal inhibitors. Nonetheless, the formation of more

organized Sph1A inclusion bodies requires proteasomal inhibition. There is limited information concerning Sph1A but it is able to interact with Sph1 (possibly through the ankyrin-like domains), α-syn and SIAH, recruiting them to its inclusion bodies. Upon recruitment of Sph1 to Sph1A-containing inclusions, SIAH-mediated degradation of Sph1 decreases, promoting inclusions formation within cells. Furthermore, Sph1A is also able to reduce SIAH ubiquitin-ligase activity, decreasing SIAH auto-ubiquitylation and ubiquitylation of SIAH substrates. This negative modulation performed by Sph1A promotes SIAH substrates accumulation, facilitating protein aggregation[74, 79, 80].

Despite the lack of studies concerning Sph1A, this isoform has reinforced Sph1 relevance for LBs formation as it is an aggregation-prone protein potentially able to take part in LBs seeding. Thence, Sph1A may be involved in the early events of LBs formation. Nevertheless, Sph1A studies display two main limitations: (1) all of them are overexpression based which could be limiting as Sph1A has low abundance within cells in comparison with Sph1; and (2) it is not known if all LBs contain Sph1A as such studies have not been performed so far[74, 79, 80].

1.5.3 Synphilin-1 aggregation properties and modulation by Sph1 interactors

Many studies have documented the ability of Sph1 to form inclusions upon overexpression in cell culture models, both alone or with α-syn[53, 81]. This process usually begins with the production of multiple small highly mobile aggregates which are dispersed through the cytoplasm. Nevertheless, small aggregates can be rapidly translocated into aggresomes if proteasome inhibitors are used, being the translocation dependent on Sph1 aggresome-targeting signal[40, 41]. Furthermore, Sph1 aggregation could be enhanced through K63-linked polyubiquitylation[82].

After these observations, efforts have been made in order to understand Sph1 aggregation patterns and aggresomes formation. Its relevance for PD pathogenesis and LBs formation lies in three main ideas: (1) Sph1 is able to produce aggresomes in cell culture models and LBs formation is thought to be an aggresome-related process[42, 43, 53, 81]; (2) Sph1 is able to interact with α-syn and its co-expression greatly enhances aggresomes production[53, 83]; (3) Sph1 appears mainly in the central core of LBs[84]. In light of this, Sph1 has gathered great attention as it could be related with LBs seeding within cells, potentially functioning as a scaffold protein. Obviously, LBs formation is perhaps even more complex, being Sph1 just another element. Nevertheless, many of its interactors have been discovered (besides α-syn) and they have provided a huge amount of information concerning modulation of Sph1-containing aggresomes production. Interestingly, some of the most relevant interactors are: (1) E3 ubiquitin-ligases, (2) UPS components and (3) some kinases. Furthermore, many of them are also PD-related proteins.

Modulation through E3 ubiquitin ligases

At normal conditions, Sph1 is highly ubiquitylated and degraded by UPS, being its ubiquitylation performed by four major E3 ubiquitin ligases: parkin, SIAH-1, SIAH-2 and dorfin[84-86]. However, the ubiquitylation patterns of SIAH and parkin are distinct. Indeed, SIAH attaches polyubiquitin chains via lysine 48 (K48) residues which act as a proteasomal targeting signal. On the other hand, parkin attaches ubiquitin chains to lysine 63 (K63) residues which is a mechanism of signal transduction instead of degradation[82]. Ubiquitylation by dorfin is still poorly understood[85]. Therefore, any UPS dysfunction will lead to the accumulation of SIAH-ubiquitylated Sph1, eliciting inclusion bodies formation within cells[87]. However, also K63 ubiquitylation is able to produce inclusion bodies[82]. Ubiquitylation seems to be important for aggregation as a mutant Sph1 unable to be ubiquitylated by SIAH does not form inclusions[87].

Modulation through UPS components

Besides ubiquitin ligases, Sph1 also interact with some proteasomal components decreasing proteasomal function[86, 88]. Among them, the regulatory protein S6-ATPase is particularly important, being inhibited by Sph1 when they are co-expressed. This leads to high number of inclusion bodies within cells[77]. On the other hand, the interaction between Sph1 and NUB1 decreases the number of Sph1 inclusions as NUB1 accelerates Sph1 degradation by unknown mechanism[89].

Modulation through protein kinases

Protein reversible phosphorylation is a major post-translational modification known to modulate a variety of cellular processes, including ubiquitylation of diverse proteins. Thence, there are several protein kinases able to interact with Sph1[86], particularly casein kinase II (CKII)[90] and glycogen synthase kinase 3 β (GSK3β)[88, 91]. CKII phosphorylates Sph1 *in vivo*, increasing Sph1/α-syn interaction without affecting its ubiquitylation state[90]. Thence, the number of inclusion bodies increases. On the other hand, GSK3β phosphorylation of Sph1 decreases its ubiquitylation and inclusion bodies formation[88, 91].

New Sph1-specific interactors and new conclusions

The yeast two hybrid technique that led to Sph1 discovery has also uncovered some Sph1-specific interactors that could not be included in the protein classes previously stated. Two important ones are periphilin[92] and kalirin-7[41].

Periphilin is a multifunctional protein involved in many cellular functions *in vivo*, being expressed during embryogenesis and in adult brains. Periphilin was recently associated to PD for four main reasons: (1) periphilin interacts and co-localizes with Sph1 within cells; (2) is present in LBs; (3) has functional implications in controlling cell death through caspase-3; and (4) a missense mutation in periphilin gene was discovered in two PD patients[92]. Thence, this study has reinforced the relevance of caspase-3 for the Sph1 cellular effects and PD.

On the other hand, kalirin-7 is a guanine nucleotide exchange factor (GEF) able to modify Sph1 aggregation and transport into aggresomes. Indeed, upon co-expression of Sph1 and kalirin-7, there is a dramatic increase of Sph1-containing aggresomes formation. Interestingly, this feature was not dependent on kalirin-7 GTP-GDP exchange activity as mutant forms of the protein conserved the ability to enhance aggresomes production. This observation was further attributed to an interaction between kalirin-7 and HDAC6 and to kalirin-7-promoted activation of HDAC6 deacetylation activity, promoting Sph1 aggregates transport into aggresomes[41]. Perhaps the most prominent conclusion of this study was the relation between a Sph1-interacting protein and HDAC6.

1.6 LINKING PROTEIN PHOSPHORYLATION AND SYNPHILIN-1 AGGREGATION

Protein reversible phosphorylation is the best described mechanism involved in activation and inactivation of enzymes and modulation of molecular interactions in signaling pathways. In eukaryotic cells, reversible phosphorylation usually occurs on the three hydroxyl-containing amino acids, serine, threonine and tyrosine. In order to phosphorylate a specific substrate, a phosphate group must be transferred from a donor molecule (frequently ATP), reaction which is performed by specific enzymes named kinases. On the other hand, phosphatases revert the reaction by removing phosphate groups which are eliminated as inorganic phosphates.

Although kinases and phosphatases work in a regulated syntony, a great dissimilarity exists between them in terms of number. Indeed, the human genome encodes for 518 putative protein kinases[93], being the phosphatases around 147[94, 95]. Nevertheless, it is thought that combinatorial formation of phosphatase holoenzymes from a shared catalytic subunit and a large number of regulatory subunits guarantees a great level of substrate specificity *in vivo*. In light of this, great

attention has been given to phosphatase-interacting proteins as they regulate phosphatases activity (for review see[96]).

1.6.1 Protein phosphatase 1 isoforms and Protein phosphatase 1-interacting proteins

There are two main families of protein phosphatases: protein tyrosine phosphatases (PTPs) and protein serine/threonine phosphatases (PSPs). Among the PSPs, protein phosphatase 1 (PPP1) is emphasized as a major and ubiquitously expressed phosphatase in all eukaryotic cells, being able to regulate a huge variety of cellular processes[96-98].

Similarly to other phosphatases, PPP1 exhibits broad substrate specificity *in vitro*. Nevertheless, it is thought that each assembled and functional PPP1 complex displays stringent substrate specificity and elicits specific biological responses. The PPP1 holoenzyme consists of a catalytic subunit (PPP1c) and a regulatory (R) subunit. PPP1c is a 35-38 kDa protein which exists as three isoforms: α (PPP1CA), β/δ (PPP1CB) and γ (PPP1CC) sharing 90% homology. Moreover, two PPP1CC splice variants were described (PP1γ1/PPP1CC1 and PP1γ2/PPP1CC2), being PPP1CC2 a testis-specific and sperm-enriched isoform[99]. Within cells, several mechanisms are responsible for regulation of PPP1 activity: reversible phosphorylation of R subunits, dissociation of the R and PPP1c subunits, allosteric regulation of R subunits and inducible expression of R subunits[96, 98].

As R subunits modulate PPP1 activity, they were heavily investigated and about 200 of these PPP1-interacting proteins (PIPs) have been identified so far[96, 100]. Indeed, each PIP may target PPP1 for specific subcellular compartments, modulate substrate specificity or serve as substrate, influencing PPP1 physiological functions[96]. In order to interact with PPP1, PIPs must possess critical consensus motifs, being the RVxF-motif the most frequent. The RVxF-motif comprises the consensus sequence [KRL]-[KRSTAMVHNQ]-[VI]-[FIMYDP]-[FW], and binds with high affinity to a hydrophobic region remote from PPP1 catalytic site (L288-M290-C291)[101-103]. Substrate binding to PPP1 through RVxF-motifs does not significantly affect PPP1 conformation but promotes the occupation of secondary, lower affinity binding sites able to alter PPP1 activity[101]. Moreover, this motif should be in an exposed and flexible loop in order to interact with PPP1[101]. Interestingly, the RVxF-motif is rather common among eukaryotic proteins but only a small fraction can be classified as PIP.

1.6.2 Protein phosphatase 1α interactome and Sph1A

Among all mammalian tissues, the brain expresses the highest levels of protein kinases and phosphatases. Moreover, all PPP1 isoforms are ubiquitously expressed but PPP1CC1 and PPP1CA are expressed at higher levels in several brain regions[99, 104]. In order to identify the proteins expressed in human brain able to interact with PPP1CA a yeast two hybrid was performed, resulting in 66 PIPs of which 39 represented novel interactions. Among the novel interactors, Sph1A was particularly prominent because 6 positive clones were obtained, uncovering a possible functional association between Sph1A and PPP1[105].

Interestingly, this finding opens several new hypotheses to fill the huge gap that exists concerning the modulation of Sph1A aggregation. Indeed, as previously stated, several kinases are thought to modulate Sph1 aggregation and even Sph1/α-syn interaction but the phosphatases involved in such mechanisms are largely unknown. Moreover, many structural features of Sph1A suggest a possible link with PPP1: (1) Sph1A possesses a PPP1 BM (RVTF)[105]; (2) Sph1A possesses ankyrin-like repeats as other established PPP1-associated proteins (53BP2, TIMAP, MYPT1/M_{110}/MBS and ANKRD42/SARP)[106]. However, in the previously stated ankyrin-containing proteins, PPP1 BMs immediately precede the start of the first ankyrin domain, except for SARP in which the binding motif partially lies within the first ankyrin repeat[106]. This is entirely different in Sph1A as the RVTF motif is located C-terminally (residues 449-452), far from the ankyrin-repeats (residues 28-123).

Thence, Sph1A fulfill several criteria to be studied as a putative PIP. Clarifying such protein-protein interaction could be mutually interesting, providing a new PIP for the growing list of PPP1 associated proteins, and filling a gap concerning phosphatases able to regulate Sph1 aggregative properties and able to play a role in PD pathogenesis.

2. Aims

PD is a neurodegenerative disorder in which cells develop inclusion bodies termed LBs. However, the mechanism behind LBs formation remains poorly understood. Interestingly, Sph1 and its major splice variant, Sph1A, are two proteins already identified in LBs composition and both are suggested to play a role in their formation. Indeed, (1) both are α-syn-interacting proteins[53]; (2) both are able to produce inclusion bodies resembling LBs, upon overexpression in cell models[53]; and (3) a Sph1 mutation has been associated to increased susceptibility to develop PD[73]. Therefore, efforts have been made in order to clarify the regulatory mechanisms behind Sph1/Sph1A aggregation because it could be a way to unravel new aspects of LBs formation.

Nevertheless, a huge gap exists because, although many modulators of Sph1 aggregation are known (see Introduction – Section 1.5), no phosphatases able to act on Sph1 and/or Sph1A have been properly described until now. In light of this, Sph1A was recently identified as a putative PIP in human brain, through yeast two hybrid[105]. This putative interaction between Sph1A and PPP1 must be validated by other means, besides yeast two hybrid, but could represent an important event for Sph1A regulation.

Therefore, the aim of this study is to confirm Sph1A/PPP1 interaction and to clarify the role of this complex in inclusion bodies formation. In order to achieve these aims, several tasks will be performed:

a) To evaluate the effect of Sph1A in PPP1CA and PPP1CC targeting and subcellular localization, in HEK293 cells overexpressing Sph1A;
b) To compare the aggregation properties of wild type Sph1A and a Sph1A mutant in which the PPP1 BM has been mutated by quantification and morphometric characterization of the aggregates formed;
c) To assess if Sph1A is able to form *bona fide* aggresomes upon Sph1A overexpression in HEK293 cells;
d) To evaluate if Sph1A/PPP1 interaction modulates Sph1A association with α-syn, interfering with α-syn recruitment for Sph1A aggregates.

3. Materials and Methods

3.1 CELL LINES

The cell model applied in the present study was human embryonic kidney 293 cells (HEK293) with 3 to 20 passages. HEK293 cells were purchased from ATCC and were tested for *Mycoplasma* contamination before performing any experiment.

3.2 REAGENTS AND ANTIBODIES

Nocodazole was purchased from Sigma-Aldrich and was reconstituted in DMSO to a final concentration of 33.2 mM (stock). Nocodazole stock solution was stored at -20ºC.

Commercial primary antibodies used in the present work included: (1) mouse anti- γ-tubulin monoclonal antibody (Sigma-Aldrich); (2) rabbit anti-α-synuclein polyclonal antibody (Sigma-Aldrich); and (3) mouse anti-GFP monoclonal antibody (Sigma-Aldrich). Besides commercial antibodies, two homemade antibodies were also used: anti-PPP1CA (CBC2C) and anti-PPP1CC (CBC3C). Anti-PPP1CA was raised in rabbits against the PPP1CA C-terminal peptide, NKGKYGQFSGLNPGG. Anti-PPP1CC was raised in rabbits against the PPP1CC C-terminal peptide, KKPNATRPVTPPRGMITKQAKK, and detects PPP1CC1 and PP1CC2.

Secondary antibodies used included Texas Red anti-mouse (Sigma-Aldrich), Texas Red anti-rabbit (Sigma-Aldrich), Alexa Fluor 594 anti-mouse (Sigma-Aldrich) and Alexa Fluor 594 anti-rabbit (Sigma-Aldrich). For detection in Li-Cor's Odyssey Infrared Imaging System, infrared IRDye-labeled anti-rabbit and anti-mouse were applied.

3.3 PLASMID CONSTRUCTS

Constructs used in transfection protocols, Sph1A-GFP (WT Sph1A) and Sph1A-RVTA-GFP (MT Sph1A), were made by Dr.ª Sara Esteves (for more details on constructs used see Appendix). Sph1A-RVTA-GFP is a mutant form of Sph1A-GFP in which the PP1 BM was mutated from RVTF to RVTA using the QuickChange Site-Directed Mutagenesis Kit (Stratagene now Agilent Technologies). A midi preparation was performed for both constructs using PureYield^{TM} Plasmid Midiprep System (Promega, UK), in accordance with the manufacturer's instructions (Sph1A-GFP: 268 ng/µL, ratio 1.89; Sph1A-RVTA-GFP: 304 ng/µL, ratio 1.89). Constructs were ethanol precipitated to achieve approximately 1 µg/µL of concentration.

3.4 CELL THAWING

Human embryonic kidney 293 (HEK293) cryopreserved cells (with 3 passages) were removed from the liquid nitrogen store and placed into a 37ºC water bath. The 1 mL vial was gently swirled in the 37ºC water bath for 2 minutes and transferred to a laminar flow hood, after vial decontamination with 70% ethanol. Then, thawed cells were transferred to a centrifuge tube and the cell suspension was centrifuged at 200 x g for 5 minutes. Finally, the supernatant was discarded, the pellet was resuspended in 1 mL of pre-warmed complete medium and the cell suspension was gently transferred drop by drop to a 60 mm culture dish with 9 mL of pre-warmed complete medium. The medium was changed after 6 hours.

3.5 CELL COUNTING WITH TRYPAN BLUE

In order to equalize cell number across transfection experiments, HEK293 cells were counted using Trypan Blue (Sigma-Aldrich). Briefly, 10 µL of 0,4% Trypan Blue were mix with 90 µL of cell suspension and viable (unstained) cells were counted using a Haemocytometer.

3.6 CELL MAINTENANCE

HEK293 cells were maintained with Dulbecco's Modified Eagle's Medium (DMEM) – high glucose with L-glutamine (D5648-1L, Sigma), supplemented with 10% (v/v) fetal bovine serum (FBS, Gibco), 100 U/mL penicillin/100 mg/mL streptomycin (p/s) and 3,7 g/L $NaHCO_3$ at 37ºC and 5% CO_2.

3.7 TRANSFECTION WITH TURBOFECT TRANSFECTION REAGENT

Cells were transfected in 6 well plates using TurboFect reagent (Roche), according to the manufacturer's instructions. Briefly, the culture medium was replaced with serum- and antibiotic/antimycotic-free DMEM. Then, 2 µg of DNA were diluted in 100 µL of serum- and antibiotic/antimycotic-free DMEM and 4 µL of TurboFect reagent were added to each tube. After gentle bubbling with the pipette, the mixtures were allowed to rest for 25 minutes at room temperature. Finally, the complexes were added into the cell medium, drop by drop with gentle rocking of the plate. The cells were incubated at 37ºC with 5% CO_2 for 24 hours/48 hours and the medium replaced after the first 5 hours of incubation.

3.8 NOCODAZOLE TREATMENT

Nocodazole treatment was started 24 hours after transfection, was performed for 6 hours and the inhibitor concentration was kept at 5 µM.

3.9 IMMUNOCYTOCHEMISTRY

Before seeding cells, an 18 mm coverslip was introduced in each well of a 6 well plate. Poly-L-ornithine (Sigma-Aldrich), 0.1 mg/mL, was added to each well for 5 minutes. At that point, poly-L-ornithine was removed and two washing steps were performed with autoclaved water. Wells and coverslips were left to dry before use.

After surface activation, HEK293 cells were cultured in pre-coated glass coverslips and transfected as described above. Immunocytochemistry was performed at two time points: 24 hours and 48 hours. Each well was washed two times with 1X PBS and a 4% paraformaldehyde fixative solution was added and left to stand for 30 minutes. Then, cells were washed three times with 1X PBS. For permeabilization, a 0,2% TRITON X-100 solution was added for 10 minutes, followed by 3 washes with 1X PBS. Blocking was carried out for 30 minutes with PBS/3% BSA. After blocking, the primary antibody diluted in PBS/3% BSA was added and incubated at room temperature for 1hour and 30 minutes to 2 hours, depending on the antibody used. After three washes with 1X PBS, the appropriate secondary antibody was added using the same methodology and incubated for 1 hour. Finally, three washes were performed and coverslips were stained using 50 µL of Hoechst (PolyScience) staining for 4 minutes. For mounting onto microscope glass slides, a 3Mowiol:1n-propyl-galate (ROTH) solution was used.

3.10 IMAGE ACQUISITION AND AGGREGATES COUNTING

Epifluorescence microphotographies were acquired with an Olympus IX-81 inverted epifluorescence microscope, equipped with EGFP (Chroma 41020) and Texas Red (Chroma 41004) filter cubes for fluorophore microscopy visualization. Cells were blindly counted in randomly chosen fields to have more than 50 aggregate-containing cells in each coverslip.

3.11 STATISTICAL ANALYSIS

In the present study, several experiments required aggregates counting: (1) quantification of cells bearing aggregates after transfection with WT Sph1A and MT Sph1A; (2) quantification of cells bearing aggregates with predefined sizes, after transfection with WT Sph1A and MT Sph1A;

and (3) quantification of transfected cells bearing aggregates with predefined sizes, after Nocodazole treatment in comparison with untreated conditions. In all cases, at least three independent experiments (replicas) were performed for each condition to assure reproducibility of the results. It is worthy to note that all these experiments were based in the quantification of specific, categorical events (e.g. having aggregates vs. not having aggregates; having small aggregates vs. having big aggregates) which produce categorical data. Therefore, statistical analysis was adjusted to this type of results.

For a more general analysis, quantitative data from aggregates counting were expressed as arithmetic mean ± standard deviation (SD) based on the three replicas performed for each condition. Then, means were compared and the overlap between standard deviations was used to address the significance of the differences identified.

However, a more detailed analysis was also performed using statistical tests but to compare corresponding replicas within the same experiment. That means that, for each experiment, control replica 1 was compared with treatment replica 1 and so on, as if they were paired samples. Then, contingence tables were produced and p-values were calculated using Pearson's chi-squared test and Cochran-Mantel-Haenzel chi-squared test. These tests evaluate if there is an association between a specific treatment and a specific effect produced and are suitable to apply in cases of categorical data.

3.12 CELL COLLECTION AND SAMPLE FRACTIONATION

Transfected HEK293 cells were collected, lysed and subjected to fractionation in order to separate triton-soluble components from triton-insoluble components. Briefly, transfected HEK293 cells (plated in 60 mm dishes, $6.0x10^5$ cells/dish) were washed 2 times with cold PBS 1X and harvested by scrapping in 200 µL of non-denaturating cold lysis buffer containing 1% TRITON X-100 (50 mM Tris-HCl, pH 7.5; 175 mM NaCl; 5 mM EDTA; 1% TRITON X-100; protease and phosphatase inhibitors mix). Then, cell suspensions were ice incubated for 30 minutes. In order to pellet triton-insoluble components, cell suspensions were centrifuged at 16000 x g for 30 minutes (4ºC). Finally, the supernatants were transferred to new microtubes (and labeled as Triton-soluble fractions) while pellets were resuspended in 200 µL of 2% SDS-containing buffer (similar to lysis buffer but containing 2% SDS instead of TRITON) and sonicated for 10 seconds (being labeled as Triton-insoluble fractions).

3.13 BCA PROTEIN ASSAY

The protein content of HEK293 lysates was determined using BCA protein assay (Pierce). Reactions were performed by addition of 200 µL of Working Reagent to 25 µL of each sample and the standards were prepared as described in the appendix. Both standards and samples were incubated at 37ºC for 30 minutes. The absorbances were then measured at 562 nm and a standard curve was constructed by plotting the absorbance value of each BSA standard against its concentration. The standard curve allowed the estimation of the protein concentration of each sample from its absorbance value.

3.14 SDS-PAGE AND IMMUNOBLOTTING

Samples were subjected to SDS polyacrylamide gel electrophoresis (SDS-PAGE). For that, 50 µg of each sample were mixed with 4x loading buffer and resolved on a 15% polyacrylamide gel. Gels were run at 200 volts for approximately 1 hour.

After electrophoresis, proteins were transferred to nitrocellulose membranes at 200 miliampers for approximately 1 hour and 30 minutes. Membranes blocking was carried out by immersion in 5% non-fat milk/1X TBST, for 1 hour with shaking. Blocked membranes were then washed 3 times with 1X TBST and incubated with primary antibody diluted in 3% non-fat milk/1X TBST solution, for 2 hours with shaking. The 3 washing steps were repeated with 1X TBST and membranes were incubated with the appropriate secondary antibody also diluted in 3% non-fat milk/1X TBST solution, for 1hour with shaking. Bands detection was performed at 700 nm (anti-rabbit) or 800 nm (anti-mouse) using Odyssey CLx as secondary antibodies were conjugated with infrared fluorescent reporters.

4. Results

4.1 PPP1CA AND PPP1CC ARE TARGETED TO INCLUSION BODIES BY SPH1A AND THEIR TARGETING IS DEPENDENT ON SPH1A RVTF MOTIF

As Sph1A was found to interact with PPP1CA by an yeast two hybrid screen of a human brain library, it was important to validate this interaction by other methodologies[105]. Some previous results from our laboratory have already demonstrated the existence of a conserved PPP1 BM (RVTF) in Sph1A amino acid sequence. Also, Sph1A/PPP1 interaction was previously validated using yeast co-transformation and overlay blot assay[107]. Thence, in the present work, Sph1A/PPP1 interaction is addressed through immunofluorescence studies in HEK293 cells.

In order to evaluate Sph1A/PPP1 co-localization and its specificity, HEK293 cells were transfected with WT Sph1A and MT Sph1A. The first construct corresponds to wild type Sph1A fused with GFP. The second is a Sph1A mutant, in which the PPP1 BM (RVTF) was disrupted by mutagenesis of the phenylalanine residue to adenine, originating the motif RVTA that does not bind PPP1[107]. After 48 hours of transfection, endogenous PPP1CA and PPP1CC were stained with specific antibodies and the subcellular localization of Sph1A and the two PPP1 isoforms were compared (Figures 7 and 8).

Upon WT Sph1A overexpression in HEK293 cells, transfected cells develop a diffuse green signal across the cytoplasm, corresponding to soluble, GFP-tagged WT Sph1A. Furthermore, some, but not all, of the transfected cells also display GFP-positive aggregates with an exclusive cytoplasmic distribution (Figure 7). These aggregates may be isolated (Figure 7A) or may be multiple and dispersed across the cytoplasm (Figures 7B). When HEK293 cells overexpressing WT Sph1A are stained with PPP1CA and PPP1CC specific antibodies, both endogenous PPP1CA and PPP1CC reveal nuclear and cytoplasmic distribution, which is in accordance with previous studies (Figure 7)[108]. Actually, both PPP1CA and PPP1CC are known to be present in the cytoplasm and nucleus of mammalian cells but while nuclear PPP1CA mainly occurs in a diffuse nuclear pool, nuclear PPP1CC accumulates predominantly within the nucleolus[108]. Although Sph1A and the two PPP1 isoforms maintain their expected subcellular localization, according to literature[74, 108], in aggregate-containing cells, a marked co-localization between both PPP1CA (Figure 7A) and PPP1CC (Figure 7B) and WT Sph1A inclusions can be detected. Therefore, WT Sph1A seems to interact with PPP1CA and PPP1CC, recruiting them to the cytoplasmic inclusions formed.

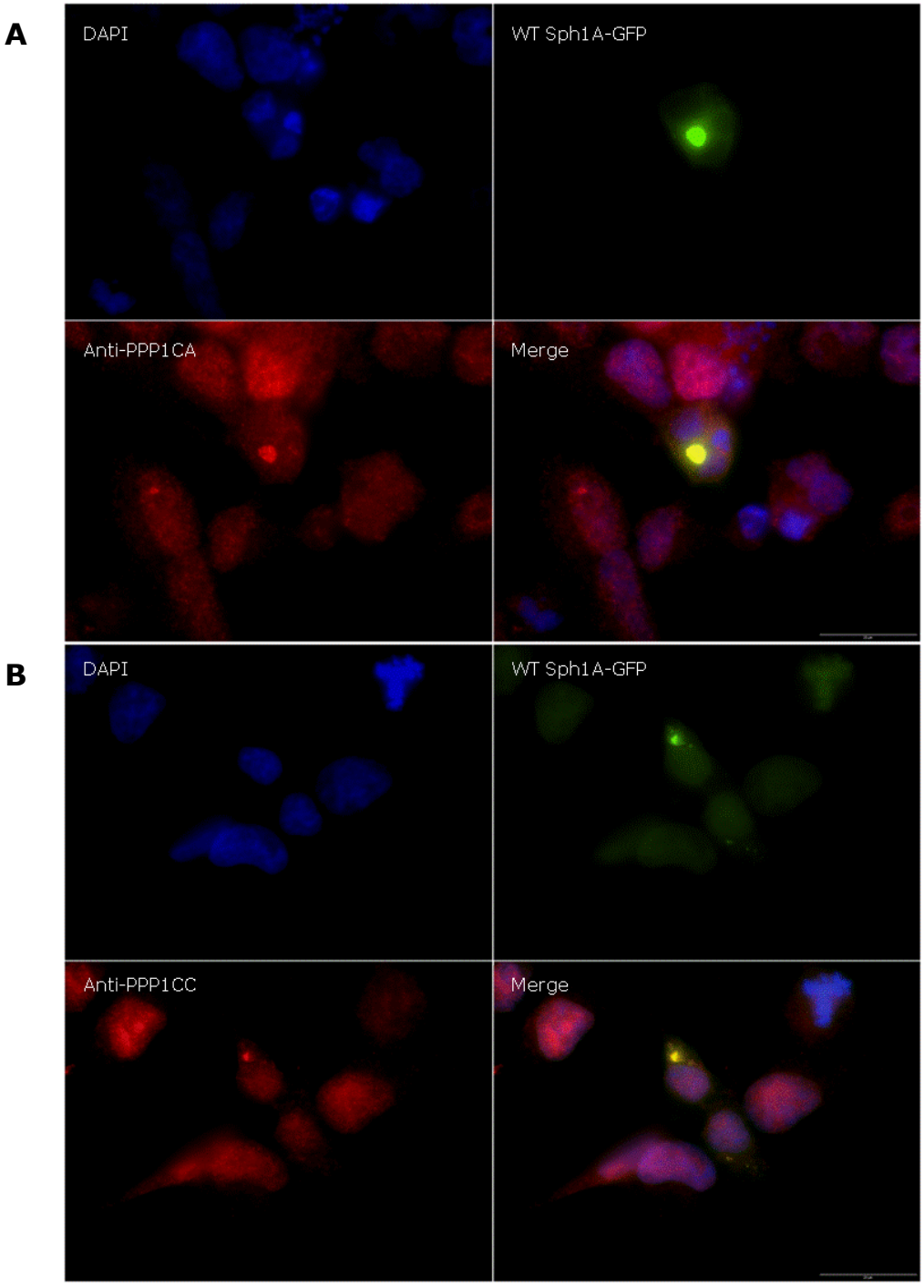

Figure 7 - Wild type synphilin-1A co-localizes with endogenous PPP1CA and PPP1CC in cytoplasmic inclusion bodies. A: Fluorescence microscopy visualization and intracellular localization of the Sph1A-GFP fusion protein (WT Sph1A-GFP) and endogenous PPP1CA (Anti-PPP1CA) within HEK293 cells overexpressing wild type synphilin-1A. B: Fluorescence microscopy visualization and intracellular localization of the Sph1A-GFP fusion protein (WT Sph1A-GFP) and endogenous PPP1CC (Anti-PPP1CC) within HEK293 cells overexpressing wild type synphilin-1A. Both panels display cells with and without aggregates. Cells transfected with EGFP-N1 served as negative control being not able to form cytoplasmic aggregates (data not shown). Scale bars: 20 µm.

Although PPP1CA and PPP1CC co-localize with WT Sph1A in cytoplasmic inclusions, this co-localization could represent an unspecific aggregative process, in which cytoplasmic PPP1 is

simply sequestered, along with many other inclusion body-related proteins. On the other hand, PPP1 could be specifically recruited by Sph1A. Many PIPs are known to modulate PPP1 targeting, specifically recruiting PPP1 isoforms to specific subcellular compartments[96]. A common feature among PIPs is that PPP1 targeting requires a conserved PPP1 BM contained in the PIP primary structure[96]. These motifs are easily found through *in silico* studies and Sph1A contains a conserved RVTF motif. Nevertheless, many proteins possess PPP1 BM but are not able to participate in PPP1 targeting and modulation, not being real PIPs. In light of that, it was necessary to address if Sph1A/PPP1 co-localization (Figure 7) is dependent on the Sph1A RVTF motif, or if is an unspecific sequestering process. This was achieved through mutation of the RVTF motif to RVTA in Sph1A, as described above.

When HEK293 cells are transfected with the Sph1A-RVTA-GFP construct instead the non-mutated construct (Sph1A-GFP), no major changes can be seen in MT Sph1A, PPP1CA and PPP1CC subcellular localization (Figure 8). Indeed, Sph1A forms a cytoplasmic, soluble pool and some cytoplasmic aggregates in transfected cells while endogenous PPP1CA and PPP1CC remain in the cytoplasm and nucleus, as expected (Figure 8). Moreover, not all transfected cells develop Sph1A inclusions and the inclusions formed can be isolated or multiple (Figure 8). However, there is a striking decrease in Sph1A/PPP1 co-localization within inclusion bodies for both PPP1 isoforms (Figure 8A and 8B), independently of aggregates morphology and size. Therefore, a unique point mutation in the Sph1A RVTF motif is sufficient to abolish PPP1CA and PPP1CC recruitment for Sph1A inclusion bodies.

Taken together, these findings confirm Sph1A/PPP1 interaction for two PPP1 isoforms (PPP1CA and PPP1CC), in *ex vivo* conditions. Indeed, PPP1CA and PPP1CC are recruited to Sph1A cytoplasmic inclusions, independently of the morphology and size of the aggregates. Nevertheless, this interaction is specific as requires a conserved PPP1 BM contained in Sph1A structure (RVTF). A single point mutation in this motif is sufficient to interrupt PPP1 recruitment to inclusion bodies and this effect is also independent of the morphology and size of the inclusions. Hence, Sph1A displays many features of a common PIP as it contains a conserved PPP1 BM, it is able to interact and target PPP1 isoforms to a specific subcellular compartment (inclusion bodies) and this targeting specifically depends on the conserved RVTF motif. Interestingly, as PPP1 is targeted to Sph1A inclusion bodies with different morphologies and sizes, PPP1 recruitment might be an early event in Sph1A inclusions formation.

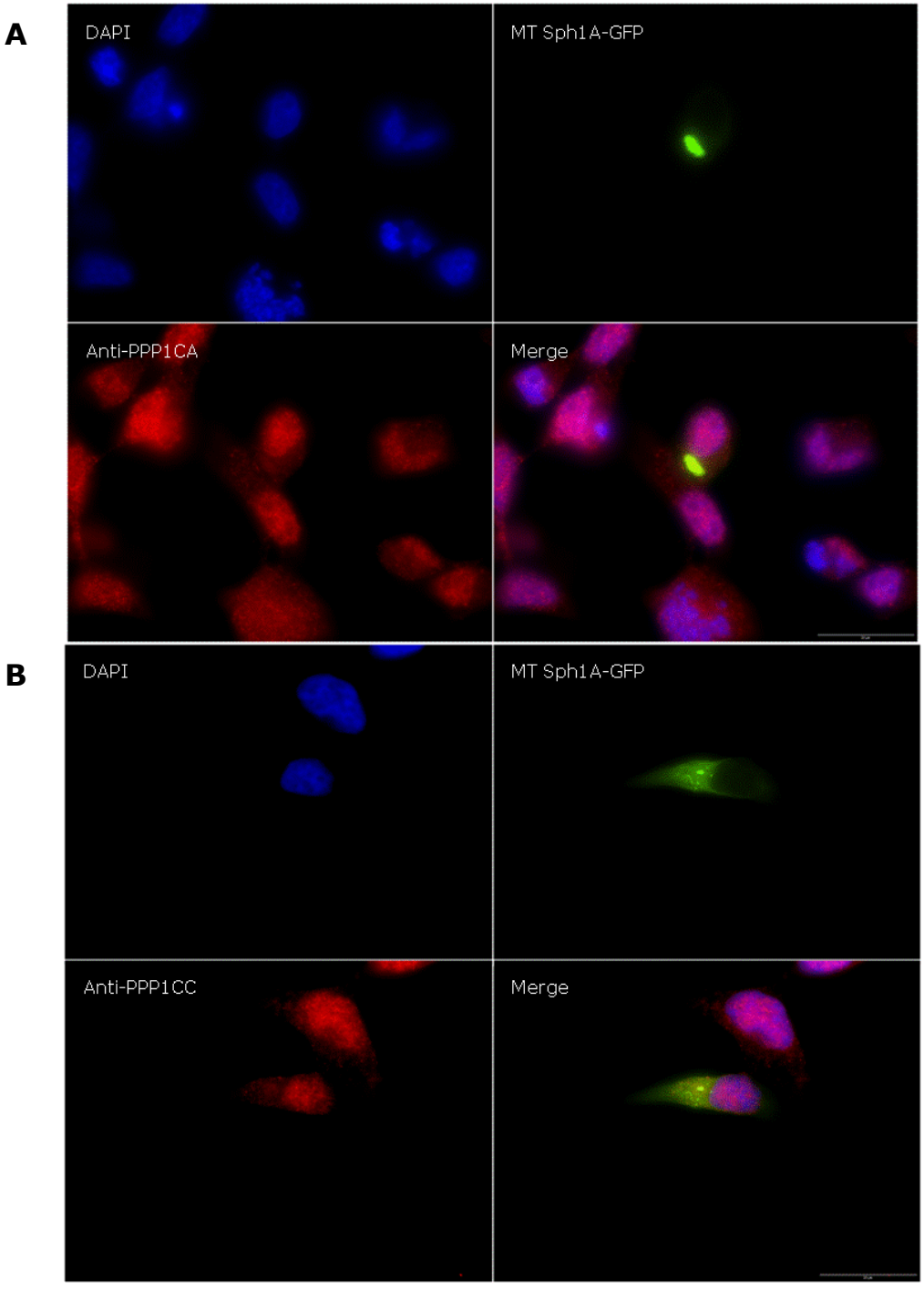

Figure 8 - Mutant synphilin-1A, in which the RVTF motif is mutated to RVTA, shows decreased co-localization with endogenous PPP1CA and PPP1CC in cytoplasmic inclusion bodies. A: Fluorescence microscopy visualization and intracellular localization of the mutant Sph1A-GFP fusion protein (MT Sph1A-GFP) and endogenous PPP1CA (Anti-PPP1CA) within HEK293 cells overexpressing the synphilin-1A mutant. B: Fluorescence microscopy visualization and intracellular localization of the mutant Sph1A-GFP fusion protein (MT Sph1A-GFP) and endogenous PPP1CC (Anti-PPP1CC) within HEK293 cells overexpressing the synphilin-1A mutant. Cells with and without aggregates are shown. Cells transfected with EGFP-N1 served as negative control being not able to form cytoplasmic aggregates (data not shown). Scale bars: 20 µm.

4.2 DISRUPTION OF SPH1A/PPP1 INTERACTION FAVORS THE FORMATION OF BIG AGGREGATES AND ACCELERATES SPH1A AGGREGATION IN HEK293 CELLS

In the previous section, data suggest that Sph1A could be a novel, putative PIP, specifically targeting PPP1CA and PPP1CC to Sph1A cytoplasmic inclusions. Nevertheless, PIPs could have other functions besides PPP1 targeting for subcellular compartments, such as modulation of PPP1 substrate specificity or may serve as PPP1 substrates[96]. Therefore, the actual Sph1A role as PIP and the functional significance of PPP1 recruitment for inclusion bodies should be clarified.

In terms of Sph1A role as putative PIP, it is clear that Sph1A is able to specifically target PPP1 to inclusion bodies. However, the consequences of such targeting may be quite diverse. Indeed, PPP1 could be recruited in order to dephosphorylate Sph1A itself, in which case Sph1A will be also a substrate. Nevertheless, Sph1A could also modulate PPP1 substrate specificity, in which case PPP1 will be recruited for dephosphorylation of other inclusion body-related proteins. Whatever the actual consequence(s) of Sph1A/PPP1 interaction and PPP1 recruitment for inclusion bodies, it is likely that it may affect the process of inclusions formation.

Actually, it is not the first time in which Sph1 phosphorylation state is referred as having a modulatory activity on Sph1 aggregation. Indeed, previous studies have suggested that Sph1 phosphorylation by GSK3β regulates its ubiquitylation and proteasomal degradation, while phosphorylation by CKII regulates its interaction with α-syn[88, 90]. Thence, Sph1 phosphorylation state could be important to regulate Sph1 degradation or interaction with other inclusion body-related proteins. In both cases, Sph1 aggregation pattern is altered. Despite that information, there are no data available concerning Sph1A and the phosphatases implicated in these mechanisms are unclear.

In light of that, it seems legit to ask whether blockage of PPP1 targeting to Sph1A aggregates has any functional consequence, altering (or not) Sph1A aggregation pattern and inclusion bodies formation. In order to answer this question, HEK293 cells were transfected with Sph1A-GFP and Sph1A-RVTA-GFP, the previously described Sph1A mutant with a mutated PPP1 BM. After that, immunocytochemical experiments were performed and both conditions (WT Sph1A and MT Sph1A) were compared in terms of percentage of cells bearing aggregates (Figure 9)[83] and in terms of morphometric features (number, size and distribution) of these aggregates [40, 41]. To gather more details on the aggregation patterns of WT Sph1A and MT Sph1A, aggregate counting and morphometric analysis were performed at two different time points: 24 hours and 48 hours, after transfection.

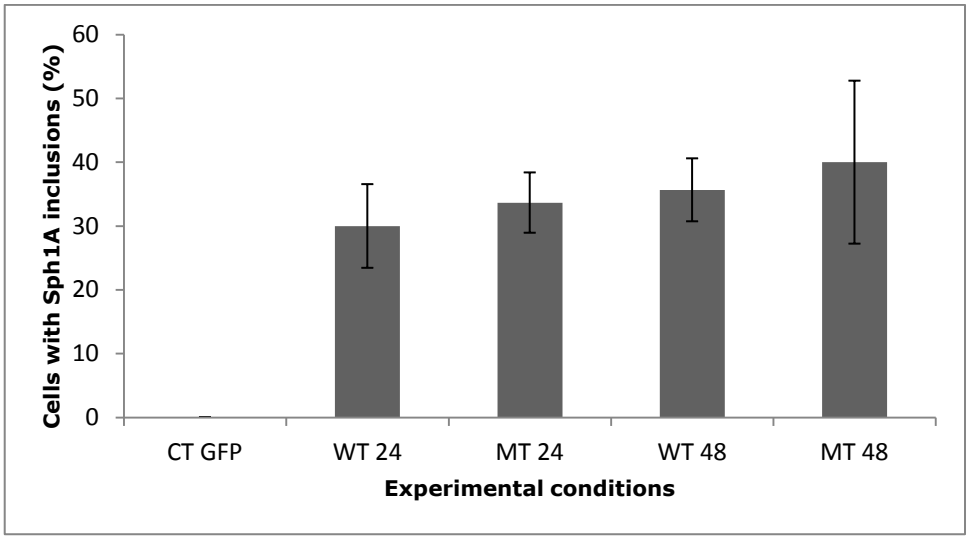

Figure 9 - Mutation of the Sph1A PPP1 BM RVTF does not significantly change the percentage of HEK293 cells bearing inclusions. The graph shows the average number of transfected HEK293 cells containing inclusions when transfected with wild type Sph1A (WT) and mutant Sph1A (MT), determined by fluorescence microscopic visualization at two time points: 24 (24) and 48 hours (48), after transfection. HEK293 cells transfected with empty EGFP-N1 vector (CT GFP) were used as negative control and do not show inclusion bodies. All data represent the mean ± SD of at least three independent experiments (n = 300 cells per condition). When Pearson's chi-squared test and Cochran-Mantel-Haenszel chi-squared test are applied to compare the proportion of cells with inclusions in corresponding replicas (WT 24 vs. MT 24 and WT 48 vs. MT 48), no differences can be detected at a significance level of 5% (p-values >0,05). Therefore, there is no association between the percentage of cells with inclusions and the type of protein (WT vs. MT) used in transfection.

The percentage of cells with inclusions is used in the present study as a broad measure of the aggregative properties of both WT Sph1A and MT Sph1A. Twenty-four hours after transfection, nearly 30% of HEK293 cells show cytoplasmic Sph1A inclusion (Figure 9). Forty-eight hours after transfection, this number increases to nearly 36% but this rise is not statistically significant (Figure 9). Furthermore, mutation of the Sph1A PPP1 BM does not significantly alter the percentage of cells bearing inclusions, for any studied time point (24 and 48 hours) (Figure 9). Therefore, when the aggregative properties of WT Sph1A and MT Sph1A are compared in terms of percentage of cells bearing aggregates, they are not significantly different (Figure 9). Interestingly, inclusions are formed in the absence of any treatment for proteasome inhibition and with expression of Sph1A alone, which is in accordance with previous studies postulating Sph1A aggregation-prone properties[74]. Furthermore, the percentage of cells bearing inclusions for WT Sph1A overexpression (nearly 30%) also matches with previous observations[74].

Broad quantification of cells displaying aggregates was not able to reveal any functional consequence of blocking PPP1 recruitment for inclusion bodies. Thence, a more detailed morphometric analysis was performed (Figure 10) based on aggregates size and number, in accordance with the following criteria: (1) aggregates with 1 µm or less in diameter were classified as small aggregates; (2) aggregates with more than 1 µm to 3 µm in diameter were classified as

medium aggregates; (3) aggregates with more than 3 µm in diameter were classified as big aggregates; (4) cells bearing multiple small aggregates and just one small aggregate were separately counted; and (5) in cells bearing multiple aggregates with different size, just the bigger was considered for classification purposes. The 5th criterion was established in order to emphasize the dynamics of protein aggregation. Therefore, and as an example, if a given cell contain a medium aggregate mixed with multiple small inclusions, it should be sorted in class 2 (medium aggregates) because, in terms of aggregation process, it reached the second stage, in which small aggregates begin to assemble to form the medium ones.

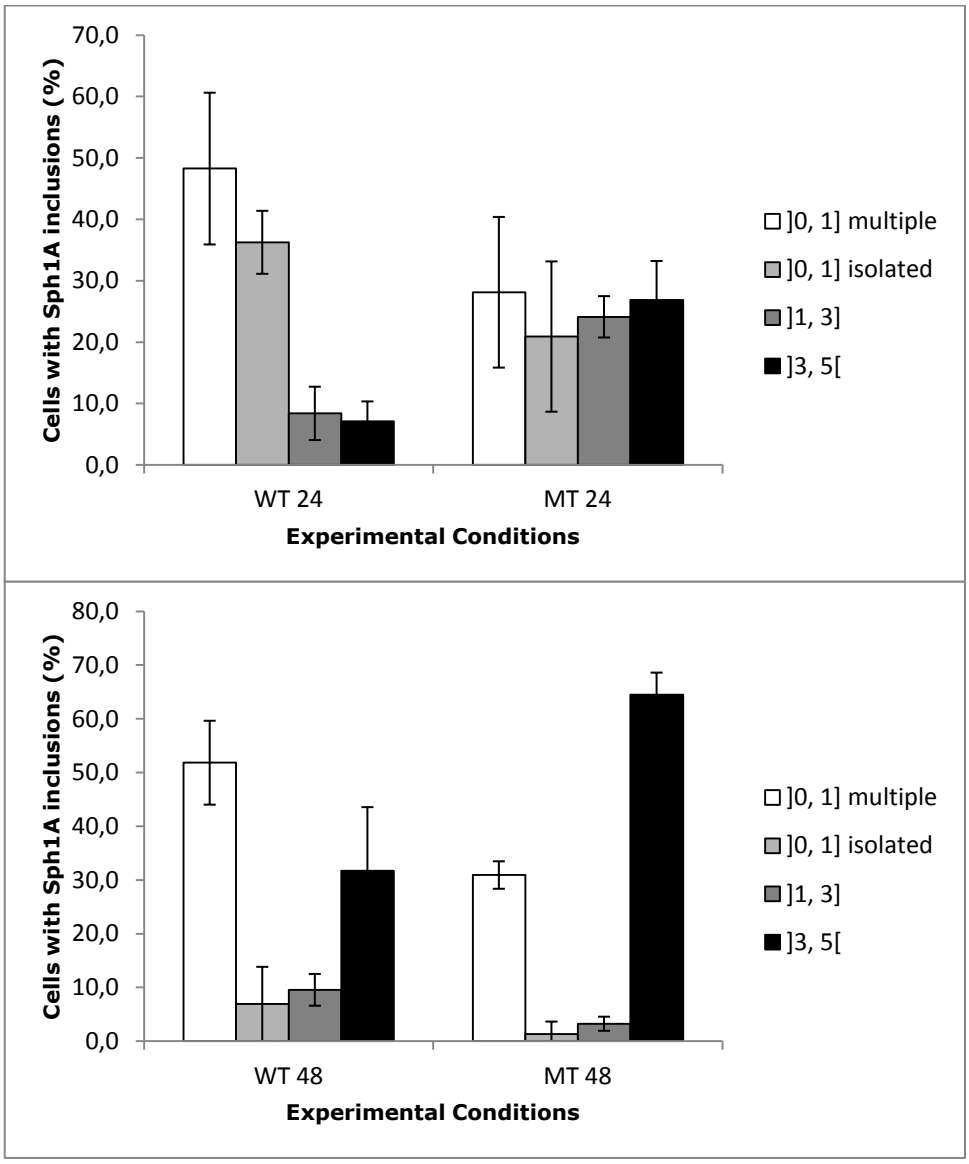

Figure 10 - Mutation of the Sph1A PPP1 BM RVTF favors the formation of medium and big aggregates in HEK293 cells. The graph shows the average number of transfected HEK293 cells displaying small multiple (]0,1] multiple), small isolated (]0,1] isolated), medium (]1,3]) and big aggregates (]3,5[), when transfected with wild type Sph1A (WT) and mutant Sph1A (MT) for 24 (24) and 48 (48) hours, determined by fluorescence microscopic visualization. All data represent the mean ± SD of at least three independent experiments (n = 150 cells per condition).

When Pearson's chi-squared test is applied to compare the aggregation pattern of corresponding replicas (WT 24 vs. MT 24 and WT 48 vs. MT 48), the differences are statistically significant which means that there is an association between the transfected protein (WT Sph1A or MT Sph1A) and the aggregation pattern produced, for both time points at a significance level of 5% (p-values <0,05).

According to the morphometric analysis, 24 hours after transfection with WT Sph1A, the majority of the transfected HEK293 cells develops small multiple (48.3%) and small isolated inclusions (36.3%) (Figure 10). Contrarily, only 15.5% develop medium and big aggregates (Figure 10). Interestingly, when the post-transfection period is extended from 24 hours to 48 hours, this propensity is maintained as small multiple aggregates continue to dominate the pool of inclusions displayed by transfected cells. However, a marked decrease in the number of cells bearing small isolated inclusions occurs, while the number of cells containing medium and, most significantly, big aggregates increases (Figure 10). Therefore, WT Sph1A has a clear propensity to form small (multiple and isolated) aggregates within HEK293 cells, both 24 and 48 hours after transfection. Moreover, as the post-transfection period is extended, there is an increase in the number of cells containing medium and big aggregates, which certainly reflects a process of assembly of small aggregates to form the bigger ones, as is described in literature[40, 41].

On the other hand, 24 hours after transfection, cells overexpressing MT Sph1A develop small, medium and big inclusions nearly in the same proportion (Figure 10). Furthermore, the proportion of cells containing big aggregates is extremely high upon transfection with the mutant form of Sph1A in comparison to cells transfected with the wild type protein, for both time points studied (Figure 10). Additionally, when the post-transfection period is extended from 24 hours to 48 hours, a marked decrease in small isolated and medium aggregates occurs, while a huge increase in big aggregates can be observed (Figure 10). Thence, MT Sph1A displays a clear propensity to form big aggregates. Moreover, comparing the two time points for MT Sph1A, a decrease in the proportion of cells containing small isolated and medium aggregates can be observed, concomitantly with an increase in the proportion of cells containing big inclusions. Then again, these differences between time points may be due to the assembly of small aggregates to form bigger ones as the post-transfection period is increased.

All together, these data suggest that, upon mutation of the Sph1A PPP1 BM, Sph1A suffers an alteration of its aggregation pattern, in which the formation of big aggregates is favored (Figure 10). Furthermore, the disruption of Sph1A/PPP1 interaction also seems to accelerate the process of inclusion bodies formation as the more advanced inclusion types (big aggregates) are massively produced earlier. Thence, blocking PPP1 targeting to inclusion bodies seems to enhance Sph1A aggregative properties and accelerate the conversion of small aggregates in big aggregates, as well as aggregates assembling and growing mechanisms.

4.3 WT SPH1A AND MT SPH1A DO NOT PRODUCE *BONA FIDE* AGGRESOMES UPON OVEREXPRESSION IN HEK293 CELLS WITHOUT ANY TREATMENT

According to data described in section 4.2, WT Sph1A and MT Sph1A are able to form cytoplasmic aggregates but display different aggregation patterns. Interestingly, some of the medium and big aggregates formed within HEK293 cells overexpressing Sph1A seem to have certain similarities with aggresomes. Indeed, these bigger Sph1A inclusions (1) seem to have perinuclear localization, (2) increase in proportion as the post-transfection periods are extended from 24 hours to 48 hours (suggesting assembling of small cytoplasmic aggregates to form bigger inclusions, perhaps true aggresomes) (Figure 10) and (3) some cells display small multiple aggregates aligned as if they are associated with microtubules (Figure 11). All together, these cues have raised the hypothesis of some of these Sph1A inclusions be *bona fide* aggresomes.

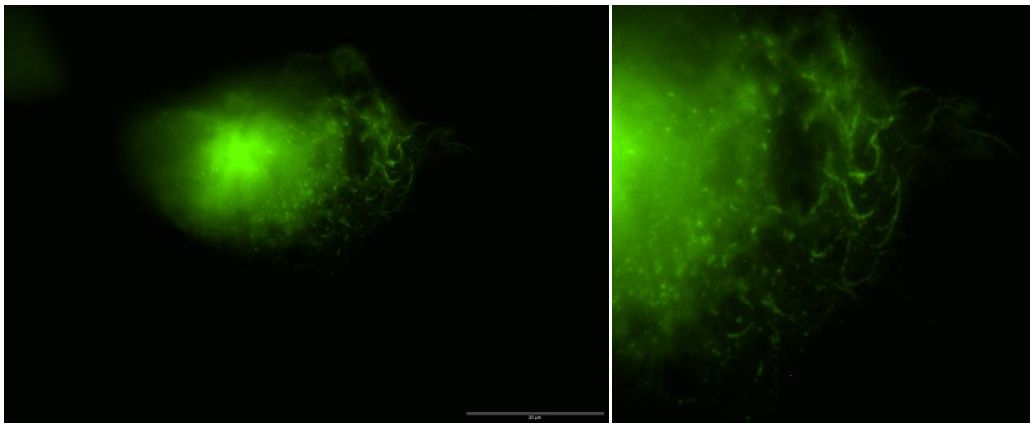

Figure 11 –HEK293 cell transfected with WT Sph1A displaying aligned small multiple aggregates, suggesting a putative association with the microtubules network. Scale bar: 20 µm. Right image emphasizes the presence of dispersed GFP-positive aggregates which are also present together with the aligned inclusions. Right image is not at scale.

Currently, there are no studies addressing if the inclusions formed by Sph1A are *bona fide* aggresomes. However, the ability of Sph1 to form aggresomes within transfected cells is well established in literature, although requires cell treatment with proteasome inhibitors. As no proteasome inhibitors were used in the present work and giving the cues described above, it is interesting to address if any Sph1A inclusions produced upon Sph1A overexpression in HEK293 cells are true aggresomes.

Two main criteria allow inclusions classification as aggresomes: (1) the dependence on microtubules for their formation; and (2) localization at the MTOC. Both these criteria were assessed.

In order to assess if microtubules are required for the formation of Sph1A bigger aggregates, HEK293 cells transfected with Sph1A-GFP and Sph1A-RVTA-GFP were treated with Nocodazole, as described (see Materials and Methods), for microtubule polymerization blocking. Then, the morphometric analysis of Sph1A aggregates was performed again, for evaluation of WT Sph1A and MT Sph1A aggregation patterns under conditions of microtubule polymerization blocking. This morphometric analysis was performed through immunofluorescence experiments (Figure 12). If Sph1A big aggregates are, indeed, aggresomes, upon Nocodazole treatment it is expected a decrease in the proportion of cells displaying big aggregates and an increase in the proportion of cells containing small aggregates.

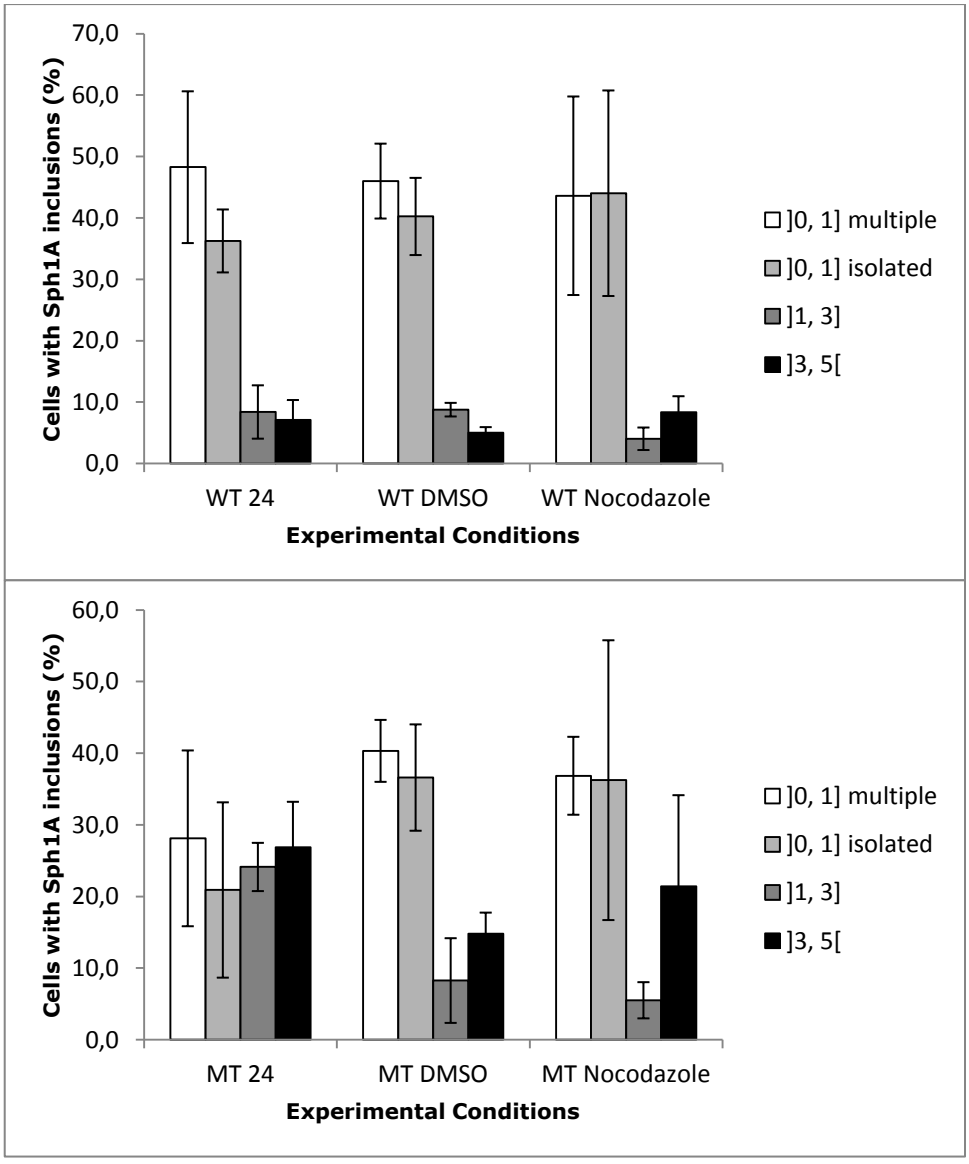

Figure 12 – HEK293 cells overexpressing WT Sph1A and MT Sph1A do not display significant sensitiveness to Nocodazole treatment. The graphs show the average number of transfected HEK293 cells displaying small multiple (]0,1] multiple), small isolated (]0,1] isolated), medium (]1,3]) and big aggregates (]3,5[), when transfected with wild type Sph1A or mutant Sph1A for 24 hours, in the absence of any treatment (WT 24 and MT 24)) and upon treatment with

Nocodazole (WT Nocodazole and MT Nocodazole). Transfected HEK293 cells treated with DMSO (WT DMSO and MT DMSO) were used as negative control. All data represent the mean ± SD of at least three independent experiments (n = 150 cells per condition). When Pearson's chi-squared test is applied to compare the aggregation pattern of corresponding replicas (WT 24 vs. WT Nocodazole; WT 24 vs. WT DMSO; MT 24 vs. MT Nocodazole and MT 24 vs. MT DMSO), there are statistically significant differences in the aggregation pattern of MT Sph1A after treatment with Nocodazole and also with DMSO, in comparison with the non-treated control. Therefore, in cells transfected with MT Sph1A the aggregation pattern is significantly altered by Nocodazole and by DMSO, at significance level of 5% (p-values <0,05). Contrarily, Nocodazole does not produce significant differences in the aggregation pattern of WT Sph1A at a significance level of 5% (p-values >0,05).

As described in section 4.2 (Figure 10), WT Sph1A produces mainly small cytoplasmic aggregates (multiple and isolated) under untreated conditions. Upon treatment with Nocodazole, this tendency does not change and the different types of aggregates continue to be formed nearly in same proportion in WT Sph1A transfected cells (Figure 12).

On the other hand, HEK293 cells overexpressing MT Sph1A display nearly the same proportion of the 4 classes of aggregates under untreated conditions (Figure 12). Interestingly, when incubated with Nocodazole (5 μM), a slight change in MT Sph1A aggregation pattern seems to occur, with a slight increase in the proportion of small aggregates and a decrease in the number of cells bearing medium and big aggregates (Figure 12). The interpretation of these differences promoted by Nocodazole becomes even more difficult as all effects seen under Nocodazole treatment also occur in the negative control, in which MT Sph1A transfected HEK293 cells were treated with DMSO alone (Nocodazole's solvent) (Figure 12).

Taken together, these results do not allow the clear classification of Sph1A inclusion bodies documented in the present work as aggresomes. Actually, in cells overexpressing WT Sph1A, treatment with Nocodazole does not produce any visible change in Sph1A aggregation. On the other hand, cells overexpressing MT Sph1A form more small aggregates and less medium and big aggregates when treated with Nocodazole, matching the expected results if some of the MT Sph1A aggregates were real aggresomes. However, as DMSO alone (negative control) is able to produce the same effect, these results are inconclusive for MT Sph1A. The manipulation produces the desired effect but could not be attributed specifically to Nocodazole.

Although Nocodazole treatment produced inconclusive results, the classification of inclusion bodies as aggresomes also requires their co-localization with MTOC markers, as stated above. Thence, HEK293 cells transfected with Sph1A-GFP and Sph1A-RVTA-GFP were also stained with anti-γ-tubulin monoclonal antibody, as γ-tubulin is a well known MTOC marker.

In non-transfected HEK293 cells, γ-tubulin is clearly enriched near the nucleus, co-localizing with two perinuclear, large dots, corresponding to the centrosome (Figure 13). Furthermore, in some non-transfected cells, these two large, perinuclear dots appear in opposite poles, suggesting mitosis occurrence, in which cases also the mitotic fuse can be distinguished. Nevertheless, the

images taken also display some background, which turns more difficult the clear identification of the centrosome in all cells. This background problem could not be solved even after a prolonged optimization period, in which many dilutions were tested for both the primary and secondary antibodies.

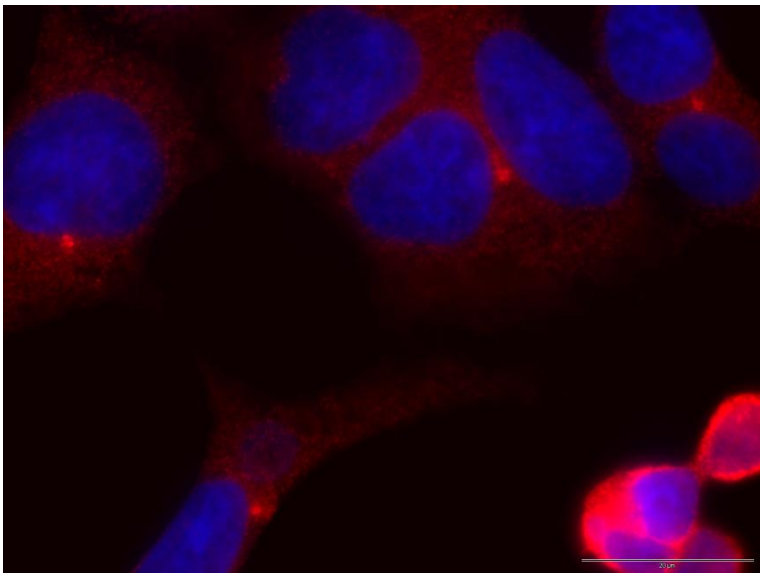

Figure 13 - Non-transfected HEK293 cells stained with anti-γ-tubulin antibody display centrosomes at the perinuclear space. Scale bar: 20 µm.

Contrarily to non-transfected cells, which display a visible centrosome at the perinuclear space, in cells transfected with WT Sph1A and MT Sph1A and containing aggregates the two large, perinuclear dots could not be clearly distinguished, for the majority of cells present in each coverslip (Figure 14). Indeed, centrosome identification is sporadically possible but not in a consistent way to be significant or conclusive (Figure 14). Furthermore, in some of these cells in which the centrosome is identified, some degree of co-localization with big Sph1A aggregates is found, but is also a sporadic event (Figure 14). It is possible that the punctuate background signal produced some degree of confounding, does not allowing an unequivocal centrosome observation for the majority of transfected cells. Nevertheless, an astonishing event is the clear identification of centrosomes in a great number of non-transfected cells and in cells not bearing aggregates, localized around the transfected ones in the same coverslip.

In light of that, for classification purposes as aggresomes, the γ-tubulin experiment is also inconclusive. Actually, some transfected cells (for both Sph1A constructs) display co-localization between big aggregates and the centrosome but in an inconsistent and sporadic way, insufficient to be considered clearly significant. Nevertheless, the clear identification of centrosomes in non-transfected cells contrarily to the transfected ones is a curious event. It is unclear whether this is

caused by confounding due to high background, transfection artifacts or some true cellular event related with protein aggregation or even apoptosis. The clarification of this topic requires further evaluation.

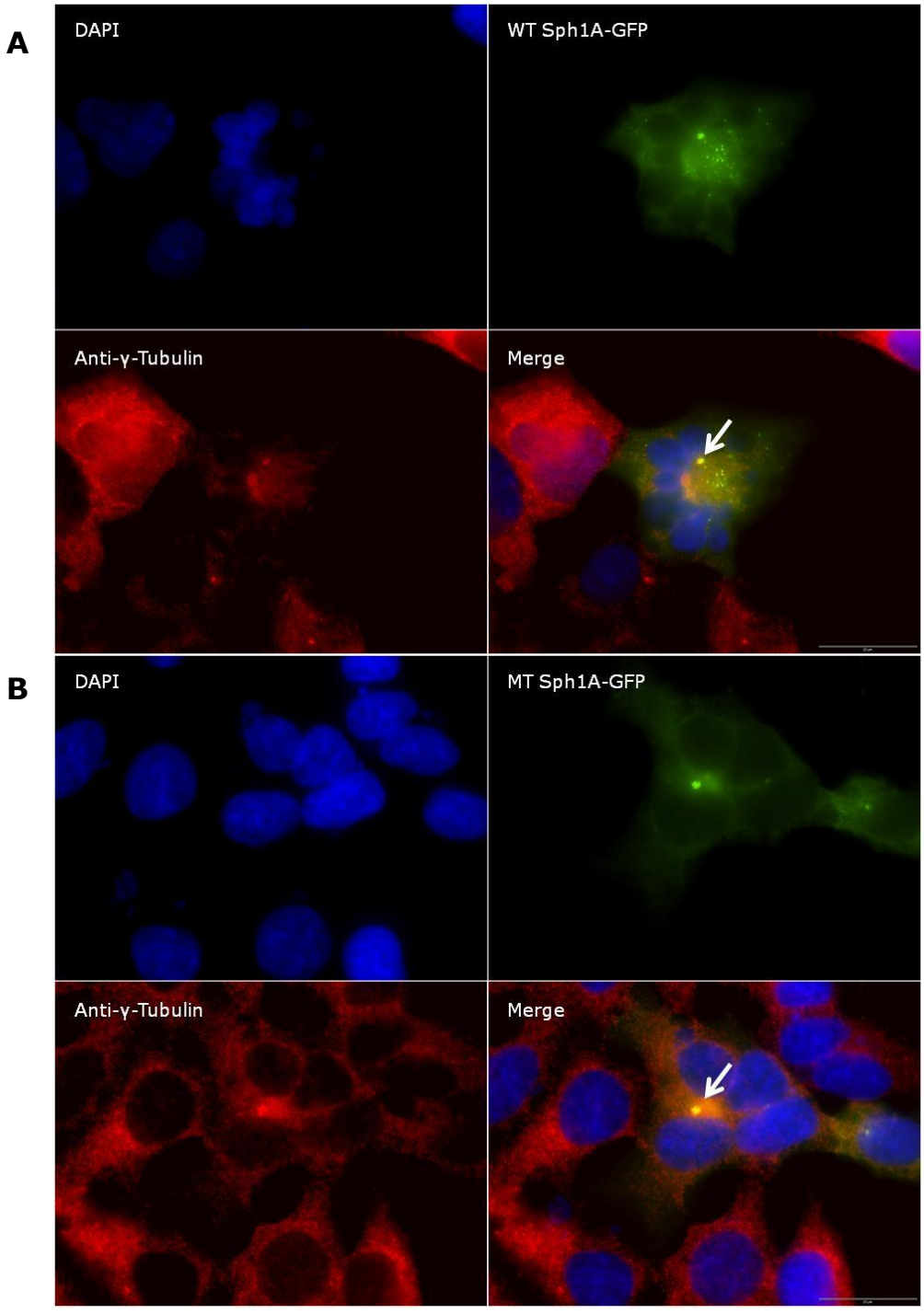

Figure 14 – Evaluation of aggresomes formation by transfected HEK293 cells through co-localization between inclusion bodies and the MTOC. A: Fluorescence microscopy visualization of Sph1A inclusion bodies (WT Sph1A-GFP) and the MTOC (Anti-γ-tubulin) in cells overexpressing WT Sph1A. One transfected cell clearly displays a perinuclear bigger aggregate co-localizing with the centrosome. Two non-transfected cells also show perinuclear dots corresponding to the MTOC. B: Fluorescence microscopy visualization of Sph1A inclusion bodies (MT Sph1A-GFP) and

the MTOC (Anti-γ-tubulin) in cells transfected with MT Sph1A. At the center of the figure, one transfected cell shows a big perinuclear aggregate co-localizing with the MTOC. Two other cells contain visible centrosomes but are non-transfected. Scale bars: 20 µm. White arrows indicate centrosome localization.

Taken together, Nocodazole treatment and γ-tubulin staining fail to demonstrate the true nature of Sph1A inclusion bodies as aggresomes. Thence, the aggregation patterns described in the previous section for WT Sph1A and MT Sph1A seem to be mainly due to passive events rather than active events mediated by the cytoskeleton. However, cytoskeleton intervention could not be completely excluded as Nocodazole produced a slight effect and aggregates co-localized with MTOC in some residual cells. Furthermore, no proteasome inhibitors were used in the present work.

4.4 COMPARISON BETWEEN TRITON-SOLUBLE FRACTIONS AND TRITON-INSOLUBLE FRACTIONS OF HEK293 CELLS TRANSFECTED WITH WT SPH1A AND MT SPH1A – A PRELIMINARY STUDY

The final aim of the present work was to address if Sph1A/PPP1 interaction was able to modulate Sph1A/α-syn interaction, affecting α-syn recruitment to inclusion bodies. This question came from the idea that Sph1A and/or α-syn phosphorylation state could modulate Sph1A/α-syn interaction. In order to answer this question, HEK293 cells transfected with WT Sph1A and MT Sph1A were subjected to fractionation using triton-containing lysis buffer and PPP1CA and α-syn recruitment to the triton-insoluble fractions were compared in both cases.

Although this is a preliminary work, needing further replication and quantification, the results were interesting. Indeed, when transfected cells (with WT Sph1A and MT Sph1A) are fractionated using 1% TRITON X-100, Sph1A (75 kDa) appears both in the triton-soluble and triton-insoluble fractions for both constructs used (Figure 15). Furthermore, more bands can be seen in soluble fractions besides the 75 kDa band, although it is unclear if they are unspecific or by-products of GFP-tagged Sph1A. As expected, GFP-tagged Sph1A is absent in negative controls (non-transfected cells) (Figure 15). Thence, upon overexpression in HEK293 cells, both WT Sph1A and MT Sph1A exist in soluble form and in a triton-insoluble form, certainly corresponding to aggregated Sph1A, confirming the previous results of this work.

In terms of PPP1CA, this phosphatase can be detected both in the triton-soluble and triton-insoluble fractions after fractionation of non-transfected HEK293 cells, but it is more abundant in the soluble fraction in control conditions (Figure 15). Therefore, PPP1CA naturally occurs in triton-insoluble forms, probably associated with cytoskeletal components within non-transfected HEK293 cells. Curiously, when cells overexpress WT Sph1A and MT Sph1A, this tendency is not

changed but the PPP1CA bands in the insoluble fractions become less intense for both constructs used (Figure 15). Moreover, it is important to emphasize that PPP1CA recruitment to insoluble fractions is even more reduced when cells are transfected with MT Sph1A, in which the Sph1A PPP1 BM is disrupted (ratio 0.4 %, Figure 15). A less PPP1CA recruitment to the insoluble fraction in cells overexpressing MT Sph1A suggests a decreased Sph1A/PPP1 interaction due to RVTF motif disruption. Nevertheless, explaining the global reduction in PPP1CA recruitment to the insoluble fractions observed for both constructs is not so easy. Indeed, loading problems and other experimental problems could not be excluded as no loading control was used and this experiment needs further replication. Other possible explanations include a true reduction in endogenous PPP1CA levels in transfected cells due to decreased PPP1CA expression and/or enhanced degradation. Other tests should be performed in order to clarify this result.

Finally, concerning α-syn, a 16 kDa band with low intensity can be clearly detected in the triton-soluble fraction of non-transfected cells (Figure 15). Moreover, a quite diffuse and fade band can also be identified in the insoluble fraction, under control conditions (Figure 15). Unluckily, the band corresponding to α-syn is maintained in the soluble fraction, even after WT Sph1A and MT Sph1A overexpression, and no clear recruitment can be observed to the triton-insoluble fractions (Figure 15). This absence of α-syn recruitment to Sph1A inclusions is not positive but is not surprising. Indeed, in the present work, Sph1A was overexpressed alone, without α-syn co-transfection, contrarily to literature that consistently documents Sph1/α-syn co-transfections as a means to produce α-syn/Sph1-positive inclusions.

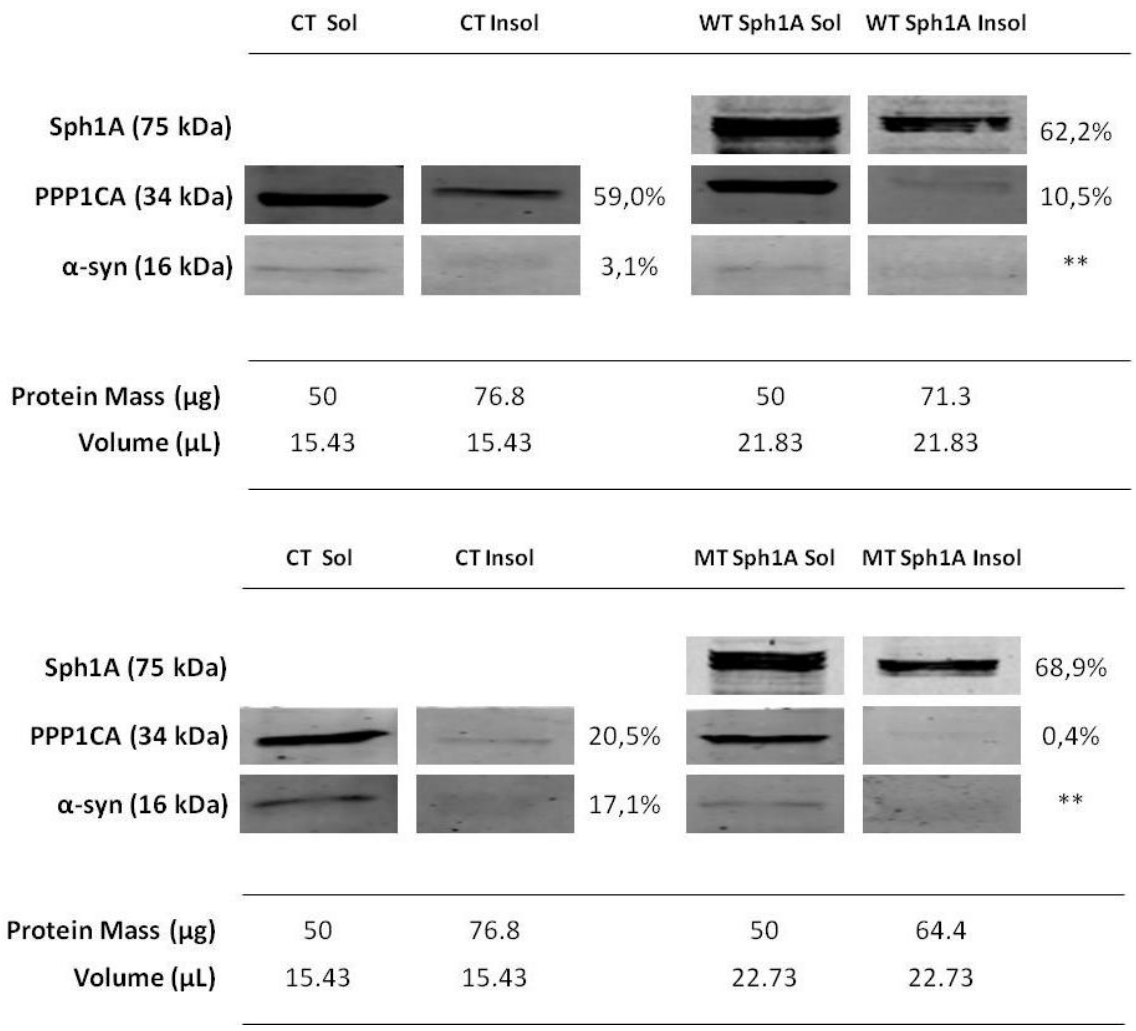

Figure 15 - PPP1CA and α-syn recruitment to Sph1A inclusion bodies in HEK293 cells. Upper panel: Western blot analysis of triton-soluble (Sol) and triton-insoluble (Insol) fractions produced through fractionation of non-transfected HEK293 cells (CT) and HEK293 cells overexpressing WT Sph1A (WT Sph1A). Lower panel: Western blot analysis of triton-soluble (Sol) and triton-insoluble (Insol) fractions produced through fractionation of non-transfected HEK293 cells (CT) and HEK293 cells overexpressing MT Sph1A (MT Sph1A). For both panels, the proteins detected and their molecular weight are depicted at the left. It is also indicated the protein mass and sample volume loaded in each lane. Soluble and insoluble fractions of the same experimental condition were loaded with equal volume to assure the maintenance of the relative proportion of each protein in the two fractions. The percentage values are the ratios between the volume of each insoluble band and the volume of its corresponding soluble band, being a measure of protein recruitment to the insoluble fraction. Ratios depicted as ** could not be determined.

Taken together, these preliminary results suggest that, upon transfection, Sph1A exists in a soluble and in an insoluble, aggregated form, and this tendency is not altered by RVTF mutation. Furthermore, in cells overexpressing Sph1A, endogenous PPP1CA suffers redistribution, being its recruitment to the insoluble fractions decreased upon mutation of Sph1A PPP1 BM in comparison to cells transfected with WT Sph1A. Finally, α-syn is mainly retained in the soluble fraction both in non-transfected cells and in cells transfected with Sph1A (WT or MT), does not suggesting its recruitment to Sph1A inclusion bodies in cells overexpressing Sph1A alone.

5. Discussion

Sph1A is a Sph1 isoform and an aggregation-prone protein able to interact with α-syn and Sph1, being relevant for LBs formation[74]. Nevertheless, the regulatory mechanisms that modulate Sph1A aggregation are poorly understood. Previous studies have already documented the regulatory role of GSK3β and CKII for Sph1 aggregation[88, 90, 91]. As Sph1A displays similarities with Sph1, its major isoform, it seems legit to assume that reversible phosphorylation may also modulate Sph1A aggregation as well.

Thence, the present work confirms the novel interaction between Sph1A and PPP1 and addresses the influence of PPP1 activity on Sph1A aggregation and inclusion bodies formation. Nevertheless, it is also discussed the putative effect of Sph1A in modifying PPP1 activity, through PPP1 targeting to specific subcellular compartments.

5.1 ESTABLISHING SYNPHILIN-1A AS A PPP1-INTERACTING PROTEIN

Protein reversible phosphorylation is the most studied post-translational modification able to modulate protein activity in eukaryotic cells. Nevertheless, there are no studies documenting the effect of phosphorylation on Sph1A aggregation. In light of that, after a yeast two hybrid screen from a human brain library, Sph1A was identified as a putative PPP1 interactor, establishing the first link between a Sph1 isoform and a phosphatase[105]. This interaction was further confirmed by biochemical methods[107]. Interestingly, it was shown in an independent study that Sph1 phosphorylation state is sensitive to okadaic acid treatment, a well established phosphatases inhibitor, but no reference or association to PPP1 were made[90].

Aiming the development of the work initiated by Dr.ª Sara Esteves, in the present work, Sph1A/PPP1 interaction is confirmed in *ex vivo* conditions and the potential role of Sph1A as a PIP is discussed[96]. Indeed, the present work shows that, upon Sph1A overexpression in HEK293 cells, PPP1CA and PPP1CC interact with and are targeted to Sph1A inclusion bodies. Furthermore, this targeting could be blocked by mutation of Sph1A PPP1 BM, RVTF, thus being dependent on the conserved Sph1A PPP1 BM. This evidence suggests Sph1A putative role as a PIP.

In order to discuss Sph1A classification as a PIP, PIP features must be clearly defined. To be considered a PIP, proteins must (1) interact with PPP1 both *in vitro* and *in vivo*, (2) containing a conserved and functional PPP1 BM responsible for the interaction with PPP1[102]. Moreover, PIP/PPP1 interaction must be significant for cell function, being the PIP a PPP1 substrate itself, a PPP1 activity modulator or a protein able to alter PPP1 subcellular localization[96]. Taking into account the previous PIP definition, Sph1A may be considered a PIP as (1) is able to interact with PPP1 (PPP1CA and PPP1CC) both *in vitro*[107] and *ex vivo*, (2) this interaction is dependent on the

Sph1A PPP1 BM (RVTF) and (3) alters PPP1 subcellular localization, targeting the phosphatase to Sph1A inclusion bodies. Although it is clear that Sph1A targets PPP1 to inclusion bodies, the present study does not address other relevant questions, particularly if Sph1A is also a PPP1 substrate or if it is able to modulate PPP1 activity, besides targeting the phosphatase.

Assuming that Sph1A is actually a functional PIP, new hypotheses arise as Sph1A/PPP1 interaction may uncover new regulatory aspects behind Sph1A aggregation and LBs formation, relevant for PD pathogenesis. Nevertheless, Sph1A should also interact and target PPP1 in non-pathologic conditions. Therefore, this interaction could also determine some aspects of Sph1A physiology within normal neurons.

Despite the solid evidence that Sph1A could be classified as a PIP, the present study has some pitfalls. Indeed, Sph1A/PPP1 interaction was confirmed through a Sph1A overexpression approach in HEK293 cells. HEK293 cells are not a neuronal lineage and protein overexpression produces non-physiologic conditions. However, this approach was useful in the present work because the objectives were mainly at the molecular level, particularly the study of protein interactions, which is often sufficiently general to be addressed in different cell types. Moreover, according to literature, all the initial studies concerning Sph1A were performed in HEK293 cells due to their advantages in terms of manipulation, resilience and transfection efficiency. On the other hand, protein overexpression is non-physiologic but is a useful tool to produce some PD-related features, as PD is characterized by the accumulation of abnormal proteins. Finally, the overexpression approach was not overused as PPP1 levels were not manipulated in any experiment of this work. PPP1 endogenous levels were always considered.

5.2 PPP1 TARGETING TO SPH1A INCLUSION BODIES – IMPLICATIONS FOR INCLUSION BODIES FORMATION

Assuming that Sph1A is a novel PIP able to, at least, target PPP1 to Sph1A inclusion bodies, it seems legit to expect some molecular/cellular effect of this targeting to inclusion bodies formation.

In order to address this hypothesis, the present study has investigated Sph1A aggregation pattern, in conditions that allow Sph1A/PPP1 interaction and upon blockage of PPP1 recruitment to Sph1A inclusion bodies, through Sph1A PPP1 BM mutagenesis. According to the results obtained, PPP1 recruitment influences Sph1A inclusion bodies formation, at least in HEK293 cells. Indeed, when PPP1 is not targeted to Sph1A aggregates, the formation of bigger aggregates is favored. On the other hand, when PPP1 is allowed to interact with Sph1A, small aggregates are the most

abundant inclusions. Furthermore, the global process of Sph1A aggregation seems to be accelerated by PPP1 targeting blockage as bigger aggregates are massively formed earlier. Taken together, these evidences suggest that PPP1, through Sph1A targeting, is able to modulate inclusion bodies formation within HEK293 cells overexpressing Sph1A.

PPP1 ability to modulate and, most specifically, to decrease Sph1A aggregation within cells is something expected and possibly could be explained taking into account Sph1A ability to target PPP1 to inclusion bodies. This explanation is attractive because reinforces the classification of Sph1A as a PIP by demonstrating a cellular effect of Sph1A/PPP1 interaction. Actually, during Sph1A aggregation in transfected HEK293 cells, PPP1 (possibly PPP1CA and PPP1CC) may be targeted to the forming inclusion bodies due to Sph1A RVTF motif. This change in PPP1 subcellular localization may concentrate PPP1 within Sph1A aggregates, favoring protein dephosphorylation within Sph1A inclusion bodies. By decreasing protein phosphorylation state in aggregates, PPP1 could decrease protein-protein interactions leading to reduced Sph1A aggregation. This could explain why WT Sph1A has a slower aggregation pattern in which the bigger aggregates only significantly appear for higher post-transfection periods. On the other hand, if PPP1 could not be recruited, for example due to Sph1A PPP1 BM disruption, this protective effect may be lost because there is an imbalance between phosphorylation and dephosphorylation within inclusion bodies. In that case, Sph1A aggregate-related proteins become hyperphosphorylated, enhancing protein-protein interactions and Sph1A aggregation. Thence, bigger aggregates are formed earlier, explaining the phenotype seen in cells overexpressing MT Sph1A.

Two main arguments corroborate the process suggested above. First, there are several PIPs known to target PPP1 to specific subcellular compartments and for specific targets, enhancing PPP1 activity in these compartments and in the close proximity of the desired targets[96]. Second, a previous study concerning CKII and Sph1 has already demonstrated that higher Sph1 phosphorylation by CKII is able to enhance inclusion bodies formation[90]. In order to explain that observation, authors suggested that Sph1/α-syn interaction could be influenced by the phosphorylation state of these proteins. Most particularly, higher phosphorylation levels were associated to an enhanced Sph1/α-syn interaction[90]. Thence, it is not surprising if the inverse process happens for Sph1A, a Sph1 isoform, and PPP1, a phosphatase. Actually, it is likely that PPP1 decreases Sph1/α-syn interaction by decreasing the phosphorylation state of the proteins referred.

Despite the attractiveness of this hypothesis, actually the fractionation experiment documented in the present work failed to demonstrate bigger α-syn recruitment to inclusion bodies

in cells transfected with a Sph1A form incapable of interact with PPP1. Nevertheless, this was just a preliminary test. Indeed, the absence of α-syn recruitment to inclusion bodies could be caused by an experimental limitation, probably the overexpression of Sph1A alone without α-syn co-transfection.

5.3 NOCODAZOLE EXPERIMENT – INTERESTING TOPICS

After the identification of alterations in Sph1A aggregation pattern in conditions of PPP1 BM disruption, Sph1A ability to produce aggresomes was evaluated because some of the big aggregates formed seem to be perinuclear. The ability of Sph1 to form aggresomes upon proteasome inhibition is well established[40]. Nevertheless, there is no information concerning Sph1A and, in the present study, no proteasome inhibitors were used. Thence, if Sph1A was really able to form true aggresomes without proteasome inhibition, this could be a quite interesting topic, demonstrating a crucial difference between Sph1A and Sph1 and strengthening the definition of Sph1A as an aggregation-prone isoform.

Unluckily, both Nocodazole treatment and γ-tubulin staining fail to clearly demonstrate the true nature of Sph1A aggregates as aggresomes. However, some ideas and observations resulting from these experiments should be emphasized.

First, it is worthy to refer that Nocodazole and, most prominently, DMSO did produce a slight effect and, interestingly, this only occurred in cells transfected with MT Sph1A, being unequivocally absent in cells transfected with WT Sph1A. Therefore, cells overexpressing MT Sph1A display some sensitiveness to manipulation, although it could not be specifically attributed to cytoskeleton dynamics. It is not easy to explain the alterations observed. Indeed, DMSO is a solvent and probably could interfere with Sph1A passive aggregation but it was massively diluted in the cells medium. Thence, a more general effect might be happening, perhaps on cytoskeleton dynamics or even in some cellular mechanism. Indeed, literature suggested the ability of DMSO to promote hyperphosphorylation of cytoskeletal components, although this event requires DMSO concentrations higher than the one applied in the present study[109]. Whatever the true explanation for that, it is certain that Nocodazole and DMSO treatment induce sufficient stress in MT Sph1A overexpressing cells to disrupt the formation of medium aggregates, most prominently under DMSO treatment.

Second, in terms of γ-tubulin staining, it is rather odd that non-transfected cells reveal readily identifiable centrosomes while transfected cells containing aggregates fail to do so, in the same coverslip. Although it could be an artificial result due to transfection or even excessive

background, one could not exclude that centrosomes actually might not be accessible in cells containing aggregates. Indeed, protein aggregation may hinder antibody interaction with γ-tubulin by surrounding the epitope. Even more likely is the possibility that transfection and protein aggregation cause sufficient stress to HEK293 cells to induce apoptosis in a large proportion of them. In this case, it is not strange that centrosomes could not be detected.

Despite all problems and inconclusive results from this section of the study, Figure 11 should be emphasized because it suggests the association between Sph1A and the microtubules network. This observation was interpreted as Sph1A aggregates transport to the perinuclear space to form aggresomes but other interesting explanations may be suggested. Indeed, Sph1A could be a protein able to interact with microtubules under physiological conditions, which is consistent with previous observations[110] as well as with Sph1 role as scaffold protein and a putative modulator of synaptic vesicle trafficking[75].

5.4 WHAT HAVE WE LEARNED ABOUT SYNPHILIN-1? WHAT COULD WE EXPECT?

This work studies the functional consequences of Sph1A/PPP1 interaction, particularly in inclusion bodies formation, suggesting that PPP1 targeting to aggregates acts like a protective mechanism, decreasing protein aggregation. Nevertheless, besides the implications for inclusion bodies formation, Sph1A/PPP1 interaction should also occur under physiological conditions, probably having astonishing consequences in presynaptic terminals dynamics.

Indeed, Sph1 is a well known α-syn-interacting protein thought to modulate α-syn function in the presynaptic terminals with possible implications in neurotransmitters release[75]. If PPP1 modulates Sph1/α-syn interaction, has it seems to occur for inclusion bodies formation, the same event should occur under physiological conditions, possibly regulating dopamine release[75]. This hypothesis becomes even more attractive if one considers that α-syn does not have a PPP1 BM but Sph1, one of its major interacting proteins, has[107].

Another interesting idea that should be emphasized is spinophilin function in PD. Spinophilin is a PIP known to play a crucial function in PPP1c targeting for dendritic spines within medium spiny neurons[111]. Furthermore, it is suggested that spinophilin associates with the cytoskeleton, particularly F-actin, regulating PPP1 specificity toward ion channels and other substrates[111]. Upon dopamine depletion, there is an enhancement of spinophilin/PPP1 interaction, which leads to a decreased PPP1 activity in dendritic spines, producing hyperphosphorylation of several substrate proteins, impairing plasticity in PD patients[112].

Taking into account the data concerning spinophilin, there are astonishing similarities between spinophilin and Sph1. Indeed, Sph1 is thought to be a scaffold protein without catalytic activity just as spinophilin. Moreover, the present work clearly demonstrates Sph1 ability to participate in PPP1 targeting within cells and some studies also suggest Sph1 ability to interact with lipid rafts, synaptic vesicles and also with cytoskeletal components. Therefore, Sph1 could be faced as a putative "pre-synaptic spinophilin", targeting PPP1 to the presynaptic terminals, with implications in α-syn functions, neurotransmitters release and perhaps other trafficking and signaling mechanisms. Hence, the observations described in this study have provided cues that probably will help to uncover the true Sph1 functions in dopaminergic neurons.

Finally, the findings present in this study may have important implications not only to better understand inclusion bodies formation but also other events of PD pathogenesis. Indeed, PD is a neurodegenerative disorder and a major hallmark of such pathologies is protein hyperphosphorylation, which could be due to decreased phosphatases activity. When it comes to PD, it is already described a decreased activity of PPP2 caused by phosphatase sequestering into LBs, leading to hyperphosphorylation of several PPP2 substrates, with implication in the pathological process[113]. As Sph1A proved its ability to recruit PPP1 to inclusion bodies, it is attractive to think that a massive recruitment of PPP1 to Sph1A aggregates could lead to an alteration in PPP1 intracellular localization, becoming enriched within aggregates and decreasing its availability in other subcellular compartments. In such a case, PPP1 activity would become higher within aggregates and decreased in other compartments, with putative implications in the phosphorylation state of numerous PPP1 substrates in a way similar to PPP2.

In summary, aiming the study of Sph1A/PPP1 interaction and its implications for inclusion bodies formation, the present study has uncovered novel cues, probably relevant to understand Sph1A/PPP1 interaction in a physiological context and in PD pathogenesis.

6. Conclusions and Future Work

The Sph1A/PPP1 complex, recently identified in human brain, is a novel complex with unknown function and was the starting point for the present work. Indeed, after its identification through a high throughput technique (yeast two hybrid), it was important to validate its existence as well as to explore its functional implications, particularly for inclusion bodies formation, as Sph1A has long been referred as a key player in PD.

Interestingly, Sph1A/PPP1 interaction proved to actually occur in *ex vivo* conditions as both PPP1CA and PPP1CC are recruited to Sph1A inclusion bodies within cells overexpressing Sph1A. Moreover, this interaction is specific and dependent on the Sph1A PPP1 BM RVTF as a point mutation in this motif is sufficient to hamper the interaction and block PPP1 targeting to Sph1A-containing aggregates.

After validation of the existence of Sph1A/PPP1 complex in HEK293 cells, the present study also uncovered an important role of this complex for inclusion bodies formation. Actually, disruption of the PPP1 BM present in Sph1A produced a more aggregative phenotype in HEK293 cells, favoring the formation of bigger aggregates and accelerating protein aggregation. Such event was attributed to decreased PPP1 targeting to inclusion bodies upon PPP1 BM disruption, leading to hyperphosphorylation of inclusion body-related proteins, enhancing protein-protein interactions. Thus, Sph1A/PPP1 complex seems to be implicated in the regulation of the interactions between some proteins present in inclusion bodies, probably Sph1 and α-syn.

As Sph1A is known to be an aggregation-prone Sph1 isoform, its ability to form aggresomes without proteasome inhibitors administration to cells was also studied. Unluckily, Sph1A does not seem able to form *bona fide* aggresomes, at least without proteasome inhibition, as Sph1A aggregation does not show significant sensitiveness to drugs that block microtubule dynamics. Also, a marked co-localization between Sph1A aggregates and MTOC could not be clearly demonstrated in our tests.

Therefore, as conclusion, Sph1A is indeed able to interact with PPP1CA and PPP1CC within cells and Sph1A/PPP1 complex plays a role in PPP1 targeting, at least to inclusion bodies, enhancing protein aggregation when disrupted. This suggests that Sph1A/PPP1 complex modulates the interaction between some PD-related proteins, probably Sph1 and α-syn, having implications not only for Lewy bodies formation but also for other events of PD pathogenesis and, perhaps, even in physiological conditions. Indeed, by regulating Sph1/α-syn association, the Sph1A/PPP1 complex might modulate neurotransmitters release and other presynaptic processes in the presynaptic terminals of dopaminergic neurons.

This work has uncovered so many cues concerning the regulation of Sph1 activities both under physiological and pathological conditions that a lot of questions should be addressed in the future. Therefore, in order to continue this work, some important future tasks are the following:

- To assess the effect of WT Sph1A and MT Sph1A in cells viability, possibly measuring LDH release by transfected cells;
- To perform Nocodazole experiment in conditions of proteasome inhibition and perhaps using other microtubule drugs, such as colchicine;
- To repeat the experiment that addressed the putative co-localization between Sph1A aggregates and MTOC but using other MTOC markers, such as vimentin or pericentrin;
- To evaluate Sph1A effect on PPP1 activity to address if Sph1A is a PPP1 inhibitor, using assays for phosphatase activity quantification;
- To address if PPP1 is able to directly dephosphorylate Sph1A;
- To assess if Sph1/α-syn interaction is really enhanced upon disruption of the Sph1A/PPP1 complex, using immunofluorescence or fractionation experiments in cells co-transfected with Sph1A and α-syn;
- To test Sph1A co-localization with microtubules markers;
- To establish and apply other suitable cell lineages in all these studies besides HEK293 cells, such as SH-SY5 or H4.

7. References

1. Parkinson, J., *An Essay on the Shaking Palsy*. 1817, London: Whittingham and Rowland for Sherwood, Neely and Jones. 66.
2. Inamdar, N.N., et al., *Parkinson's disease: genetics and beyond.* Curr Neuropharmacol, 2007. **5**(2): p. 99-113.
3. Foundation, T.M.Y.I.P.s.D. *About Parkinsons Disease*. 2013 [cited 2013 01-02-2013].
4. de Lau, L.M. and M.M. Breteler, *Epidemiology of Parkinson's disease.* Lancet Neurol, 2006. **5**(6): p. 525-35.
5. Valente, A.X., et al., *Rare coding SNP in DZIP1 gene associated with late-onset sporadic Parkinson's disease.* Sci Rep, 2012. **2**: p. 256.
6. Dauer, W. and S. Przedborski, *Parkinson's disease: mechanisms and models.* Neuron, 2003. **39**(6): p. 889-909.
7. Dickson, D.W., et al., *Neuropathological assessment of Parkinson's disease: refining the diagnostic criteria.* Lancet Neurol, 2009. **8**(12): p. 1150-7.
8. Braak, H., et al., *Staging of the intracerebral inclusion body pathology associated with idiopathic Parkinson's disease (preclinical and clinical stages).* J Neurol, 2002. **249 Suppl 3**: p. III/1-5.
9. Braak, H., et al., *Idiopathic Parkinson's disease: possible routes by which vulnerable neuronal types may be subject to neuroinvasion by an unknown pathogen.* Journal of Neural Transmission, 2003. **110**(5): p. 517-36.
10. Rodriguez-Oroz, M.C., et al., *Initial clinical manifestations of Parkinson's disease: features and pathophysiological mechanisms.* Lancet Neurol, 2009. **8**(12): p. 1128-39.
11. Jankovic, J., *Parkinson's disease: clinical features and diagnosis.* Journal of Neurology, Neurosurgery & Psychiatry, 2008. **79**(4): p. 368-376.
12. Pfeiffer, R.F., *Autonomic dysfunction in Parkinson's disease.* Expert Rev Neurother, 2012. **12**(6): p. 697-706.
13. Steece-Collier, K., E. Maries, and J.H. Kordower, *Etiology of Parkinson's disease: Genetics and environment revisited.* Proceedings of the National Academy of Sciences, 2002. **99**(22): p. 13972-13974.
14. Schapira, A.H., *Etiology of Parkinson's disease.* Neurology, 2006. **66**(10 Suppl 4): p. S10-23.
15. Langston, J.W., et al., *Chronic Parkinsonism in humans due to a product of meperidine-analog synthesis.* Science, 1983. **219**(4587): p. 979-80.
16. Paolini, M., A. Sapone, and F.J. Gonzalez, *Parkinson's disease, pesticides and individual vulnerability.* Trends in pharmacological sciences, 2004. **25**(3): p. 124-129.
17. Polymeropoulos, M.H., et al., *Mutation in the α-Synuclein Gene Identified in Families with Parkinson's Disease.* Science, 1997. **276**(5321): p. 2045-2047.
18. Bekris, L.M., I.F. Mata, and C.P. Zabetian, *The Genetics of Parkinson Disease.* J Geriatr Psychiatry Neurol, 2010. **23**(4): p. 228-242.
19. Chu, Y., et al., *Alterations in lysosomal and proteasomal markers in Parkinson's disease: relationship to alpha-synuclein inclusions.* Neurobiol Dis, 2009. **35**(3): p. 385-98.
20. Pan, T., et al., *The role of autophagy-lysosome pathway in neurodegeneration associated with Parkinson's disease.* Brain, 2008. **131**(Pt 8): p. 1969-78.
21. Matsuda, N. and K. Tanaka, *Does impairment of the ubiquitin-proteasome system or the autophagy-lysosome pathway predispose individuals to neurodegenerative disorders such as Parkinson's disease?* J Alzheimers Dis, 2010. **19**(1): p. 1-9.
22. Exner, N., et al., *Mitochondrial dysfunction in Parkinson's disease: molecular mechanisms and pathophysiological consequences.* EMBO J, 2012. **31**(14): p. 3038-62.
23. Winklhofer, K.F. and C. Haass, *Mitochondrial dysfunction in Parkinson's disease.* Biochim Biophys Acta, 2010. **1802**(1): p. 29-44.
24. Olanow, C.W. and K. McNaught, *Parkinson's disease, proteins, and prions: milestones.* Mov Disord, 2011. **26**(6): p. 1056-71.

25. Dobson, C.M., *Protein folding and misfolding.* Nature, 2003. **426**(6968): p. 884-90.
26. Tyedmers, J., A. Mogk, and B. Bukau, *Cellular strategies for controlling protein aggregation.* Nat Rev Mol Cell Biol, 2010. **11**(11): p. 777-788.
27. Goldberg, A.L., *Protein degradation and protection against misfolded or damaged proteins.* Nature, 2003. **426**(6968): p. 895-899.
28. Bukau, B., J. Weissman, and A. Horwich, *Molecular chaperones and protein quality control.* Cell, 2006. **125**(3): p. 443-51.
29. Horwich, A.L., et al., *Two families of chaperonin: physiology and mechanism.* Annu Rev Cell Dev Biol, 2007. **23**: p. 115-45.
30. Pickart, C.M., *Mechanisms underlying ubiquitination.* Annu Rev Biochem, 2001. **70**: p. 503-33.
31. Martinez-Vicente, M. and A.M. Cuervo, *Autophagy and neurodegeneration: when the cleaning crew goes on strike.* Lancet Neurol, 2007. **6**(4): p. 352-61.
32. McNaught, K.S. and C.W. Olanow, *Proteolytic stress: a unifying concept for the etiopathogenesis of Parkinson's disease.* Ann Neurol, 2003. **53 Suppl 3**: p. S73-84; discussion S84-6.
33. Johnston, J.A., C.L. Ward, and R.R. Kopito, *Aggresomes: a cellular response to misfolded proteins.* J Cell Biol, 1998. **143**(7): p. 1883-98.
34. Garcia-Mata, R., et al., *Characterization and dynamics of aggresome formation by a cytosolic GFP-chimera.* J Cell Biol, 1999. **146**(6): p. 1239-54.
35. Kopito, R.R., *Aggresomes, inclusion bodies and protein aggregation.* Trends Cell Biol, 2000. **10**(12): p. 524-30.
36. Kawaguchi, Y., et al., *The deacetylase HDAC6 regulates aggresome formation and cell viability in response to misfolded protein stress.* Cell, 2003. **115**(6): p. 727-38.
37. Olzmann, J.A., et al., *Parkin-mediated K63-linked polyubiquitination targets misfolded DJ-1 to aggresomes via binding to HDAC6.* J Cell Biol, 2007. **178**(6): p. 1025-38.
38. Burnett, B.G. and R.N. Pittman, *The polyglutamine neurodegenerative protein ataxin 3 regulates aggresome formation.* Proc Natl Acad Sci U S A, 2005. **102**(12): p. 4330-5.
39. Viswanathan, J., et al., *Alzheimer's disease-associated ubiquilin-1 regulates presenilin-1 accumulation and aggresome formation.* Traffic, 2011. **12**(3): p. 330-48.
40. Zaarur, N., et al., *Triggering aggresome formation. Dissecting aggresome-targeting and aggregation signals in synphilin 1.* J Biol Chem, 2008. **283**(41): p. 27575-84.
41. Tsai, Y.C., et al., *The Guanine nucleotide exchange factor kalirin-7 is a novel synphilin-1 interacting protein and modifies synphilin-1 aggregate transport and formation.* Plos One, 2012. **7**(12): p. e51999.
42. Olanow, C.W., et al., *Lewy-body formation is an aggresome-related process: a hypothesis.* Lancet Neurol, 2004. **3**(8): p. 496-503.
43. McNaught, K.S., et al., *Aggresome-related biogenesis of Lewy bodies.* Eur J Neurosci, 2002. **16**(11): p. 2136-48.
44. Lewy, F., *Paralysis agitans.* Handbuch der Neurologie, 1912. **III**: p. 920-933.
45. Tanaka, M., et al., *Aggresomes formed by alpha-synuclein and synphilin-1 are cytoprotective.* J Biol Chem, 2004. **279**(6): p. 4625-31.
46. Beyer, K., M. Domingo-Sabat, and A. Ariza, *Molecular pathology of Lewy body diseases.* Int J Mol Sci, 2009. **10**(3): p. 724-45.
47. Campbell, B.C., et al., *The solubility of alpha-synuclein in multiple system atrophy differs from that of dementia with Lewy bodies and Parkinson's disease.* Journal of Neurochemistry, 2001. **76**(1): p. 87-96.
48. Gomez-Tortosa, E., et al., *alpha-Synuclein immunoreactivity in dementia with Lewy bodies: morphological staging and comparison with ubiquitin immunostaining.* Acta Neuropathol, 2000. **99**(4): p. 352-7.

49. Takahashi, H. and K. Wakabayashi, *Controversy: is Parkinson's disease a single disease entity? Yes.* Parkinsonism Relat Disord, 2005. **11 Suppl 1**: p. S31-7.
50. Wakabayashi, K., et al., *The Lewy body in Parkinson's disease: molecules implicated in the formation and degradation of alpha-synuclein aggregates.* Neuropathology, 2007. **27**(5): p. 494-506.
51. Bennett, M.C., *The role of alpha-synuclein in neurodegenerative diseases.* Pharmacol Ther, 2005. **105**(3): p. 311-31.
52. Dev, K.K., et al., *Part I: parkin-associated proteins and Parkinson's disease.* Neuropharmacology, 2003. **45**(1): p. 1-13.
53. Engelender, S., et al., *Synphilin-1 associates with alpha-synuclein and promotes the formation of cytosolic inclusions.* Nat Genet, 1999. **22**(1): p. 110-4.
54. Maroteaux, L., J.T. Campanelli, and R.H. Scheller, *Synuclein: a neuron-specific protein localized to the nucleus and presynaptic nerve terminal.* J Neurosci, 1988. **8**(8): p. 2804-15.
55. Uversky, V.N., *A protein-chameleon: conformational plasticity of alpha-synuclein, a disordered protein involved in neurodegenerative disorders.* J Biomol Struct Dyn, 2003. **21**(2): p. 211-34.
56. Iwai, A., et al., *The precursor protein of non-A beta component of Alzheimer's disease amyloid is a presynaptic protein of the central nervous system.* Neuron, 1995. **14**(2): p. 467-75.
57. Bellani, S., et al., *The regulation of synaptic function by alpha-synuclein.* Commun Integr Biol, 2010. **3**(2): p. 106-109.
58. Celej, M.S., et al., *Toxic prefibrillar alpha-synuclein amyloid oligomers adopt a distinctive antiparallel beta-sheet structure.* Biochem J, 2012. **443**(3): p. 719-26.
59. Davidson, W.S., et al., *Stabilization of alpha-synuclein secondary structure upon binding to synthetic membranes.* J Biol Chem, 1998. **273**(16): p. 9443-9.
60. Jensen, P.H., et al., *Binding of alpha-synuclein to brain vesicles is abolished by familial Parkinson's disease mutation.* J Biol Chem, 1998. **273**(41): p. 26292-4.
61. Jensen, P.H., et al., *alpha-synuclein binds to Tau and stimulates the protein kinase A-catalyzed tau phosphorylation of serine residues 262 and 356.* J Biol Chem, 1999. **274**(36): p. 25481-9.
62. Lee, F.J., et al., *Direct binding and functional coupling of alpha-synuclein to the dopamine transporters accelerate dopamine-induced apoptosis.* FASEB J, 2001. **15**(6): p. 916-26.
63. Marques, O. and T.F. Outeiro, *Alpha-synuclein: from secretion to dysfunction and death.* Cell Death Dis, 2012. **3**: p. e350.
64. Bisaglia, M., S. Mammi, and L. Bubacco, *Structural insights on physiological functions and pathological effects of alpha-synuclein.* FASEB J, 2009. **23**(2): p. 329-40.
65. Dev, K.K., et al., *Part II: alpha-synuclein and its molecular pathophysiological role in neurodegenerative disease.* Neuropharmacology, 2003. **45**(1): p. 14-44.
66. Kawamata, H., et al., *Interaction of alpha-synuclein and synphilin-1: effect of Parkinson's disease-associated mutations.* Journal of Neurochemistry, 2001. **77**(3): p. 929-934.
67. Engelender, S., et al., *The alpha-synuclein-associated protein, synphilin-1: Gene structure and localization, and presence of synphilin-1 protein in Lewy bodies.* American Journal of Human Genetics, 1999. **65**(4): p. A270-A270.
68. Wakabayashi, K., et al., *Synphilin-1, an alpha-synuclein-associated protein, shows immunoreactivity in Lewy bodies and glial cytoplasmic inclusion.* Brain Pathology, 2000. **10**(4): p. 520-520.
69. Wakabayashi, K., et al., *Synphilin-1 is present in Lewy bodies in Parkinson's disease.* Ann Neurol, 2000. **47**(4): p. 521-523.
70. Engelender, S., et al., *Organization of the human synphilin-1 gene, a candidate for Parkinson's disease.* Mamm Genome, 2000. **11**(9): p. 763-6.

71. Bandopadhyay, R., et al., *No pathogenic mutations in the synphilin-1 gene in Parkinson's disease.* Neurosci Lett, 2001. **307**(2): p. 125-7.
72. Farrer, M., et al., *Genetic analysis of synphilin-1 in familial Parkinson's disease.* Neurobiol Dis, 2001. **8**(2): p. 317-23.
73. Marx, F.P., et al., *Identification and functional characterization of a novel R621C mutation in the synphilin-1 gene in Parkinson's disease.* Hum Mol Genet, 2003. **12**(11): p. 1223-31.
74. Eyal, A., et al., *Synphilin-1A: an aggregation-prone isoform of synphilin-1 that causes neuronal death and is present in aggregates from alpha-synucleinopathy patients.* Proc Natl Acad Sci U S A, 2006. **103**(15): p. 5917-22.
75. Ribeiro, C.S., et al., *Synphilin-1 is developmentally localized to synaptic terminals, and its association with synaptic vesicles is modulated by alpha-synuclein.* J Biol Chem, 2002. **277**(26): p. 23927-33.
76. Nagano, Y., et al., *Siah-1 facilitates ubiquitination and degradation of synphilin-1.* J Biol Chem, 2003. **278**(51): p. 51504-14.
77. Marx, F.P., et al., *The proteasomal subunit S6 ATPase is a novel synphilin-1 interacting protein--implications for Parkinson's disease.* FASEB J, 2007. **21**(8): p. 1759-67.
78. Kruger, R., *The role of synphilin-1 in synaptic function and protein degradation.* Cell and Tissue Research, 2004. **318**(1): p. 195-199.
79. Eyal, A. and S. Engelender, *Synphilin isoforms and the search for a cellular model of lewy body formation in Parkinson's disease.* Cell Cycle, 2006. **5**(18): p. 2082-6.
80. Szargel, R., et al., *Synphilin-1A inhibits seven in absentia homolog (SIAH) and modulates alpha-synuclein monoubiquitylation and inclusion formation.* J Biol Chem, 2009. **284**(17): p. 11706-16.
81. O'Farrell, C., et al., *Transfected synphilin-1 forms cytoplasmic inclusions in HEK293 cells.* Molecular Brain Research, 2001. **97**(1): p. 94-102.
82. Lim, K.L., et al., *Parkin mediates nonclassical, proteasomal-independent ubiquitination of synphilin-1: implications for Lewy body formation.* J Neurosci, 2005. **25**(8): p. 2002-9.
83. Buttner, S., et al., *Synphilin-1 Enhances alpha-Synuclein Aggregation in Yeast and Contributes to Cellular Stress and Cell Death in a Sir2-Dependent Manner.* Plos One, 2010. **5**(10).
84. Chung, K.K.K., et al., *Parkin ubiquitinates the alpha-synuclein-interacting protein, synphilin-1: implications for Lewy-body formation in Parkinson disease.* Nature Medicine, 2001. **7**(10): p. 1144-1150.
85. Ito, T., et al., *Dorfin localizes to Lewy bodies and ubiquitylates synphilin-1.* Journal of Biological Chemistry, 2003. **278**(31): p. 29106-29114.
86. Szargel, R., R. Rott, and S. Engelender, *Synphilin-1 isoforms in Parkinson's disease: regulation by phosphorylation and ubiquitylation.* Cellular and Molecular Life Sciences, 2008. **65**(1): p. 80-88.
87. Liani, E., et al., *Ubiquitylation of synphilin-1 and alpha-synuclein by SIAH and its presence in cellular inclusions and Lewy bodies imply a role in Parkinson's disease.* Proc Natl Acad Sci U S A, 2004. **101**(15): p. 5500-5.
88. Avraham, E., et al., *Glycogen synthase kinase 3beta modulates synphilin-1 ubiquitylation and cellular inclusion formation by SIAH: implications for proteasomal function and Lewy body formation.* J Biol Chem, 2005. **280**(52): p. 42877-86.
89. Tanji, K., et al., *NUB1 suppresses the formation of lewy body-like inclusions by proteasomal degradation of synphilin-1.* American Journal of Pathology, 2006. **169**(2): p. 553-565.
90. Lee, G., et al., *Casein kinase II-mediated phosphorylation regulates alpha-synuclein/synphilin-1 interaction and inclusion body formation.* Journal of Biological Chemistry, 2004. **279**(8): p. 6834-6839.

91. Tanji, K., et al., *Glycogen synthase kinase-3 beta phosphorylates synphilin-1 in vitro.* Neuropathology, 2003. **23**(3): p. 199-202.
92. Soehn, A.S., et al., *Periphilin is a novel interactor of synphilin-1, a protein implicated in Parkinson's disease.* Neurogenetics, 2010. **11**(2): p. 203-215.
93. Johnson, S.A. and T. Hunter, *Kinomics: methods for deciphering the kinome.* Nat Methods, 2005. **2**(1): p. 17-25.
94. Moorhead, G.B., L. Trinkle-Mulcahy, and A. Ulke-Lemee, *Emerging roles of nuclear protein phosphatases.* Nat Rev Mol Cell Biol, 2007. **8**(3): p. 234-44.
95. Alonso, A., et al., *Protein tyrosine phosphatases in the human genome.* Cell, 2004. **117**(6): p. 699-711.
96. Fardilha, M., et al., *The physiological relevance of Protein Phosphatase 1 and its interacting proteins to health and disease.* Current Medicinal Chemistry, 2010. **17**: p. 22.
97. Silva, E.d.C.e., *As fosfatases e a linguagem intracelular.* Colóquio/Ciências, 1998. **22**: p. 43-56.
98. Cohen, P.T., *Protein phosphatase 1--targeted in many directions.* J Cell Sci, 2002. **115**(Pt 2): p. 241-56.
99. da Cruz e Silva, E.F., et al., *Differential expression of protein phosphatase 1 isoforms in mammalian brain.* J Neurosci, 1995. **15**(5 Pt 1): p. 3375-89.
100. Moorhead, G.B., et al., *Displacement affinity chromatography of protein phosphatase one (PP1) complexes.* BMC Biochem, 2008. **9**: p. 28.
101. Wakula, P., et al., *Degeneracy and function of the ubiquitous RVXF motif that mediates binding to protein phosphatase-1.* J Biol Chem, 2003. **278**(21): p. 18817-23.
102. Gibbons, J.A., D.C. Weiser, and S. Shenolikar, *Importance of a surface hydrophobic pocket on protein phosphatase-1 catalytic subunit in recognizing cellular regulators.* J Biol Chem, 2005. **280**(16): p. 15903-11.
103. Hendrickx, A., et al., *Docking motif-guided mapping of the interactome of protein phosphatase-1.* Chem Biol, 2009. **16**(4): p. 365-71.
104. Silva, E.d.C.e., et al., *Differential expression of protein phosphatase 1 isoforms in mammalian brain.* J. Neurosci, 1995b. **15**: p. 3375-89.
105. Esteves, S., et al., *Protein phosphatase 1alpha interacting proteins in the human brain.* Omics: A journal of integrative biology, 2012. **16**(1-2): p. 1-15.
106. Browne, G.J., et al., *SARP, a new alternatively spliced protein phosphatase 1 and DNA interacting protein.* Biochem J, 2007. **402**(1): p. 187-196.
107. Esteves, S., *PP1 interactomes as a means of characterizing protein functions*, in *Biology Department*2012, Aveiro: Aveiro. p. 282.
108. Trinkle-Mulcahy, L., J.E. Sleeman, and A.I. Lamond, *Dynamic targeting of protein phosphatase 1 within the nuclei of living mammalian cells.* J Cell Sci, 2001. **114**(Pt 23): p. 4219-28.
109. Julien, C., et al., *Dimethyl Sulfoxide Induces Both Direct and Indirect Tau Hyperphosphorylation.* Plos One, 2012. **7**(6): p. e40020.
110. Iseki, E., et al., *Immunohistochemical study of synphilin-1 in brains of patients with dementia with Lewy bodies - synphilin-1 is non-specifically implicated in the formation of different neuronal cytoskeletal inclusions.* Neurosci Lett, 2002. **326**(3): p. 211-5.
111. Hsieh-Wilson, L.C., et al., *Characterization of the neuronal targeting protein spinophilin and its interactions with protein phosphatase-1.* Biochemistry, 1999. **38**(14): p. 4365-73.
112. Brown, A.M., et al., *Association of protein phosphatase 1 gamma 1 with spinophilin suppresses phosphatase activity in a Parkinson disease model.* J Biol Chem, 2008. **283**(21): p. 14286-94.
113. Wu, J., et al., *Lewy-like aggregation of alpha-synuclein reduces protein phosphatase 2A activity in vitro and in vivo.* Neuroscience, 2012. **207**: p. 288-97.

8. Appendix

Cell Culture Solutions and Immunocytochemistry:

PBS (1X)

For a final volume of 500 mL, dissolve one pack of Modified Dulbecco's Phosphate Buffered Saline Pack (Pierce) in deionized H_2O. Final composition:

- 8 mM Sodium Phosphate
- 2 mM Potassium Phosphate
- 40 mM NaCl
- 10 mM KCl

Sterilize by filtering through a 0.2 µm filter and store at 4ºC.

1 mg/mL Poly-L-ornithine solution (10X)

To a final volume of 10 mL, dissolve in deionized H_2O 100 mg of poly-L-ornithine (Sigma-Aldrich, Portugal).

4% Paraformaldehyde fixative solution

For a final volume of 100 mL, add 4 g of paraformaldehyde to 25 mL of deionized H_2O. Dissolve by heating the mixture at 58 ºC while stirring. Add 1-2 drops of 1 M NaOH to clarify the solution and filter (0.2 µm).

DMEM medium

For a final volume of 1L, dissolve one pack of DMEM powder (with L-glutamine and 4500 mg glucose/L, Sigma-Aldrich) in deionized H_2O and add:

- $NaHCO_3$ (Sigma-Aldrich) 3.7 g

Adjust to pH 7.4. Sterilize by filtering through a 0.2 µm filter and store at 4 ºC.

Complete DMEM

For a final volume of 1L, when preparing DMEM medium adjust to pH 7.4 and before sterilizing add:

- 100 mL (10% v/v) Fetal Bovine Serum (FBS)
- Antibiotics (5 mL)
    - 100 U/mL penicillin
    - 100 mg/mL streptomycin

DMEM Components

Amino acids:	Concentration (mg/L)
L-Arginine hydrochloride	84
L-Cystine	62.6
L-Glutamine	584
Glycine	30
L-Histidine hydrochloride	42
L-Isoleucine	105
L-Leucine	105
L-Lysine hydrochloride	146
L-Methionine	30
L-Phenylalanine	66
L-Serine	42
L-Threonine	95
L-Tryptophan	16
L-Tyrosine	103.79
L-Valine	94
Vitamins:	
Choline chloride	4
D-Calcium pantothenate	4
Folic acid	4

Niacinamide	4
Riboflavin	0.4
Thiamine hydrochloride	4
Myo-Inositol	7.2

Inorganic Salts:

Calcium Chloride ($CaCl_2.2H_2O$)	200
Magnesium Sulfate ($MgSO_4.7H_2O$)	97.67
Potassium Chloride	400
Sodium Chloride	6400
Sodium Phosphate monobasic ($NaH_2PO_4.2H_2O$)	109

Other components:

D-Glucose	4500
Phenol Red	15.9

SDS-PAGE and Immunoblotting Solutions:

LGB (Lower Gel Buffer)

To 900 mL of deionized H_2O add:

- 181.65 g Tris
- 4 g SDS

Mix until the solutes have dissolved. Adjust pH to 8.9 and adjust volume to 1 L with deionized H_2O.

UGB (Upper Gel Buffer)

To 900 mL of deionized H_2O add:

- 75.69 g Tris

Mix until the solute has dissolved. Adjust pH to 6.8 and adjust volume to 1L with deionized H_2O.

30% Acrylamide/ 0.8% Bisacrylamide

To 70 mL of deionized H_2O add:

- 29.2 g Acrylamide
- 0.8 g Bisacrylamide

Mix until the solutes have dissolved. Adjust volume to 100 mL with deionized H_2O. Store at 4 ºC.

Loading Gel Buffer

- 250 mM Tris-HCl (pH 6.8)
- 8% SDS
- 40% Glycerol
- 2% 2-mercaptoethanol
- 0.01% Bromophenol blue

1X Running Buffer

- 25 mM Tris-HCl (pH 8.3)
- 250 mM Glycine
- 0.1% SDS

1X Transfer Buffer

- 25 mM Tris-HCl (pH 8.3)
- 192 mM Glycine
- 20% Methanol

1X TBS

- 10 mM Tris-HCl (pH 8.0)
- 150 mM NaCl

Adjust pH to 8.0 with HCl and adjust volume to 1L with deionized H_2O.

1X TBST

- 10 mM Tris-HCl (pH 8.0)

- 150 mM NaCl
- 0.05% Tween

Adjust pH to 8.0 with HCl and adjust volume to 1L with deionized H_2O.

II. ANTIBODIES

Table 1 - Antibodies and dilutions. (IF, Immunofluorescence; WB, Western Blot; Rb, Rabbit; Ms, Mouse; IR, Infrared)

Antibodies	Dilutions	Company	Secondary antibody	Dilutions
PPP1CA	1:500 (IF)	Homemade	Texas Red Anti-Rb	1:300
	1:2500 (WB)		Anti-Rb (IR) Odyssey CLx	1:5000
PPP1CC	1:1000 (IF)	Homemade	Texas Red Anti-Rb	1:300
	1:5000 (WB)		Anti-Rb (IR) Odyssey CLx	1:5000
β-Tubulin	1:1000 (WB)	Sigma-Aldrich	Anti-Ms (IR) Odyssey CLx	1:5000
GFP	1:1000 (WB)	Sigma-Aldrich	Anti-Ms (IR) Odyssey CLx	1:5000
α-Synuclein	1:1000 (WB)	Sigma-Aldrich	Anti-Rb (IR) Odyssey CLx	1:5000
γ-Tubulin	1:5000 (IF)	Sigma-Aldrich	Alexa 594 Anti-Ms	1:500

III. BCA STANDARDS

Table 2 - BCA standards.

Standards	BSA (µL)	1% SDS (µL)	Protein mass (µg)	WR (µL)
P0	-	25	0	200
P1	0.5	24.5	1	200
P2	1	24	2	200
P3	2.5	22.5	5	200
P4	5	20	10	200
P5	10	15	20	200

The BSA solution used for standards preparation has a concentration of 2 mg/mL.

The Working Reagent (WR) is prepared adding 1 mL of Solution B to each 50 mL of Solution A.

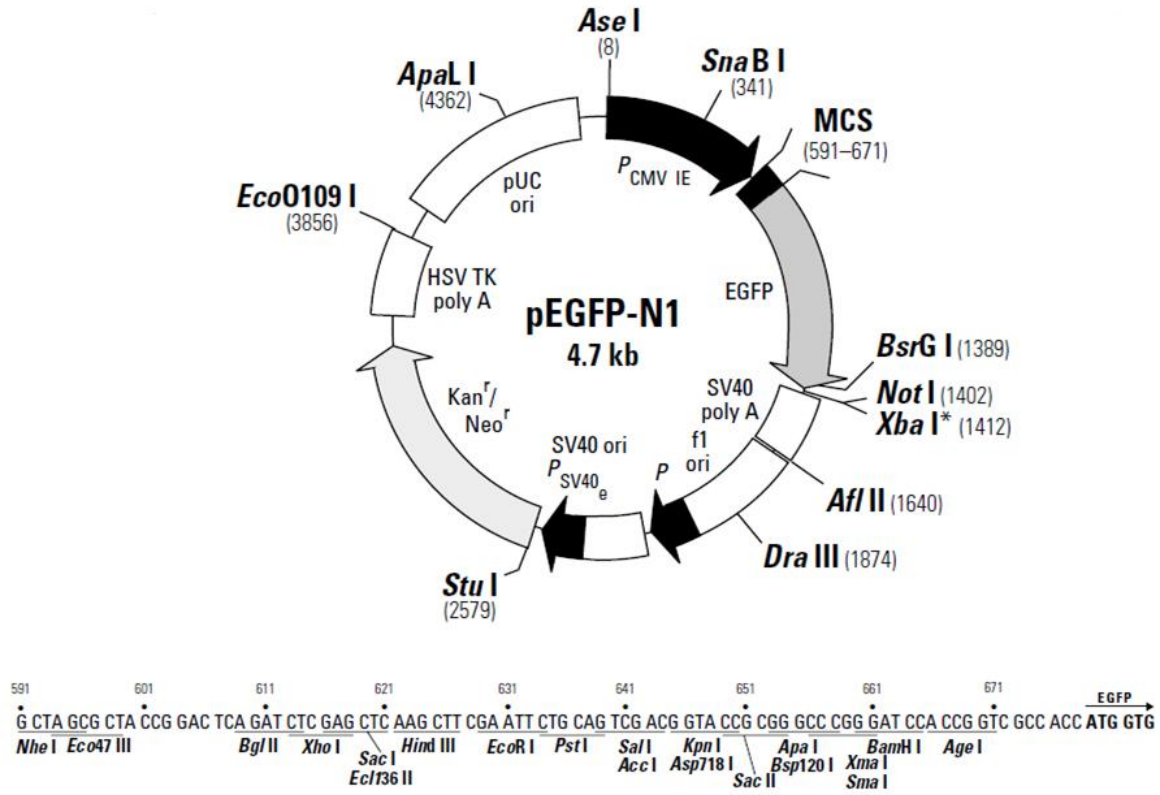

Figure 16 - pEGFP-N1 vector map and MCS (Clontech). This eukaryotic expression vector was used to express GFP-tagged WT Sph1A and GFP-tagged MT Sph1A in mammalian cells (HEK293 cells). As depicted, the fusion protein is expressed with an EGFP tag at the C-terminus of the target protein.