UNIVERSIDADE DE LISBOA

FACULDADE DE CIÊNCIAS DA UNIVERSIDADE DE LISBOA

DEPARTAMENTO DE BIOLOGIA VEGETAL

DISSERTAÇÃO

Alpha-synuclein phosphorylation role on Parkinson's disease

Filipa Magalhães Silva

Mestrado em Biologia Molecular e Genética

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Dissertação orientada por:

Professor Doutor Tiago Fleming Outeiro
Cell and Molecular Neuroscience Unit – Instituto de Medicina Molecular (IMM)

Professora Doutora Maria Margarida Blasques Telhada
Faculdade de Ciências da Universidade de Lisboa

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Resumo

A doença de Parkinson (DP) é uma doença neurodegenerativa caracterizada pela presença de inclusões proteicas designadas por corpos de Lewy (LB). Os LB são agregados intracelulares que se formam nos neurónios dopaminérgicos, localizados na *substantia nigra,* mas que podem ocorrer em outras áreas do cérebro à medida que a doença progride. Os LB são constituídos principalmente pela proteína alfa-sinucleína (aSyn), sendo esta a principal característica desta doença. A aSyn é uma proteína envolvida tanto nos casos de doença idiopática, como familiar da DP. Vários resíduos de aSyn são fosforilados quer na forma solúvel quer na forma agregada da proteína. Sendo que, aproximadamente 90% da aSyn presente nos agregados é fosforilada no resíduo serina 129 (S129), em contraste com apenas 4% de fosforilação neste resíduo em cérebros saudáveis. Apesar da fosforilação no resíduo S129 ser a mais bem estudada, pouco se compreende sobre o seu papel na doença. Por outro lado, estudos recentes realizados em culturas de células, em *Drosophila* e em cerébros humanos demonstraram a existência de fosforilação no resíduo tirosina 125 (Y125). Sabe-se que a fosforilação deste resíduo suprime a oligomerização. Para além disso, durante o envelhecimento, a fosforilação Y125 é naturalmente reduzida, sendo essa redução mais notável nos pacientes com DP. A falta de informação sobre a fosforilação no resíduo Y125 está relacionada com a dificuldade de detectar aSyn fosforilada neste resíduo em cérebros de humanos. Para além disso, é difícil estabelecer a ligação entre a progressão da doença e os níveis de aSyn fosforilados no resíduo de Y125. Devido a todas estas razões torna-se importante estudar o papel da fosforilação da aSyn nos resíduos S129 e Y125 na patogénese da DP.

A levedura *Saccharomyces cerevisiae* (*S. cerevisiae*) é um organismo eucariota e unicelular versátil que apresenta uma organização intracelular complexa e cujas vias metabólicas são conservadas. Deste modo, este modelo é amplamente utilizado para compreender processos biológicos complexos encontrados em eucariotas superiores. *S. cerevisiae* é um modelo de estudo validado para a investigação da DP, sendo que já foi demonstrado que aSyn humana expressa em células de levedura induz toxicidade e formação de inclusões citoplasmáticas à semelhança do que foi observado noutros modelos biológicos, nomeadamente em modelos celulares de mamífero e em modelos animais de PD. Para além disso, existem várias vantagens para a utilização da levedura, como um organismo modelo, tais como a facilidade de manipulação em laboratório; o fato do seu genoma estar totalmente sequenciado; de apresentar um tempo de geração curto e também devido ao fato de ser possível manipular geneticamente através da aplicação simples de métodos clássicos de genética molecular.

O principal objetivo do presente trabalho foi estudar o papel da fosforilação de aSyn na etiologia da DP, no que diz respeito ao efeito a nível de toxicidade e propensão para formar inclusões, utilizando como modelos *S. cerevisiae* e células humanas. Para isso, foram utilizadas três formas de aSyn: a forma selvagem (WT) e as formas não fosforiladas nos resíduos Y125 (Y125F) e S129 (S129A ou S129G), respetivamente. Observou-se que o bloqueio da fosforilação no resíduo Y125 (Y125F aSyn) diminui a percentagem de células com inclusões, enquanto, que o bloqueio da fosforilação no resíduo S129 (S129A aSyn ou S129G aSyn) aumenta a percentagem de células com inclusões. Em paralelo, observou-se que a mutação Y125F não altera a toxicidade da aSyn expressa na levedura *S. cerevisiae* enquanto, que a mutação S129G aumenta a toxicidade desta.

Para validar os resultados em levedura, foi utilizado um modelo de agregação de aSyn, previamente descrito, numa linha celular humana de neuroglioma (H4). Neste modelo, a co-transfecção de uma forma modificada de aSyn (fundida a uma forma truncada de proteína verde fluorescente (GFP), e designada por SynT) juntamente com sinfilina-1, uma proteína neuronal também identificada nos LBs, resulta na formação de inclusões citoplasmáticas, as quais foram observadas através do método de imunocitoquimica. Utilizou-se este modelo estabelecido de agregação de aSyn para investigar o papel das mutações Y125F SynT e S129G SynT na formação de inclusões, em comparação com a situação controlo, WT SynT. Observou-se que as mutações Y125F SynT e S129G SynT resultaram no aumento do número de células com inclusões, o que indica que o bloqueio da fosforilação nos resíduos Y125 e S129 aumenta a propensão da aSyn para agregar no modelo de agregação H4. Além disso, observou-se que o padrão de formação de inclusões na mutação Y125F SynT era diferente do observado na WT SynT e S129G, apresentando agregados mais pequenos.

Neste estudo mostrou-se que existem evidências de uma correlação entre fosforilação, agregação e toxicidade celular de aSyn usando o modelo simples, mas poderoso da levedura *S. cerevisiae*, e um modelo de células neurogliais humanas. Os resultados obtidos também contribuíram para a compreensão da base molecular da doença de Parkinson, tal como de outros distúrbios neurodegenerativos (isto é, outras sinucleionopatias, como, *multiple system atrophy* (MSA), *pure autonomic failure* (PAF), Doença de Lewy bodies). É importante referir que a forma não fosforiladado do resíduo Y125, neste caso, a mutação Y125F, deve ser considerada para estudos posteriores, pois obtivemos resultados diferentes quando se bloqueou a sua fosforilação quer em células de levedura quer em células neurogliais humanas. Isto pode significar que não há uma conservação de mecanismos entre espécies ou então que os resultados obtidos dependem do facto da sinfilina-1 não estar presente em levedura; será algo que terá interesse aprofundar. Também se pode explicar a discrepância de resultados com o facto de o modelo

H4 se basear na co-expressão de sinfilina-1, uma proteína que não tem homologo em levedura mas quando expressa em levedura se sabe aumentar a propensão da aSyn para agregar. A fosforilação de Y125 aSyn, apesar de pouco conhecida e estudada considera-se muito importante, pois muitos estudos sugerem que uma alteração neste resíduo pode estar relacionada com a causa da doença neurodegenerativa e que pode ter uma influência negativa sobre a oligomerização, e consequentemente na agregação de aSyn.

Por outro lado, a mutação S129G aSyn conduz ao aumento da formação de *inclusões* em levedura e a formação de inclusões nas células H4. Assim, concluímos que S129 é um resíduo que tem efeitos semelhantes nos dois modelos usados, sendo que provavelmente a sua fosforilação é um mecanismo conservado entre espécies. Seria de extremo interesse de futuro, estudar em mais detalhe as cinases e as fosfatases envolvidas na regulação da fosforilação deste resíduo. Em conjunto, estes novos resultados demonstram que diferentes resíduos fosforilados na aSyn resultam em diferentes níveis de agregação e toxicidade, sendo que os efeitos da fosforilação de S129 se mostraram indubitavelmente consistentes entre espécies. Por outro lado, a fosforilação de Y125 não apresentou essa mesma consistência. Uma explicação possível pode estar relacionada com o fato das cinases/fosfatases que regulam tirosinas em aSyn nas células humanas poderem não estar tão conservadas em células de levedura como as vias que fosforilam/desfosforilam serinas. Deste modo, torna-se interessante de futuro estudar os dois resíduos em simultâneo e verificar o que acontece quando os dois locais estão disponíveis ou não para serem fosforilados.

O presente trabalho contribuiu para esclarecer o papel de uma modificação pós-traducional, a fosforilação, no processo de agregação de aSyn, no contexto da DP. Conhecer melhor estes mecanismos moleculares pode vir a permitir o desenvolvimento de novas estratégias para intervenção na DP, tal como noutras sinucleinopatias.

Palavras-chave: aSyn; Doença de Parkinson; Agregação; Mutações; Fosforilação.

Abstract

Alpha-synuclein (aSyn) is a protein involved in both idiopathic and familial Parkinson´s disease (PD) cases, being the major component of Lewy body inclusions (LBs), the typical pathological hallmark of this disorder. It is estimated that ~90% of the aggregated aSyn in LBs is phosphorylated in S129 residue, in contrast with only 4% of phosphorylation in the same residue in normal brain.

Saccharomyces cerevisiae is a versatile eukaryotic organism that has being extensively used to understand the complex biological processes found in higher eukaryotes and it is a validated model in PD. Indeed, it has already been shown that aSyn induces toxicity and inclusion formation in a similar manner to that observed in other cell and animal model systems.

The objective of this work was to study the role of phosphorylation of aSyn in the etiology of PD. For this, we used three forms of aSyn: wild shape (WT) and non-phosphorylated forms in the residues Y125 (Y125F) and S129 (S129A or S129G), respectively. We observed that blocking the phosphorylation at residue Y125 reduced the percentage of cells with foci while blocking the phosphorylation at residue S129 increased the percentage of cells with foci. In parallel, we observed that the Y125F mutation did not change the toxicity of aSyn expressed while the S129G mutation increased its toxicity. Subsequently, the aggregation resulting from the expression of Y125F aSyn and S129G aSyn was analyzed in the mammalian cell aggregation model based on co expression SynT and synphilin-1. Both mutations increased the formation of aSyn inclusions in those cells.

Taken together, these new results provide an important insight into the role of phosphorylation in the aggregation process. A better understanding of these molecular mechanisms may enable the development of new strategies for intervention in PD and other synucleinopathies.

Keywords: aSyn, Parkinson's disease, Aggregation; Mutations; Phosphorylation.

List of Abbreviations

aa	amino acid
AD	Alzheimer's disease
aSyn	alpha-synuclein
BCA	bicinchoninic acid
bp	base pairs
BSA	bovine serum albumin
C	Celsius
CO_2	carbon dioxide
CYC1	cytochrome-c oxidase
DLB	dementia with LBs
DNA	desoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
E.coli	*Escherichia coli*
FBS	Fetal Bovine Serum
GAL	Galactose
GAPDH	glyceraldehyde-3-phosphate dehydrogenase protein
GFP	green fluorescent protein
HD	Huntington's disease
H4	neuroglioma cells
kDa	kilo Dalton
LB	Luria-Bertani Broth
LBs	Lewy bodies
LB/Amp	Luria-Bertani Broth/ampicilin
LB/Agar	Luria-Bertani Broth/agar
LiAc	lithium acetate
mM	Millimolar
MCS	multiple cloning site
mL	Milliliter
MSA	multiple system atrophy
NAC	non-amyloid componente
Ng	nanogram
NGS	normal goat serum
$OD_{600\ nm}$	optical density read at 600 nm
ODs	optical densities

PAGE	Polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PD	Parkinson's disease
PEG	polyethyleneglicol 4000
PFA	Paraformaldehyde
pS129 aSyn	aSyn phosphorylated at S129
pY125 aSyn	aSyn phosphorylated at Y125
Raf	rafinose
rpm	revolutions per minute
RT	room temperature
SC	synthetic complete
SDS	Sodium dodecyl sulfate
SN	*substantianigra pars compacta*
SynT	aSynuclein fused with a non-fluorescent GFP fragment
S129	serine 129
S129A	129 replaced by alanine
S129G	129 replaced by glycine
TAE	Tris acetate EDTA
TCA	trichloroacetic acid
TE	Tris-EDTA
WT	wild type
YEP	Yeast Extract Peptone
YPD	Yeast extract Peptone Dextose
Y125	tyrosine 125
Y125F	125 replaced by phenylalanine
µg	Micrograma

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1. Introduction

1.1. Parkinson's Disease and other synucleinopathies

Parkinson's disease (PD), originally described by James Parkinson in 1817, is the second most common late-onset neurodegenerative disorder after Alzheimer's disease [1]. The neuropathological hallmark of the disease is represented by the deposition of alpha-synuclein (aSyn) in the Lewy Bodies (LB), which are intracellular aggregates characteristically found in the dopaminergic neurons affected by the disorder [2]. PD is known to affect around 1% of the population over the age of 65 [3] and 4-5% of the population by the age of 85 [2]. It is clinically characterized by tremor, rigidity, akinesia (reduction in movement) and bradykinesia (slowed movement) correlated to the degeneration of dopaminergic innervation involved in the movement [4]. In fact, the initial defects in the motor system result from the progressive loss of the dopaminergic neurons in the *substantia nigra pars compacta* (SNpc), which consequently cause a reduction in the dopamine levels. In the late stage of the disease the neurodegeneration affects also the other brain regions, with patients experiencing non-motor symptoms such as autonomic dysfunction, sleep disturbances and neuropsychiatric symptoms [5, 6].

The majority of the PD cases are sporadic (about 95%) with unknown aetiology, demonstrating that the disease may result from a combination of environmental, epigenetic and genetic factors [6, 7]. Indeed, environmental factors such as pesticides, have been implicated as risk factors for PD pathogenesis [8, 9]. The genetic factors may then represent susceptibility to disease that, either acting alone or in combination with other environmental factors, might predispose an individual to PD [10, 11] . Only 5% of the cases are familiar and are associated to specific gene mutations [12]. In the last years several mutations responsible for the disease were discovered [10]. These studies suggest the presence of common mechanisms underlying both familial and sporadic forms, like the presence of specific protein within the LBs. It is believed that these bodies may present a protective mechanism of the disease, where dysfunctional and misfolded proteins are sequestered, although there are increasing evidences suggesting a toxic role for aSyn intermediate oligomers species formation [13, 14].

In this scenario, synucleinopathies constitute a distinct group of neurodegenerative disorders that share a common pathologic hallmark, the presence of aSyn aggregates in surviving neurons. These disorders are characterized by the deposition of intracellular inclusions composed largely of fibrillar aSyn, although the distribution of aSyn aggregates is significantly different according to the disease [14-16]. This group of neurodegenerative disorders includes Parkinson's disease (PD), dementia with Lewy bodies (DLB), Lewy body

variant of Alzheimer's disease, multiple system atrophy (MSA) and pure autonomic failure (PAF) [17-19].

These disorders are characterized by different molecular and cellular pathological mechanisms, however, they point out to a key role for aSyn misfolding that may allow us to understand if aSyn intracellular inclusions are a cause or a consequence of neuronal death, and help us to design new therapeutic strategies [20].

1.2. Alpha-synuclein

aSyn was initially associated with PD pathogenesis by being characterized as a primary component of the LB, and by the subsequent discovery of early onset familial PD cases caused by three different autosomal dominant missense mutations (A30P, E46K and A53T) present in the gene encoding aSyn [2, 21, 22]. Recently, new missense mutations, H50Q and G51D, were discovered, but few is known about it [23, 24]. Also duplications and triplications of its locus were associated with the early onset of PD pathogenesis [25, 26].

aSyn is a 14.5 kDa protein of 140 amino acids, with three major domains [27]. The N-terminal is an amphipatic lysine rich domain that is characterized by containinng a highly conserved region of seven 11-residue imperfect repeats with a hexameric motif (KTKEGV) [28, 29]. The hydrophobic central region, comprising residues 61-95, also known as NAC domain, with two additional KTKEGV repeats [30, 31]. The carboxy terminal (C-terminal) domain enriched in acidic residues is negatively charged, comprises the majority of post-translational modification sites and has been involved in the majority of aSyn proteins interactions [32-34] (Fig.1).

The function of aSyn is not yet truly understood, but several studies suggest that this protein might have a role in synaptic development, function and plasticity [35, 36]. aSyn found only in vertebrates, is expressed throughout the central nervous system and particularly enriched in neural pre-synaptic terminals, where may function as a chaperone-like protein or may be involved in synaptic vesicular regulation [37]. Numerous factors have been reported to affect the aggregation process of aSyn, however, few proteins and compounds have been identified as able to inhibit its aggregation propensity [38]. In addition aSyn is known to interact with several proteins, such as synphilin-1, in synaptic vesicles [39].

Figure 1. Schematic representation of aSyn and the positions of genetic mutations. The N-terminal (shown in blue) presents the mutations associated with familial PD (shown in red). The imperfect KTKEGV

repeats are represented in yellow. In purple the NAC domain. In green the C-terminal domain. (adapted from Tenreiro S and Outeiro, unpublished).

1.3. Alpha-synuclein phosphorylation

Several aSyn post-translational modifications, such as nitrosylation, ubiquitinylation, oxidation, truncation and phosphorylation have been identified and significant advances have been made towards the identification of new ones. Among these, phosphorylation is strongly linked to the pathogenesis of PD. In fact, among the aSyn post-translational modifications present in the LB, phosphorylation in Serine 129 (S129) is the most frequent [17, 40-42].

Phosphorylation regulates the structural and functional properties of proteins in health and disease conditions. The role of phosphorylation (a reversible post-translational modification) in the aggregation and toxicity of aSyn is currently the subject of intense investigation [41] .

Phosphorylation of aSyn at one or multiples sites may play an important role in the regulation of LB formation and neurotoxicity *in vivo*, but also on its physiological function [43, 44]. Several studies showed that aSyn is predominantly phosphorylated on serine residues. Residues S87 and S129 were identified as possible phosphorylation sites. S129 is the more phosphorylation site in the C-terminal region of aSyn [40-42, 45] . Phosphorylation of aSyn is known to occur also on tyrosine residues 125, 133 and 136 (Y125, Y133, and Y136), but very little is known about its biological significance [43, 46].

aSyn phosphorylation status influences the affinity of aSyn for other proteins and thus alters the biological processes regulated by these interactions. One way to study aSyn toxicity or aggregation related with phosphorylation, is by mimicking this modification by mutating specific serine or tyrosine residues to another amino acid (aa). Depending on the animal model used, the effect of phosphorylation of aSyn using the phospho-mimicking approach has yielded different results. *Drosophila* and several rat models have also been used to determine which form of aSyn confers toxicity [43, 46] (Fig.2).

While there are reports suggesting that pS129 phosphorylation promotes aSyn inclusion formation [47, 48], others suggest that phosphorylation prevents or does not affect inclusion formation [49]. Studies in *Drosophila* and transgenic mouse models of PD showed that phosphorylation S129 (pS129) aSyn mediates aSyn neurotoxicity and inclusion formation [46, 50]. These results suggested that modulation of pS129 aSyn had a role in inducing neuronal dysfunction [51, 52]. However studies in rats and in *C. elegans* failed to reveal any toxicity associated with the accumulation of phosphorylated aSyn [52, 53] .

Cell culture model systems have also shown that three adjacent tyrosine residues (Y125, Y133, and Y136) in the aSyn C-terminal (phosphorylation of these residues suppresses aSyn oligomerization) can be phosphorylated as well as in human and *Drosophila* brain [54].

Phosphorylation of wild type human aSyn at Y125 in *Drosophila* was shown to be protective against aSyn neurotoxicity. Interestingly, during the normal aging process both in humans and flies Y125 phosphorylation decreases. In addition, cortical tissue from patients with dementia with LB showed less phosphorylation at Y125. aSyn neurotoxicity in Parkinson disease and others synucleinopathies may result from the loss of the neuroprotective action of Y125 phosphorylation, which might inhibit toxic oligomer formation [54]. The lack of further studies on Y125 is related to the difficulty of detecting pY125 aSyn in human brains and the correlation between the progression of the disease and the levels of aSyn tyrosine phosphorylation [55].

Figure 2. aSyn phosphorylation sites. Schematic representation illustrating the various aSyn residues that can be phosphorylated *in vitro* (represented in green) and the ones detected in LBs inclusions (shown in red) (adapted from Tenreiro and Outeiro, unpublished).

1.4 Yeast as a model to study neurodegenerative disease

The yeast model *S. cerevisiae* has been extensively used as a tool in the study of neurodegenerative diseases, such as Huntington's disease (HD), Alzheimer's disease (AD) and PD, since it recapitulates key cellular pathways involved in neurodegeneration [56]. *S. cerevisiae* is an extremely powerful model for molecular biologists and extensive knowledge has been produced on its fundamental cellular mechanisms [57]. Simple yeast cells have made possible to gain insight into neurodegenerative diseases and to uncover and establish basic aspects of both normal and abnormal aSyn biology [58, 59].

Despite the absence of an aSyn ortholog in *S. cerevisiae*, it is still possible to study the human gene via its heterologous expression in yeast, generating a humanized model system [58, 60]. Heterologous expression of aSyn in yeast induces toxicity in a concentration-dependent manner and is associated with the formation of cytoplasmic protein inclusions as observed in other cellular model systems and human PD brain [58]. Key cellular processes between yeast and higher eukaryotes are highly conserved. This conserved cellular network between yeast and higher eukaryotes has been benefitial to identify and study genes known to be involved in human diseases [61, 62]. Other features

obtained through the use of multicellular model systems have also been successfully recapitulated in yeast, namely the involvement of protein quality control system, production of reactive oxygen species and apoptosis [56]. Furthermore, in yeast, several proteins with very diverse functions were identified as genetic modifiers of aSyn toxicity and alterations of these pathways, together with the accumulation of aSyn, contribute to the same cellular dysfunction observed in PD pathology [61, 62].

1.4.1 The life cycle of *Saccharomyces cerevisiae*

Yeast cells propagate as haploid **a**, haploid **α** or diploid **a/α** cell types. The haploid cells undergo a simple life cycle of mitosis and growth. Under conditions of high stress they will, in general, die. On the other hand, during its vegetative cycle, the diploid **a/α** cells (the preferential 'form' of yeast) undergo meiosis and sporulation to produce an ascus that contains four haploid spores, which can proceed on to mate. After germination, two of these spores become cell type **a** and the other two become cell type **α**; these are divided to produce a mother and a daughter cell that have the same mating type as the original spore (Fig.3). As the second mitotic division occurs, the daughter duplicates its DNA, undergoes budding and division without switching mating type, whereas the mother switches the mating type before S phase. At the end of two mitotic divisions of the original spore, four cells are generated, being two **a** and other two **α** mating types. Four cells are then generated after two mitotic divisions of the original spore (two with **a** mating type and two with **α**) [63, 64].
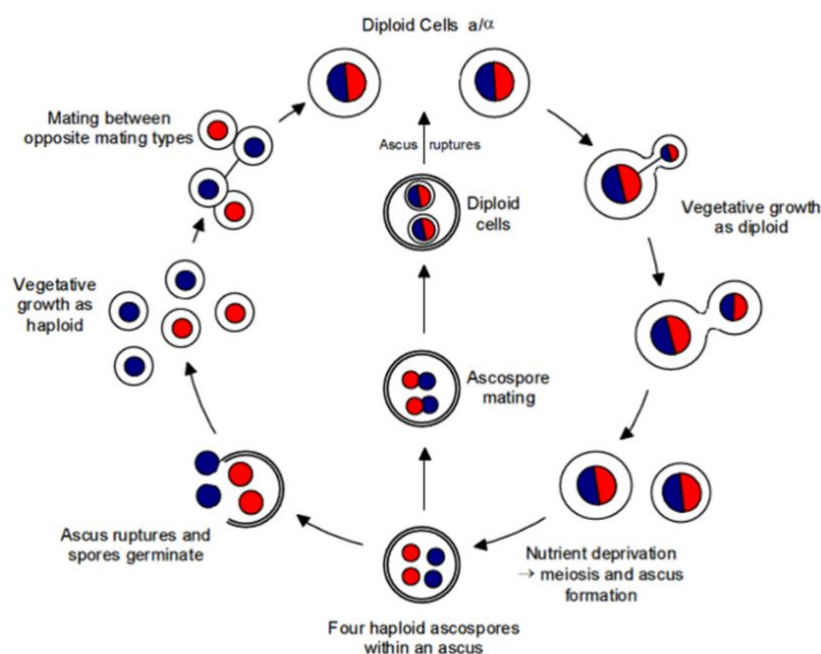
Figure 3. The life cycle of *S. cerevisiae.* The diagram shows yeast life cycle. Mating of haploids gives origin to a diploid, and meiosis of a diploid originates haploid cells. **a/α** cells produce within an ascus, which

contains the four haploid products (spores) that results from the meiosis of the diploid cell. To analyze the individual spores, the ascus wall is digested with degradation enzymes, and the individual spores are separated from each other by micromanipulation. The spores begin to grow on nutrient media and form colonies that can be tested for their mating type and for other markers (Adapted from http://www.uta.edu/search/?q=saccharomyces).

1.5. Mammalian cells model of aSyn aggregation

1.5.1. SynT and synphilin-1

The SynT aggregation model used in this study was first described by McLean and colleagues and has been extensively used since then. This model is based on the expression of aSyn tagged with a truncated, non-fluorescent fragment of GFP (SynT) (Fig.4). In this model, overexpression of SynT together with synphilin-1 in a neuroglioma cell line (H4) promotes the formation of aSyn inclusions [65].
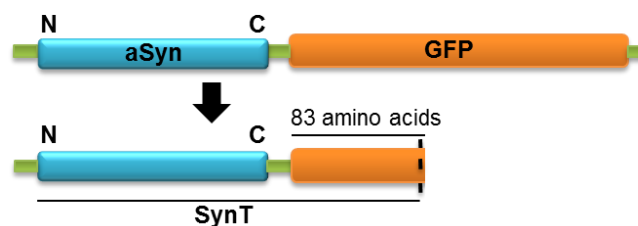
Figure 4. Schematic representation of SynT. This construction allows us to study aSyn aggregation *in vitro* in different mammalian cell lines.

Synphilin-1 is a presynaptic protein that was first identified by yeast-two-hybrid screening as a protein that interacts with aSyn and it is composed of 919 amino acid residues. aSyn interacts *in vivo* with synphilin-1 via its N-terminal and co-localizes with aSyn in LBs of PD patients. Similarly to aSyn, synphilin-1 is present in the majority of LB of patients with PD being present in almost 90% of LB from substantia nigra and other regions of the brain, suggesting its importance to the pathogenesis of PD [66, 67]. Moreover, synphilin-1 seems also to be required for inclusion formation [68].

2. Aims of the study

In order to study the effect of aSyn serine and tyrosine phosphorylation on its toxicity and aggregation we set two major aims:

- To determine the role of phosphorylation of specific residues Y125 and S129 on aSyn toxicity and inclusions formation. For this purpose, an established yeast *S. cerevisiae* model, a versatile eukaryotic organism, was has been already extensively used to understand the complex biological processes found in higher eukaryotes, involving aSyn post-translational modification in PD.

- To validate the main achievements obtained in yeast in a higher eukaryotic model of PD, using a human neuroglioma cell line (H4). For that aim, we generated the constructs Y125F and S129G to express mutant forms of aSyn and to investigate the effects of the different mutations on aSyn inclusions in cultured cells.

3. Materials and Methods

3.1. Molecular biology techniques used in *Escherichia coli*

3.1.1. Plasmids used in this study

The plasmids used in this study are listed in Annex 7.1.

Bacterial cultures of *E. coli* transformed with those plasmids were routinely preserved in glycerol stocks prepared in 2 mL vials of cryopreservation. For that, 500 µL of bacterial culture were mixed with 500 µL of glycerol (50% v/v) and stored at -80°C for future use.

The vectors chosen for the heterologous expression of aSyn in *S.cerevisiae* were pRS304 and pRS306 and were used to express human aSyn under a galactose-inducible promoter and fused with GFP (green fluorescent protein) which enables the evaluation of protein expression and subcellular localization by fluorescence microscopy. They have a yeast TRP1 and URA3 selectable marker, respectively, and *E.coli* ampicillin resistance marker.

3.1.2. Growth media

The *E. coli* were grown at 37ºC overnight in specific medium (Luria-Bertani Broth - LB). The transformed bacteria were streaked onto plates supplemented with 2% agar containing 100 µg/mL of ampicillin (LB/Amp) as selection antibiotic and incubated overnight at 37ºC. After visible growth, the plates were kept at 4ºC.

3.1.3. Transformation of *E.coli* supercompetent cells

E. coli supercompetent cells were thawed on ice. 10 µL of transforming DNA were mixed with 100 µL thawed supercompetent bacteria cells (Nzytec). After that, mixed transformation was kept in ice for 30 min. Heat-shock of bacteria cells was performed by placing the tubes at 42ºC for 45 sec and the cells were placed on ice for 2 min. The resulting solutions added to 900 µL of LB medium without antibiotics and incubated for 1 hour at 37ºC. The transformed bacteria were spread on LB/Agar plates containing 100 µg/mL of ampicillin as selection antibiotic and incubated overnight at 37ºC.

3.1.4. Extraction and purification of plasmids

Random colonies were picked from the LB/Agar plates. These selected colonies were used to inoculate in 3 mL liquid LB/Amp medium, grown overnight with agitation (200 rpm) at 37ºC. The bacterial cultures were harvest by centrifugation and the plasmid DNA purified using NZYMiniPrep Kit from Nzytech. DNA extraction was performed following the manufactured recommendations. The plasmid DNA was eluted in 30 µL of preheated (42ºC)

sterile water. DNA concentration was determined at Nanodrop® (Thermo Scientific) or stored at -20ºC for further use.

3.1.5. Restriction digestion

To prepare the DNA for cloning procedures or to analyse the resulting clones multiple restriction digestion was performed. These restriction reactions were preformed both to confirm the presence of a gene in the expression vector and also to clone genes into expression vectors.

For cloning proposes, vectors and inserts were subject to enzymatic restriction. About 2-3 µg of DNA was digested in a final volume of 40 µL. The appropriated restriction enzymes were added using 1U for each 500 ng/µL of DNA and 4 µL of the corresponding enzyme buffer. For restriction map analysis of the clones obtained from the cloning procedures, a similar enzymatic restriction reaction was performed, but then 1 µL of the miniprep DNA was digested in a final volume of 10 µL.

The reactions were incubated at 37ºC for 2-3 hours or overnight and the resulting DNA fragments were visualized in agarose gel.

3.1.6. DNA extraction from agarose gel

The agarose gel was prepared in TAE 1x buffer and ethidium bromide was added before gel solidification. For visual tracking of DNA fragments migration during electrophoresis, it was applied the Orange G loading buffer (1/3 of the total volume of the samples to the DNA samples). The resolving agarose gel allowed separation of DNA fragments at a constant voltage of 80V.

For further cloning, the bands corresponding to the DNA fragments of interest were cut from the gel and DNA was purified using a specific kit.

3.1.7. Generation of Y125F and S129G phospho-mutants in yeast and mammalian vectors

Using the aSyn WT or SynT (aSynEGF deletion mutant (WTSynGFPΔ155) [65] and synphilin-1 [69]) constructs as a template, the mutations Y125F and S129G phospho-mutants were introduced by site-directed mutagenesis (QuickChange Site-Directed Mutagenesis Kit, Agilent Technologies).Briefly, a PCR was carried out using the *PfuTurbo®* DNA *Polymerase* (Stratagene, La Jolla, CA, USA) (1.25 U), the plasmid DNA template (10 ng) and the mutagenesis primers (62.5 ng, each primer) (Table 1).

Table 1. **Primers used for site-directed mutagenesis.**

Primer	Phospho-mutants	Sequence 5' to 3'
Y125F-S	Y125F mutagenesis	5' CCTGACAATGAGGCTTTTGAAATGCCTTCTGAG 3'
Y125F-AS	Y125F mutagenesis	5' CTCAGAAGGCATTTCAAAAGCCTCATTGTCAGG 3'
S129G-S	S129G mutagenesis	5' GGCTTATGAAATGCCTGGTGAGGAAGGGTATCAAG 3'
S129G-AS	S129G mutagenesis	5' CTTGATACCCTTCCTCACCAGGCATTTCATAAGCC 3'

The following conditions were used to perform the PCR reaction: 1 min at 95ºC, 18 cycles x [50 s at 95ºC, 50 s at 60ºC, 8 min at 68ºC] and 10 min at 68ºC. The resulting products were incubated with *DpnI* at 37ºC, overnight. The *DpnI* endonuclease specifically digests methylated and hemimethylated DNA allowing the selective digestion of the DNA template (methylated) and the selection of the mutated constructs synthesized *de novo* (not methylated). Accordingly, the digested DNA solutions contained only the mutant plasmids. Then, mutant plasmids were transformed in supercompetent *E. coli* cells as described in 3.1.3.

Two independent clones of each mutagenesis reaction were sequenced by means of external DNA sequencing services to confirm the mutations introduced. The results of sequencing were analysed using the Basic Local Alignment Search Tool.

E. coli transformants of the positive clones were preserved in glycerol stocks as described above.

3.1.8 Cloning strategy of yeast integrative vectors

The vectors chosen for the heterologous expression of aSyn in *S. cerevisiae* were pRS304-GAL and pRS306-GAL. These were a shuttle integrative yeast expression vectors that have an inducible GAL (galactose) promoter, carrie a yeast TRP1 and URA3 selectable marker, respectively, and an *E. coli* ampicillin resistance marker (Fig.5).

The Y125F aSyn mutant forms were obtained by site-directed mutagenesis as shown in 3.1.7. While S129G aSyn were obtained by sub-cloning Gal1-aSyn S129G-CYCterminator from p426 yeast plasmid to either prs304 or prs306 integrative plasmids, using *SacI*/*KpnI*. The tyrosine (Y) residue at position 125 of the aSyn polypeptide chain was replaced by the amino acid phenylalanine (F) and the serine (S) residue at position 129 of the aSyn polypeptide chain was replaced by the amino acid guanine (G).

The different aSyn genes are fused to a GFP tag (green fluorescent protein) which enables the evaluation of protein subcellular localization by fluorescence microscopy.
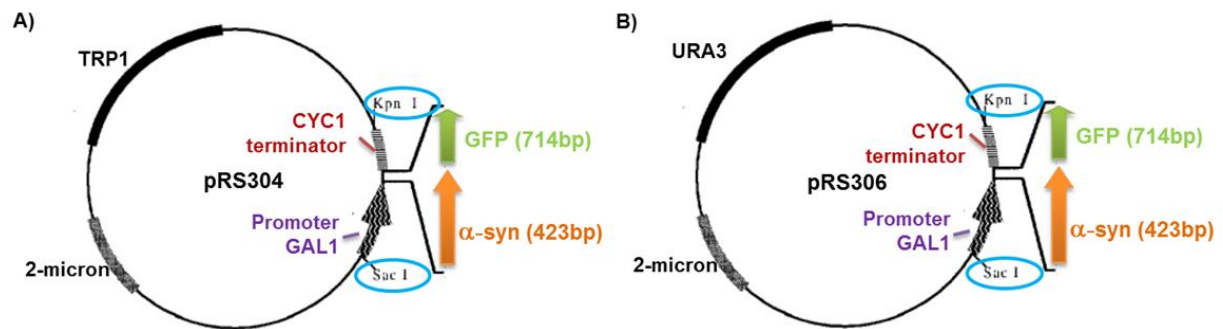
Figure 5. Maps of the yeast expression vectors (A) pRS304-GAL and (B) pRS306-GAL. These expression vectors carry a TRP1 and URA3 selection marker, respectively. The GAL promoter controls the expression of generated fusion proteins. Here are shown the restriction sites of the multiple cloning site (MCS).

3.2. Molecular biology techniques used in *S. cerevisiae*

3.2.1. Yeast strains

The yeast strains used in this study are listed in Annex 7.2. Yeast strains were stored in glycerol stocks prepared by mixing 500 µL of yeast culture added to 500 µL of glycerol (50 % v/v) and stored at -80°C for future use. For further manipulations, strains were defrost and cultivated in solid medium. These plates were incubated at 30ºC until visible cell growth. The cultures were then stored at 4ºC.

3.2.2. Growth media

For routine work, yeast strains were grown at 30ºC in YPD medium and when necessary in synthetic complete (SC) medium lacking specific amino acids in Annex 7.3.

3.2.3. Yeast transformation

Yeast transformation protocol is based in the permeabilization of the yeast cellular membrane by lithium acetate (LiAc), its destabilization by TE and added PEG 40%. These compounds allowed the entry of DNA molecules in the cell.

Yeast cell were prepared prior to manipulation: in the first day, a single colony of each was picked into 5 mL of YPD and incubated at 30ºC with 250 rpm until late afternoon. The optical densities (ODs) were measured at 600 nm and yeast pre-cultures were diluted in 10 mL per transformation to obtain an OD between 0.5-0.7 (generation time of yeast cells in YPD were 90 min). In the second day, cells were harvest by centrifugation, washed with 30 mL of sterile water and resuspended in 1mL of sterile water. Then, pellet was washed in 0.5 mL of a solution of the LiAc 10x (1M LiAc pH 7.5) mixed with TE buffer 10x (0.1 M Tris pH 7.5; 0.01 M EDTA pH 7.5). For each transformation, 50 µL of yeast cells were added to the corresponding eppendorf along with 5 µL of previously boiled denatured salmon sperm. Ten

µL of transforming DNA were added and 300 µL of a solution containing PEG 50 %, TE 10x and AcLi 10x. The mixture was homogenized and incubated at 30ºC with orbital agitation for 30 min. The eppendorf was transferred to a 42ºC bath for 20 min. Cells were washed and were resuspended in 100 µL of sterile water, and plated onto appropriate selective medium.

The plates were incubated at 30ºC for 2-3 days and the transformants obtained were streaked onto fresh plates.

3.2.4. Construction of haploid yeast strains with single genome insertions of the aSyn forms

The integrative plasmids already available and the ones here constructed (point 3.1.8) were used to construct new yeast strains either in the W303.1A or the W303.1B background, carrying in its genome single integrations of WT aSyn, S129A aSyn, or Y125F aSyn, with or without a N-terminal fusion with GFP.

Namely, 3 µg of the integrative vectors of interest either in the pRS304 or in the pRS306 backbone were linearized with EcoRV as described in point 3.1.5. Then DNA was purified using a specific Kit (Wizard SV Gel and PCR Clean-Up System). The obtained DNA was them used to transform either W303.1A or W303.1B as described in point 3.2.3.

After strains were obtained, the correct insertion of the integrative vectors was verified by PCR using a specific pairs of primers (table 2).

Table 2. Primers used for confirmation of the insertion of the integrative vectors in the expected genome locus.

Primer	Sequence 5' to 3'
Y125F-S	5' CCTGACAATGAGGCTTTTGAAATGCCTTCTGAG 3'
Y125F-AS	5' CTCAGAAGGCATTTCAAAAGCCTCATTGTCAGG 3'
S129G-S	5' GGCTTATGAAATGCCTGGTGAGGAAGGGTATCAAG 3'
S129G-AS	5' CTTGATACCCTTCCTCACCAGGCATTTCATAAGCC 3'
S129A-S	5' CTTATGAAATGCCTGCTGAGGAAGGGTATC 3'
S129A-AS	5' GATACCCTTCCTCAGCAGGCATTTCATAAG 3'

The following conditions were used to perform the PCR reaction: 5 min at 95ºC, 30 cycles x [30 s at 95ºC, 30 s at 55ºC, 2 min at 72ºC] and 10 min at 72ºC. After the PCR reaction was performed to confirm the PCR by agarose gel (1% agarose gel TAE 1x was run 60 min at 90V). Transformants were positive for vectors integrations were preserved at 4°C or used.

These haploid strains were then used to generate diploid strains by mating. Briefly, yeast cells from each haploid strain were mixed on a YPD agar plate and incubated at 30ºC for 2 days. Then, cells were picked, ressuspended in sterile watter and diploids were selected in minimal medium by URA and TRP auxotrophy.

These diploid strains were used to obtain haploid strains with double insertions of the appropriate aSyn by our collaborator Donata Wawrzycka. Briefly, diploids were by sporulation and tetrad dissection using a micromanipulator. The haploid strains obtained from each spore was analysed regarding auxotrophies and mating type.

The phenotypic characterization of these haploid strains was performed in several haploids obtained from dissected spores from independent tetrads and independent matings.

3.2.5. Growth conditions and spot assays

In the afternoon of the first day a pre-inoculum of the yeast strains of interest was prepared from fresh cultures in 5 mL YEP-Raffinose liquid media and incubated at 30ºC with orbital agitation (200 rpm) overnight. Optical density at 600 nm ($OD_{600\ nm}$) was read in the next afternoon and yeast cells were diluted into 5 mL of the same medium and incubated at 30ºC, with orbital agitation (200 rpm) until the standardized culture $OD_{600\ nm}$=0.5 was reached the next morning. The volume of yeast culture needed to inoculate a new culture with an initial standardized $OD_{600\ nm}$=0.2 was centrifugated and the cells were resuspended in YEP-Galactose liquid media and incubated at 30ºC, with orbital agitation (200 rpm), for 6 hours.

Serial dilutions (1:3) of the cell suspensions were prepared and spotted directly onto the agar plates that were then incubated at 30ºC and growth of yeast strains was monitored during 3 days.

3.3. Molecular biology techniques used in human neuroglioma cells

3.3.1. Construction of psi Y125F and S129G aSyn mammalian expression vectors

The SynT aggregation model used in this study was a starting point for the production of the Y125F and S129G phospho-mutant constructs. We generated two different phospho-mutant constructs using the SynT as a template. These mutations were introduced by site-directed mutagenesis (as described in 3.1.7).

3.3.2. Cell culture and cell transfection

The experiments were carried out in Human neuroglioma cells (H4) (ATCC HTB-148, LGC Standards, Barcelona, Spain) which were maintained in OPTI-MEM® I (Gibco, Invitrogen, Barcelona, Spain) supplemented with 10% Fetal Bovine Serum (FBS). The cells were grown at 37°C in an atmosphere of 5% CO_2, under controlled conditions. For selected method cells were counted and seeded at a specific density of cells and on different types of plates. The density was maintained among the different sizes of plates in order to obtain

comparable results with different techniques. For microscopy experiment, cells were counted and seeded at a density of 120.000 cells/cm^2 on glass- bottom 35 mm dishes (10 mm glass surface diameter, MatTek Corporation, Ashland, MA, USA) and for protein extraction cells experiment, cells were counted and seeded at a density of 175.000 cells/cm^2 in 100 mm dishes (Techno Plastic Cultures AG, Switzerland). After 24 hours prior to transfection, H4 cells were transfected with the different combinations of plasmids. So, DNA transfection (Roche diagnostics, Mannheim, Germany) was used to transiently transfect cells (the cells are 60-80% confluent upon transfection) with the different combinations of plasmids, according to the manufacturer's instructions.

3.3.3. Immunocytochemistry

The H4 cells were washed three times with PBS 1x, after 24 hours of incubation in the conditions described below. Cells were then fixed in 4% paraformaldehyde (PFA) at room temperature (RT) for 10 min. Cells were washed with PBS three times and thenwere permeabilized in 0.5% Triton X at RT for 20 min. The Triton X was removed but not washed. Cells were blocking in 1.5% normal goat serum (NGS) at RT for 1 hour. After blocking cells were incubated with primary antibody (mouse anti-aSyn, 1:1000) overnight at 4ºC. The primary antibody was removed and washed with PBS for three times. The secondary antibody was added (anti-mouse IgG-Alexa488, 1:1000, Invitrogen) and incubated at RT for 2 hours. Cells were washed more three times with PBS. Cells were maintained in PBS at 4ºC for further analyse fluorescent microscopy.

3.4. Fluorescence microscopy and image analysis

Fluorescence microscopy was performed using a Zeiss Axiovert 200 M Widefield fluorescence microscope equipped with a digital Axiocam from Zeiss (objective 40x and 63x, EC Plan-NeoFluar, Dry, NA (0.75)).

The yeast cultures grown as described in 3.2.4 were centrifuged at 3000 rpm, at 30ºC for 5 min and visualized under the microscope. The percentage of cells with aSyn inclusions was then determined by counting at least 500 cells *per* strain using ImageJ software.

Transfected H4 cells were fixed with 4 % paraformaldehyde described in 3.3.2. Slides were subjected to fluorescence microscopy. The proportion of cells with aSyn inclusions was then determined by counting at least 60-100 cells per culture using ImageJ software.

3.5. Protein extraction

For protein extraction, equal amounts of yeast cells were harvest and incubated with Trichloroacetic acid (TCA) (10%) at -20ºC for 20 min. Cells were than lysed using MRB buffer [50 mM sodium phosphate, 25 mM MES, pH 7.01 1% SDS, 3 M urea, 0.5% B-mercaptoethanol, 1mM sodium azide and proteases and phosphatases inhibitors (Roche diagnostics, Mannheim, Germany)] and disrupted with glass beads (3 cycles of 30 sec in the beadbeater and 5 min on ice). The samples were incubated at 70ºC for 10 min, and unlysed cells and membranes were removed by centrifugation at 10.000 g for 1 min at 4ºC and the supernatant containing the proteins was collected.

For H4 cells, the medium was removed. Cells were washed three times with PBS and harvest with Lysis buffer NP_4O (with proteases inhibitor cocktail tablet).The plates were scraped and the cells were collected to an eppendorf and frozen at -80ºC for 15 min to disrupt membranes. Cells were always kept on ice during the extraction procedure, to avoid protein degradation. Cells were then sonicated 10 sec at 5 mA putted in 2 min in the ice twice (Soniprep 150 sonicator (Albra, Milano, Italy). After, cells were centrifuged at 0.7 rcf for 10 min at 4ºC. Proteins that were in the supernatant were collected.

The protein concentration was determined by means of the BCA Protein Assay Reagent Kit (Thermo Fisher Scientific Inc., Rockford, IL, USA), following manufacturer's instructions.

3.6. Western blot analysis

Protein sample buffer (200 mM Tris-HCl pH 6.8, 6% 2-mercaptoethanol, 8% SDS, 40% glycerol, 0.4% bromophenol blue) was added to each protein sample that was then heated for 10 min at 100ºC. Protein samples were run in SDS-PAGE. After, resolved, proteins were transferred to a nitrocellulose membrane using a Trans-Blot Turbo transfer system (Bio-Rad). Immunoblotting was performed following standard procedures with the listed antibodies: aSyn (BD Transduction Laboratories, San Jose, CA, USA), pS129 aSyn (Wako Chemicals USA, Inc., Richmond VA, USA) and Y125F Abcam ab10789, GAPDH (Ambion, Cambridgeshire, UK) antibody served as loading control for yeast.

3.7. Data and statistical analysis

Results are shown as the averages of at least three independent experiments and are represented as the means ± SD. Differences amongst treatments were detected by analysis of variance with the Tukey HSD (honest significant difference) Multiple Comparison Test using SigmaStat 3.10 (Systat). Values of $p < 0.05$ were considered significant.

4. Results

4.1 Construction of yeast strains with double insertions of the Y125F, S129G or WT SNCA human gene

4.1.1. Construction of yeast integrative expression vectors

In this work it was decided to use integrative expression vectors in order to guarantee stable expression levels of aSyn. Accordingly, the first step consisted to construct the plasmids that were not available yet and to use them in parallel with other already available and characterized in our laboratory (UNCM/IMM).

The strain W303.1A was chosen for this study, as several strains constructed in this background were, as mentioned above, were already available in the laboratory. This strain was also used and characterized by Outeiro and Lindquist, 2003. This study showed that when the gene encoding aSyn, *SNCA* is cloned into a 2 μ vector (pRS426GAL) and is expressed in this strain (W303.1A) results toxic, decreases yeast growth and augments the number of cells presenting foci. Since 2 μ expression of aSyn results quite toxic, cells have tendency to reduce the plasmid copy number and protein expression might be considerable variable in a yeast cell population; therefore in our study it was preferred to use the aSyn integrated strains.

The aSyn mutants forms Y125F and S129G, where the phosphorylation in this resides is blocked were obtained either by site-directed mutagenesis as described in point 3.1.7. or by subcloning as described in 3.1.8. (Fig.6).
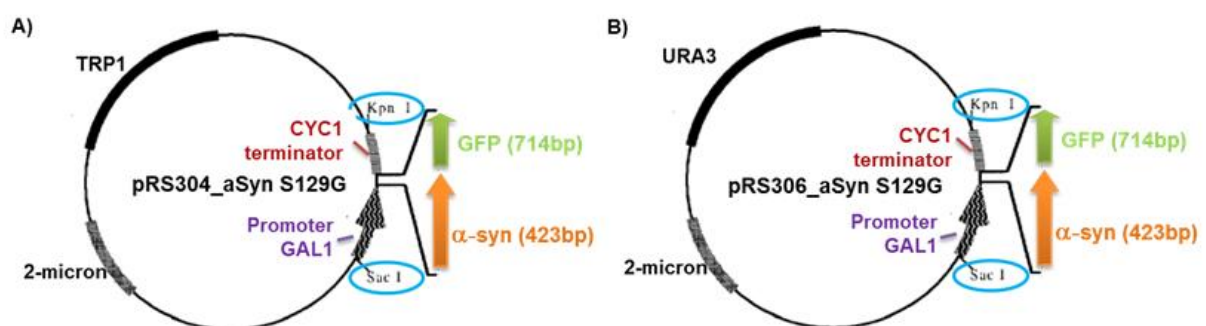

Figure 6. Maps of the yeast expression vectors of S129G aSyn in (A) pRS304-GAL and (B) pRS306-GAL plasmids. These expression vectors contain a Gal promoter which controls the expression of the fusion proteins S129G aSyn; a MCS with multiple restriction sites and a TRP1 or URA3 selection marker, respectively.

In Figure 7 there is a schematic representation of the nucleotides and aminoacid sequences of aSyn where the site-directed mutagenesis was performed.

```
SpeI
   1 ATGGATGTAT TCATGAAAGG ACTTTCAAAG GCCAAGGAGG GAGTTGTGGC
     M   D   V   F    M   K   G    L   S   K    A   K   E    G    V   V   A

  51 TGCTGCTGAG AAAACCAAAC AGGGTGTGGC AGAAGCAGCA GGAAAGACAA
     A   A   E    K   T   K   Q    G   V   A    E   A   A    G   K   T   K

 101 AAGAGGGTGT TCTCTATGTA GGCTCCAAAA CCAAGGAGGG AGTGGTGCAT
     E    G   V    L   Y   V    G   S   K    T    K   E    G   V   V   H

 151 GGTGTGGCAA CAGTGGCTGA GAAGACCAAA GAGCAAGTGA CAAATGTTGG
     G   V    A    T    V   A   E    K   T   K    E   Q   V    T   N   V   G

 201 AGGAGCAGTG GTGACGGGTG TGACAGCAGT AGCCCAGAAG ACAGTGGAGG
     G   A   V    V   T   G   V    T   A   V    A   Q   K    T   V   E   G

 251 GAGCAGGGAG CATTGCAGCA GCCACTGGCT TTGTCAAAAA GGACCAGTTG
      A   G   S    I   A   A    A   T   G   F    V   K   K    D   Q   L

                                    EcoRI
 301 GGCAAGAATG AAGAAGGAGC CCCACAGGAA GGAATTCTGG AAGATATGCC
     G   K   N   E    E   G   A    P   Q   E    G   I   L   E    D   M   P

      BamHI                  TTT Y125F      GGT S129G
 351 TGTGGATCCT GACAATGAGG CTTATGAAAT GCCTTCTGAG GAAGGGTATC
     V   D   P    D   N   E   A    Y   E   M    P   S    E   G   Y   Q

 401 AAGACTACGA ACCTGAAGCC TAA                   HindIII
      D   Y   E    P   E   A    *
```

Figure 7. Schematic representation of the aSyn nucleotide and amino acid sequences. Upper line represents the nucleotides sequence and the below one represents the protein sequence. Codons modified by site-directed mutagenesis for Y125F are shown in blue, while the ones modified by S129G are shown in green.

In more detail, Y125F mutation occurred through the replacement of tyrosine for phenylalanine, which blocks phosphorylation and allows studying the effect of phosphorylation on this residue. Indeed, S129G mutation occurred through the replacement of serine for a glycine residue. This mutation mimics aSyn unphosphorylated form and represents a novel mutant as alternative to the widely characterized S129A, where the serine was replaced by an alanine.

4.1.2. Construction of yeast strains with single insertions of the *SNCA* gene

The plasmids obtained in 4.1.1. were used to construct haploid yeast strains with single insertions of Y125F aSyn in the genome (see 3.2.3). Integrative plasmids containing the mutation Y125F and bearing URA3 or TRP1 as auxotrophic markers, were cloned. Using

this construction and S129G; aSyn without GFP, S129A without GFP (already available in the laboratory) were made single integrations in the genome of W303.1A and W303.1B. Integrative vectors were designed in order to address a specific auxotrophic marker to integrate aSyn gene by homologous recombination (Fig.8).

Different insertions in genome were performed with different mating types. The plasmids used were pRS306 and pRS304, which present different auxotrophies, URA and TRP respectively. The insertion of the integrative vectors in the expected genome locus was confirmed by PCR using specific pairs of primer. Figure 9 shows the PCR primers used and their respective products.
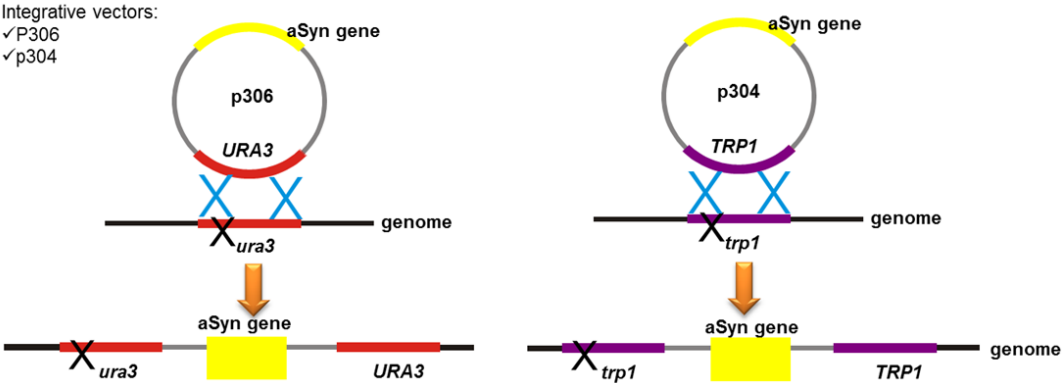
Figure 8. Construction of yeast strains with single genome insertion of the indicated phospho-mutants. Integrative yeast vector with different selective markers have been used to clone each aSyn mutant and integrated in genome.

Figure 9. Representative electrophoresis to confirm genome insertions with plasmids containing different auxotrophies. (A) PCR primers used and their respective products. **(B)** The insertion of the integrative vectors in the expected genome locus (*ura3* or *trp1*) was confirmed by PCR using specific pairs

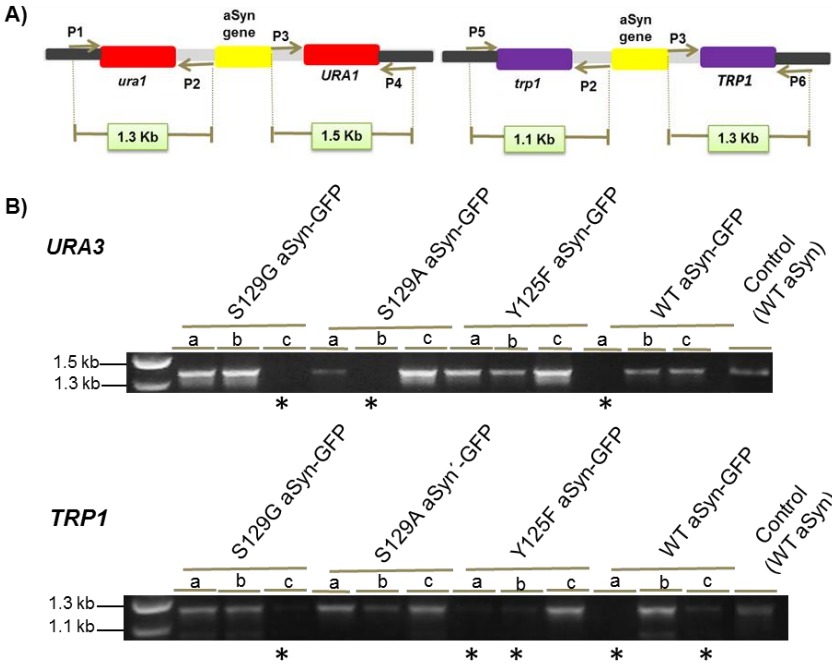of primers. S129G aSyn-GFP; S129A aSyn-GFP and Y125F aSyn-GFP represents the unphosphorylated forms and WT aSyn is the control (VSY72). a, b, c represents different of colonies. * indicates negative candidates while all the remaining strains were positive for the correct genome insertion of the integrative vectors.

4.1.3. Construction of yeast strains with double insertions of the SNCA gene

A normal cell division of yeast leads to their genetically identical progeny. However, in this study we crossed different strains of yeast in order to produce new strains with unique genetic combinations.

For this purpose yeast strains of each mating type were mated. The next step was to induce sporulation of the cells mentioned above by removing nutrients from their growth media. During this sporulation cells undergo two rounds of cell division, resulting in four spores in which the chromosomes have been distributed. This process generates four haploid yeast cells that are contained in an ascus and a single unit can be grown as an haploid cells.

W303 diploid strains were used to obtain haploid strains with double insertions of the appropriate aSyn by our collaborator Donata Wawrzycka (University of Wroclaw, Poland). Briefly, haploid cells were induced by sporulation and tetrads were dissected using a micromanipulator. The haploid strains obtained from each spore was characterized by auxotrophic marker and mating type.

The phenotypic characterization of these haploid strains was performed in several haploids obtained from dissected spores from independent tetrads and independent matings (Fig.10).
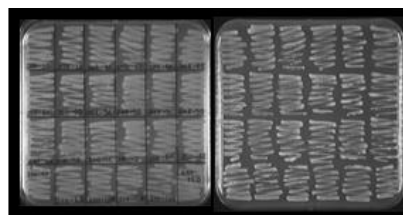
Figure 10. Haploid strains obtained from dissected spores from independent tetrads and independent matings. The picture represents the stains obtained by budding (as described in 3.2.4). Throughout the plate are observed haploid strains resulting from de germination of different tetrads obtained from the mating of, in this case the strains SC208/220 (see Annex 8.2).

The VSY strains which contain a double genome insertion of WT aSyn where used as control strains. These strains were previously described and characterized by Sancenon et al., 2010.

Then we performed the characterization of the tetrads in order to study the toxicity and formation of foci in the Y125F and S129G residues.

4.2. Using yeast as a model to study aSyn phosphorylation

4.2.1. Effect of Y125F on aSyn toxicity and foci formation

We next investigated the role of blocking phosphorylation on residue Y125 on aSyn aggregation and toxicity using the phospho-mutant Y125F aSyn. Expression of this mutant was under the regulation of a galactose-inducible promoter which was turned on upon transfer of cells from raffinose and to galactose media.

To evaluate the effect of blocking phosphorylation on Y125 on aSyn toxicity, the strains selected previously expressing one or two copies of Y125F aSyn (haploid strains obtained in 4.1.3), which resulted from the same tetrad were analysed. A replica of one of these set of strains was compared to strains expressing two copies of WT aSyn (VSY72) or S129A aSyn mutant form (VSY73) (that mimics the unphosphorylated form of aSyn) by spotting assay (Fig. 11). The control strain (VSY71) contained an empty vector.

Expression of two copies of WT aSyn was not toxic for yeast as previously described [58], whereas two copies of S129A aSyn (VSY73) caused mild toxicity. Our results showed that either one or two copies of Y125F aSyn did not cause cellular toxicity, as it presented identical growth as for the WT strains (Fig.11). Therefore, no significant phenotype was visible when phosphorylation on Y125 was blocked.

Figure 11. Effect of Y125F on aSyn toxicity in yeast cells carrying single or double insertions of the encoding gene. Spotting assay of serial dilutions of cell suspensions, initially adjusted to $OD_{600\ nm}=0.05+-0.005$, that where applied as spots (4 µl) onto the surface of the solid medium either with glucose (aSyn expression repressed) or galactose (aSyn expression induced). Results shown are from one representative experiment from at least three independent experiments.

Furthermore, we analysed cells expressing WT and Y125F aSyn-GFP by fluorescence microscopy. After 6 hours of induction of aSyn expression, cells expressing WT aSyn already showed the presence of aSyn foci (Fig.12). Surprisingly, we did not observe formation of foci in cells expressing either one or two copies of Y125F aSyn.
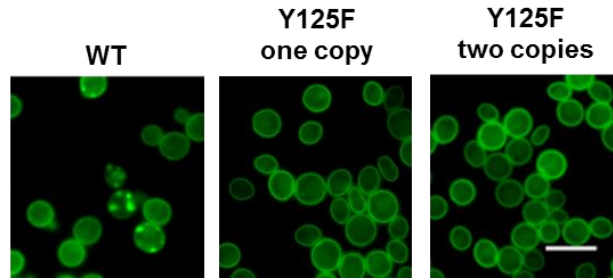
Figure 12. Effect of Y125F on aSyn foci formation in yeast cells carrying single or double insertions of the encoding gene. Fluorescence microscopic visualization and intracellular localization of the WT and phospho-mutant Y125F aSyn fused to GFP 6 hours post induction of aSyn expression. Scale bar is 10 μm.

4.2.2. Effect of S129G on aSyn toxicity and foci formation

In these experiments, we compared the effect of two mutations (S129A and S129G) that prevent the phosphorylation of S129 residue on aSyn toxicity. Expression of these mutants was under the regulation of a galactose-inducible promoter (as described in 4.3.1.).We previously showed that aSyn toxicity and foci formation was enhanced (Tenreiro et al., unpublished results) by S129A mutation that blocks aSyn phosphorylation. However, whether this was simply due to structural effects caused by the S129A substitution in aSyn or due to blocking phosphorylation was not completely clear. To clarify this, we replaced the serine 129 by glycine, which also blocks phosphorylation, following the same procedure as in 4.2.1. (haploid strains obtained in 4.1.3). The spotting assays were performed with haploid strains that resulted from the same tetrad and were compared to strains expressing two copies of WT aSyn (VSY72) or S129A aSyn mutant form (VSY73) (that mimics the unphosphorylated form of aSyn). The control strain (VSY71) contained an empty vector. We observed that the toxicity caused by expression of S129A and S129G aSyn is identical (Fig.13).
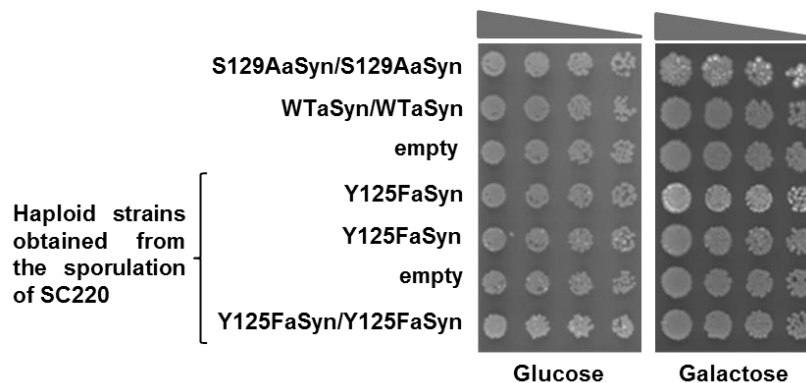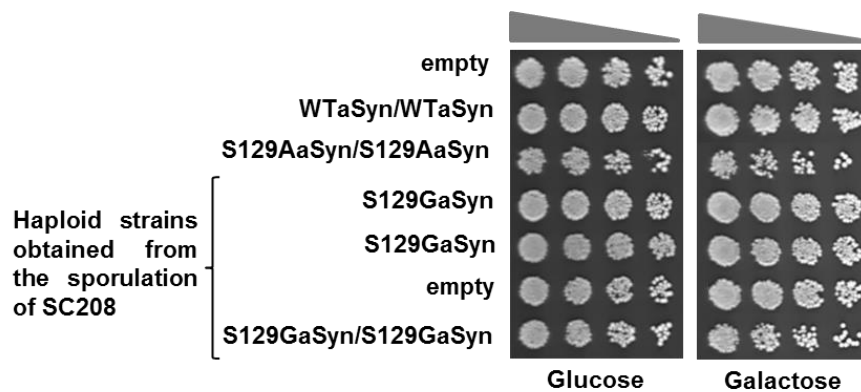

Figure 13. Effect of S129G on aSyn toxicity in yeast cells carrying single or double insertions of the encoding gene. Spotting assay of serial dilutions of cell suspensions, initially adjusted to $OD_{600\,nm}$=0.05+-

0.005, that where applied as spots (4 µl) onto the surface of the solid medium either with glucose (aSyn expression repressed) or galactose (aSyn expression induced). Results shown are from one representative experiment from at least three independent experiments.

In particular, yeast strains carrying two copies of human *SNCA* integrated in the genome, encoding either WT, S129A and S129G mutant aSyn C-terminally tagged with GFP were used to to be compared side by side and pursuit the work. Confirming the results apresented in fig. 13, the expression of S129A and S129G aSyn is more toxic for yeast cells than expression of WT aSyn, as evaluated by spotting assay (Fig.14).
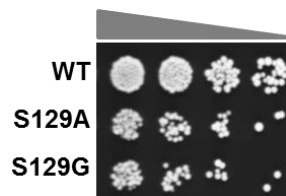

Figure 14. S129G aSyn shows a similar phenotype to S129A aSyn. Cell viability of yeast cells expressing either WT aSyn, S129A aSyn or S129G aSyn, after 6 hours of aSyn expression induction, assessed by spot assay. Cell suspensions were adjusted to $OD_{600\ nm}$=0.05±0.005 and used to prepare 1/3 serial dilutions that were applied as spots (4 µl) onto the surface of the YPD rich medium and incubated at 30ºC for 2 days. Results shown are from one representative experiment from at least three independent experiments.

Furthermore, after 6 hours of induction of aSyn expression, aSyn foci formation in cells expressing the WT, S129A or S129G was quantified by fluorescence microscopy. The majority of cells expressing either S129A or S129G aSyn displayed foci (90%). In contrast, only 70 % of cells expressing WT aSyn presented foci (Fig.15).
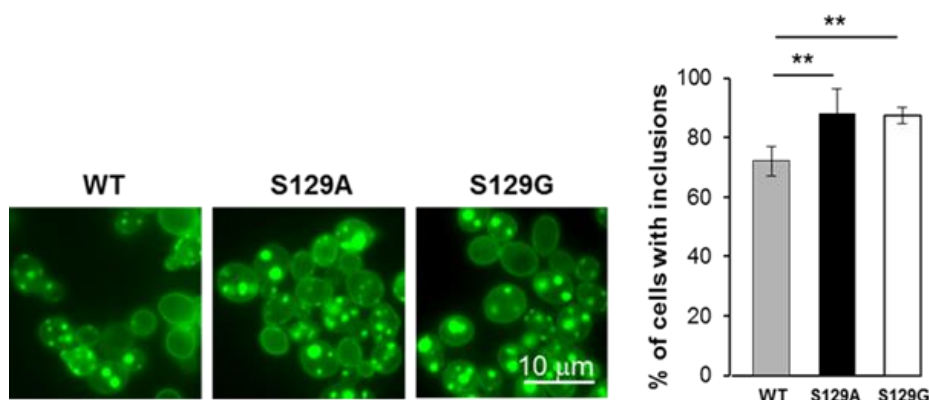

Figure 15. S129G aSyn shows similar foci formation to S129A aSyn in yeast cells. Intracellular localization of the WT, S129A or S129G aSyn-GFP (left panel) and percentage of yeast cells containing aSyn foci, after 6 hours of aSyn expression induction, assessed by fluorescence microscopy (**p < 0.01;

ANOVA and post-hoc Tukey test) (right panel). Error bars indicate the standard error. Results shown are from one representative experiment from at least three independent experiments. Values represent the mean ± SD of three independent experiments. Scale bar is 10 µm.

In conclusion, the expression of S129G aSyn was indistinguishable from expression of S129A aSyn, confirming that the previous observations obtained with the S129A mutation were due to the block of phosphorylation and not due to structural modification of the protein.

4.2.3. Effect of Y125Fand S129G on aSyn S129 phosphorylation levels

The expression levels of the different variants of aSyn tested in this work were assessed by western-blot analysis. Here we checked that aSyn expression levels were not affected by the mutations Y125F and S129G and found all variants were expressed at similar levels after 6 hours of expression induction (Fig. 16). Using an antibody that specifically recognizes pS129 aSyn, we observed that all aSyn were phosphorylated on S129 except the ones with mutations on the serine residue (S129G and S129A) (Fig.16). Importantly, S129G and S129A aSyn mutants cannot be detected by the anti-pS129 antibody, as expected.
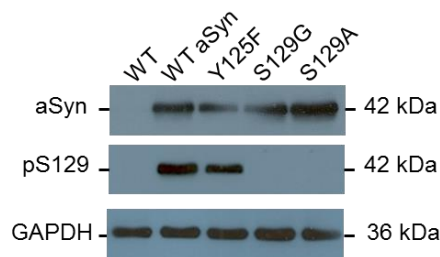
Figure 16. S129 phosphorylation levels of WT aSyn, Y125F aSyn, S129G aSyn and S129A aSyn expressed in yeast cells, determined by immunoblotting analysis. Representative immunoblotting of yeast cells expressing Y125F, S129G and S129A phosphorylation mutants. GAPDH was used as loading control.

4.3. Validation of phosphorylation effects in human cells

4.3.1. Effects of aSyn phosphorylation on inclusion formation

The SynT aggregation model was used to study the effect of Y125F and S129G mutations on aSyn inclusion formation. The SynT plasmids were constructed by site-directed mutagenesis as described for the yeast ones (see 3.1.7). H4 cells were co-transfected with WT, Y125F or S129A SynT and synphilin-1. Forty-eight hours post-transfection cells were fixed and subject to ICC as described in 3.3.2., and observed by fluorescence microscopy as described in 3.4. For this study, cells were counted and categorized according to the number

of inclusions shown. Three groups of cells were established: cells without inclusions; cells with less than ten inclusions (<10) and cells with 10 or more inclusions (≥ 10). The latter group surprisingly included only cells expressing Y125F as different types of inclusions were observed for this particular aSyn mutant (Fig.17).
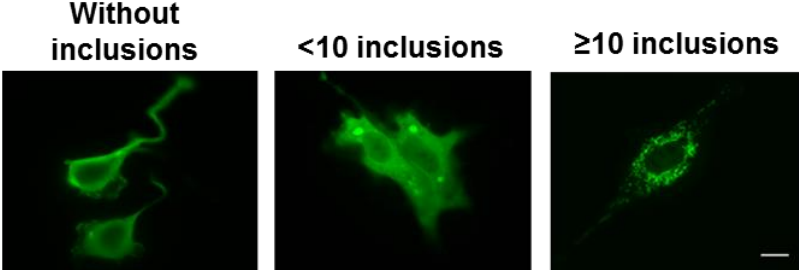
Figure 17. Representative images of the phenotypes observed in the H4 cells co-transfected with WT, Y125F or S129A aSyn and synphilin-1. The three different phenotypes were used to group and count H4 cells according to the number of inclusions. Scale bar is 20 µm.

We observed that the effect of aSyn inclusion formation may be dependent on the tyrosine phosphorylation status of aSyn. Both Y125F and S129G phosphorylation mutants showed a similar inclusion pattern compared to WT aSyn, but in almost 50% of the cells presenting the Y125F mutation, more than 10 inclusions were detected (Fig.18).
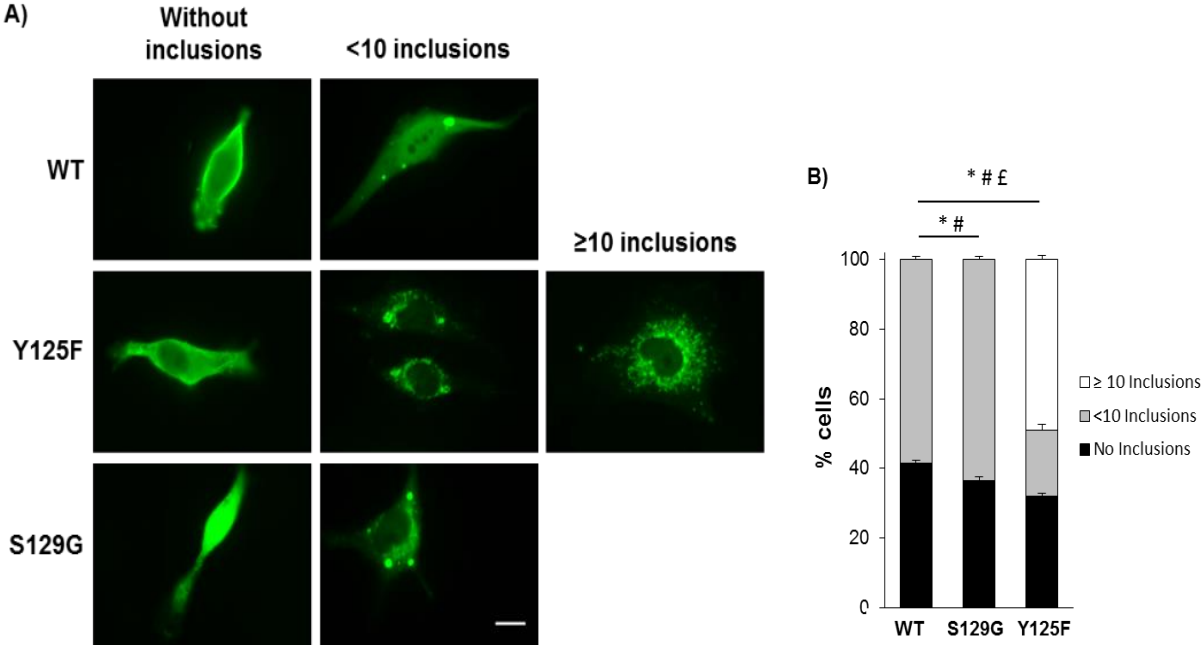
Figure 18. Effect of S129G and Y125F mutations on SynT inclusions formation in H4 cells. **(A)** Fluorescence microscopy of H4 cells co-transfected with SynT and synphilin-1, showing the WT, Y125F

and S129G SynT cellular localization (in green). Scale bar is 20 µm. **(B)** Percentage of cells showing no inclusions (black), less of 10 inclusions (grey) or 10 or more than 10 inclusions (white) per cell (*No inclusions, [#]<10 inclusions e [£]>10 inclusions ;p < 0.001; ANOVA and post-hoc Tukey test). Error bars indicate the standard error. Results shown are from one representative experiment from at least three independent experiments. Values represent the mean ± SD of three independent experiments.

In the control situation, about 60% of cells form less than 10 aggregates, while about 40% does not. However, the number of cells with aggregates increases with the expression of both S129G and Y125F in about 5% and 10%, respectively, with statistical significance. In cells expressing Y125F and S129G SynT, around 70% of cells displayed inclusions. However, these phosphorylation mutants presented a distinctive pattern of inclusions, as in the cells expressing Y125F SynT, 50% from the 70% of cells with aggregates, presented more than 10 aggregates, and, in some situations, an uncountable senary of aggregates was detected.

To further assess if the different percentage of inclusions were not caused due to different expression levels, western blot analysis was performed. Cells were transfected with WT, Y125F or S129G aSyn and synphilin-1, cell lysates were prepared and analyzed by immunoblotting. The expression levels of Y125F and S129G were similar when compared to those of WT aSyn (Fig.19).
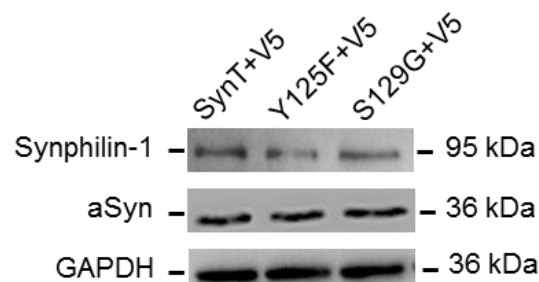

Figure 19. Immunoblotting analysis of aSyn Y125F and S219G mutants co-expressed with synphilin-1. Representative immunoblotting of H4 cells expressing Y125F and S129G phosphorylation mutants. Results shown are from one representative experiment from three independent experiments.

5. Discussion and Conclusions

Several neurodegenerative diseases display abnormal aggregation of aSyn, in the form of Lewy bodies, Lewy neurites, or glial cytoplasmic inclusions [18]. The poor understanding of the mechanisms involved in aSyn aggregation contributes to the controversy surrounding the role of aSyn aggregation in cytotoxicity and neurodegeneration.

Post translational modifications regulate the structural and functional properties of proteins in health and pathological conditions [70]. Currently, the role of phosphorylation in modulating the aggregation of aSyn is being investigated in PD. *In vivo*, usually less than 5% of aSyn is phosphorylated and this occurs predominantly at the C-terminal [71]. Phosphorylation of aSyn occurs in the serine residue 129 (S129), as well as in the tyrosine residue 125 (Y125) [72]; however, it remains unclear whether phosphorylation inhibits or promotes aSyn aggregation and toxicity.

The present work showed new evidence for a correlation between phosphorylation in two phosphorylation residues, Y125 and S129, and aSyn aggregation and cellular toxicity *in vivo* using a simple but powerful model organism, *S. cerevisiae*, and *in vitro* using a human cell model.

We used yeast strains carrying double insertions of the aSyn human gene in the genome. Specifically, we generated either the WT or phospho-resistant mutants Y125F and S129A or S129G of aSyn, with the C-terminal fused with GFP, and expressed it in yeast cells under regulation of an inducible promoter (*GAL1*).

To validate the results obtained in yeast, we used a human cell model of aSyn aggregation. Cells were co-transfected with a C-terminal tagged form of WT, Y125F or S129G aSyn (SynT) together with synphilin-1, an aSyn interacting protein that is known to potentiate aSyn aggregation [65].

Initially, the effect of Y125F mutation on the toxicity of aSyn was evaluated in yeast cells. We also analyzed the toxicity of a set of haploid strains with double and single insertions of Y125F aSyn, in parallel with a WT aSyn expressing strain. The results showed that the Y125F mutation did not significantly affect aSyn toxicity phenotype. However, it completely abolished the formation of aSyn foci in yeast cells. In contrast, in the H4 aggregation model, the Y125F mutation resulted in the formation of smaller but in a higher number of SynT aggregates per cell. Altogether, these results suggest that Y125 phosphorylation affects the formation of aSyn aggregates and may indicate that is involved in a mechanism that prevents the formation of larger aggregates.

These apparent conflicting results obtained in the two models might be due to the fact that we are using different forms of aSyn in yeast and mammalian cells. While in yeast we

are using aSyn fused to GFP, in mammalian cells we are using aSyn fused to a truncated version of GFP, which promotes aSyn aggregation. Besides this, the H4 model is based in the co-expression of SynT with synphilin-1, a protein that has no counterpart in yeast but when expressed in yeast is known to increase aSyn aggregation [68].

The results here obtained are in contradiction with the results obtained in a *Drosophila* model were it was observed that the phosphorylation of the Y125 suppresses aSyn aggregation [54]. However is important to mention that authors used a triple phospho-mutant Y125F-Y133F-Y135F [54]. Accordingly, it is not possible to exclude a role of the other tyrosine residues on the observed phenotype.

In the present work, we also studied the role of S129 phosphorylation on aSyn aggregation and toxicity. For that, we generated a new S129G aSyn yeast mutant in order to mimic the unphosphorylated form of S129, since the use of S129A aSyn mutant is controversial due to a putative effect of the mutation in the increment of aSyn aggregation [73]. Our data showed that expression of S129G aSyn results in a similar level of toxicity and foci formation when compared to S129A aSyn. Moreover, both mutants are more toxic and form more foci than WT aSyn, which it is known to be phosphorylated in yeast by endogenous kinases (Tenreiro et al., under revision). Thus, we concluded that the effects observed with the mutant S129A aSyn were due to the inability of the protein to be phosphorylated, and not due to structural differences induced by the alanine residue. Interestingly, the S129G mutation also increases the formation of SynT aggregates in the H4 model. Furthermore, we observed that the pattern of inclusions formation in S129G SynT was different from the one observed with WT SynT and Y125F SynT. Accordingly, blocking aSyn S129 phosphorylation has similar effects in both cellular models used.

Together, the new results here described demonstrate that different phosphorylated residues in aSyn result in different levels of aggregation and toxicity. From the two residues here studied known to be phosphorylated in aSyn, the one that was less characterized so far is the Y125. This is mainly due to the fact that it is difficult to detect significant levels of pY125 aSyn in human brain tissues, therefore little is known about this phosphorylation [55]. However, we believe that future work should be considered in order to better evaluate the role of phosphorylation at Y125 aSyn residue. The yeast model here developed for the first time could also be very useful for the follow up of this work, to study the role of Y125 phosphorylation as well as to identify the yeast kinases/phosphatases that are able to phosphorylate/dephosphorylate this residue. This strain could be also used to study the interplay between the residues Y125, S129 and S87 phosphorylation, another residue known to be phosphorylated in aSyn, which has also been poorly investigated [74]. In particular, the emerging opportunities to further continue the investigation might rely in more detailed studies on the toxicity and aggregation effects of Y125F SynT in H4 cells. Namely,

cytotoxicity could be evaluated using techniques such as Lactate dehydrogenase (LDH), Caspase 3, and Propidium iodide (PI). Regarding the inclusions nature formed in this cell line, this can be dissected analyzing the distribution of oligomeric species by sucrose gradient complemented by Triton X solubility assay of Y125F aSyn aggregates to check whether they are more or less insoluble than the aggregates of the WT aSyn.

The understanding of the molecular mechanisms underlying the interplay between aSyn phosphorylation and cytotoxicity might pave the way for the development of novel strategies for therapeutic intervention in PD and other synucleinopathies.

6. References

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8. Annexes

Annex 7.1

Table 3. Plasmids used in this study. During this work we used plasmids already available at the lab or produced specifically to this study.

Plasmid	Reference
Yeast Plasmids	
p426_GAL (empty)	in our lab
p426_GAL_aSynWT	in our lab
p426_GAL_aSynWT_GFP	in our lab
p426_GAL_aSyn Y125F_GFP	in our lab
pRS304_Gal_aSyn WT	in our lab
pRS304_Gal_aSyn S129G_GFP	in our lab
pRS304_Gal_aSynY125F_GFP	in our lab
pRS304_Gal_aSyn S129A	in our lab
pRS304_Gal_aSyn_A53T	in this study
pRS304_Gal_aSyn_E46K	in this study
pRS304_Gal_aSyn_A30P	in this study
pRS304_Gal_aSyn_S87A_GFP	in this study
pRS304_Gal_aSyn_S87E_GFP	in this study
pRS306_Gal_aSyn WT	in our lab
pRS306_Gal_aSyn S129G_GFP	in our lab
pRS306_Gal_aSyn S129A	in our lab
pRS306_Gal_aSyn_A53T	in this study
pRS306_Gal_aSyn_E46K	in this study
pRS306_Gal_aSyn_A30P	in this study
pRS306_Gal_aSyn_Y125F_GFP	in this study
pRS306_Gal_aSyn_S87A_GFP	in this study
pRS306_Gal_aSyn_S87E_GFP	in this study
Mammalian Plasmids	
psi synTY125F	in this study
psi synTS129G	in this study

Table 4. *S. cerevisiae* strains used in this study. W303.1A, W303.1B, VSY71, VSY72 and VSY73 were strains, already available (as shown in respective references). As to the other presented strains, they were all produced by us during this work and we hope to use them in further researches. Moreover, many other yeast strains, not presented in this table, were created and used in this work.

Yeast Strain	Description	Reference
Wild-type strains		
W303.1A	*MAT**a***; *can1-100*; *his3-11,15*; *leu2-3,112*; *trp1-1*; *ura3-1*; *ade2-1*	[75]
W303.1B	*MAT**α***; *can1-100*;*his3-11, 15*; *leu2-3, 112*; *trp1-1*; *ura3-1*; *ade2-1*	[75]
Haploid strains with single insertions		
SC149	W303.1A; ade2-1; can1-100; his3-11,15; ura3-1/GAL1pr-SNCA(S129G)-GFP::URA3	this study
SC172	W303.1A; ade2-1; can1-100; his3-11,15; ura3-1/GAL1pr-SNCA(S129A):URA3	this study
SC182	W303.1A; ade2-1; can1-100; his3-11,15;ura3-1/GAL1prSNCA(Y125F):URA3	this study
SC190	W303.1A; ade2-1; can1-100; his3-11,15; ura3-1/GAL1pr-SNCA(WT)::URA3	this study
Diploid strains with double insertions		
SC208	W303 *MAT**a**/**α**;* ade2-1/ade2-1; can1-100/can1-100; his3-11,15/his3-11,15; ura3-1/GAL1pr-SNCA(S129G)-GFP::URA3; trp1-1/GAL1pr-SNCA(S129G)-GFP::TRP1	this study
SC217	W303 *MAT**a**/**α**;* ade2-1/ade2-1; can1-100/can1-100; his3-11,15/his3-11,15; ura3-1/GAL1pr-SNCA(S129A):URA3; trp1-1/GAL1pr-SNCA(S129A)::TRP1	this study
SC220	W303 *MAT**a**/**α***; ade2-1/ade2-1; can1-100/can1-100; his3-11,15/his3-11,15;ura3-1/GAL1prSNCA(Y125F):URA3; trp1-1/GAL1pr-SNCA(Y125F)::TRP1	this study
SC212	W303 *MAT**a**/**α***; ade2-1/ade2-1; can1-100/can1-100; his3-11,15/his3-11,15; ura3-1/GAL1pr-SNCA(WT)::URA3; trp1-1/GAL1pr-SNCA(WT)::TRP1	this study

	Haploid strains with genomic double insertions	
VSY71	W303.1A *trp1-1::pRS304 TRP1+*; *ura3-1:: pRS306 URA3+*	[76]
VSY72	W303.1A *trp1-1:: pRS304 GAL1pr-SNCA*(WT)*-GFP TRP1+*; *ura3-1:: pRS306 pRS306GAL1pr-SNCA*(WT)*-GFP::URA3+*	[76]
VSY73	W303.1A *trp1-1:: pRS304 GAL1pr-SNCA*(S129A)*-GFP TRP1+*; *ura3-1:: pRS306 GAL1pr-SNCA*(S129A)*-GFP::URA3+*	[76]
	Tetrads obtained by sporulation of diploid strains	
SC244	MAT alpha; can1-100 his3-11 15 leu2-3 112 ade2-1; GAL1pr-syn S129G-GFP::TRP1; ura3-1	this study
SC245	MAT a; can1-100 his3-11 15 leu2-3 112 ade2-1; trp1-1; GAL1pr-syn S129G-GFP::URA3	this study
SC246	MAT a; can1-100 his3-11 15 leu2-3 112 ade2-1; GAL1pr-syn S129G-GFP::TRP1; GAL1pr-syn S129G-GFP::URA3	this study
SC247	MAT alpha; can1-100 his3-11 15 leu2-3 112 ade2-1; trp1-1; ura3-1	this study

Annex 7.3

Table 5. Growth media used for _S. cerevisiae_ in this study. Throughout this work, we used different growth media for _S. Cerevisiae_, each of them composed as presented in this table.

Media	Components	Composition
YPD	Glucose (Sigma)	20g/L;
	Yeast extract (Himedia)	20g/L
	Peptone (Himedia)	10g/L
YEP Raf	Raffinose 1% (Sigma)	10 g/L;
	Yeast extract 1% (Himedia)	10 g/L
	Peptone 2% (Himedia)	20 g/L
YEP Gal	Galactose 1% (Sigma)	10 g/L;
	Yeast extract 1% (Himedia)	10 g/L
	Peptone 2% (Himedia)	20 g/L
SC	Yeast nitrogen base without amino acids or (NH4)2SO4	6.7g/L;
	Glucose, raffinose* or galactose*	20g/L,10g/L*
	CSM (standard mixture of amino acids, vitamins and other components used to supplement auxothrophies)	