UNIVERSITÀ
DEGLI STUDI
DI PADOVA

PhD Programme In Molecular Medicine

Curriculum Biomedicine

Department of Molecular Medicine

Director Prof. Stefano Piccolo

32nd series

Generation of lentiviral vectors expressing chimeric NEDD4 ubiquitin ligases specifically targeting alpha-synuclein: a tool for studying Parkinson's disease pathogenesis and for the development of innovative therapeutic approaches

Tutor: Prof. Arianna Calistri

PhD student: Stefania Vogiatzis

Index

Abstract

Background. The main pathological features of Parkinson's disease (PD) are the death of dopaminergic neurons and the diffuse accumulation of alpha-synuclein (aS) aggregates in neurons. The NEDD4 E3 ubiquitin ligase has been shown to promote aS degradation by the endosomal/lysosomal route. Interestingly, NEDD4 is protective against human aS toxicity in evolutionary distant models. Furthermore, a small molecule able to activate NEDD4 functions, was neuroprotective in evolutionary distant models of aS toxicity. While activation of E3s cannot be easily obtained pharmacologically, their flexibility and the lack of “consensus” motifs for ubiquitin (Ub) conjugation allow the development of engineered Ub-ligases able to target proteins of interest. The aim of our study was to exploit chimeric Ub-ligases, named *ubiquibodies*, specifically targeting aS to prove the protective role of aS degradation pathway towards the development of innovative strategies and rescue the normal physiology of neurons.

Methods. To this end, we have developed lentiviral vectors encoding well characterized human scFvs fused in frame to the NEDD4 catalytic domain, in order to obtain enzymes that specifically ubiquitinate aS either in its monomeric and/or oligomeric form. We also generated two lentiviral vectors expressing wild type aS and a mutant form (A53T aS), known to play a role in PD pathogenesis, either alone or fused in frame with the reporter protein EGFP.

Furthermore, we adopted different in vitro models including human and murine dopaminergic cell lines and three different lines of human induced pluripotent stem cells (hiPSCs) derived from a healthy donor and from Parkinson’s patients, transduced with lentiviral particles expressing ubiquibodies, with the aim of analyzing their ability to rescue of hiPSCs-derived dopaminergic neurons physiological features.

Results And Conclusions. We are able to demonstrated that: i) the recombinant proteins are expressed in human embryonic 293T cells, as well as in the human and

mouse dopaminergic neuroblastoma, SH-SY5Y and MN9D in cell line respectively; ii) recombinant lentiviral particles transduce not only 293T, MN9D and SH-SY5Y cells, but also in human hiPSCs, neural stem cells (NSCs) and NSC-derived dopaminergic neurons; iii) ubiquibodies interacts in a specific manner with overexpressed aS also in dopaminergic cell lines; iv) a specific degradation of aS takes place in 293T cells transduced with one of the developed the chimeric Ub-ligases (Nac32HECTWT) that was selected based on the achieved results on its characteristics. Finally, experiment conducted in hiPSCs-derived NSCs indicate that aS overexpression and mutation have a negative effect on NSCs differentiation into dopaminergic neurons with an increase in cell mortality. The phenotype can be rescued by Nac32HECTWT expression.

The results achieved so far represent a starting point strongly supporting the validity of the strategy we intend to adopt in order to obtain a specific degradation of aS.

Summary

Parkinson's disease (PD) is the second most common neurodegenerative disorder after Alzheimer's (Pankratz & Foroud, 2007) and is characterized by impaired motor symptoms (resting tremor, stiffness, postural instability) accompanied by non-motor clinical manifestations (depression, apathy, sleep disorders).

Although aging is the most important single risk factor for PD (Schapira & Jenner, 2011), the disease has a complex and multifactorial etiology, resulting from the interaction of genetic and environmental factors. Some studies correlate industrialization and exposure to herbicides (e.g. paraquat) and insecticides (e.g. rotenone) with a high risk of developing PD (reviewed Dauer & Przedborski, 2003). In addition, several researches on PD have identified mutations responsible for certain familiar forms (reviewed Pankratz & Foroud, 2007). The two main changes present in patients with this disorder are the degeneration of the dopaminergic neurons of the *Substantia Nigra pars compacta* and the presence of protein aggregates, better known as Lewy bodies (LB), cytoplasmatic inclusions mainly constituted by alpha-synuclein protein (aS). The fact that these aggregates contain poorly folded aS, along with the finding that specific mutations within the aS encoding gene (*SNCA*) are responsible for rare family forms of PD, support the hypothesis of a involvement of aS in the pathogenesis of the disease (Dehay et al., 2015). aS is a protein of 140 amino acids (aa) abundantly expressed in neurons. Although its physiological functions have not been well clarified yet, its location to the presynaptic terminals strongly suggest for gives to the protein a role in synaptic transmission (Bendor, Logan, & Edwards, 2013). Moreover, aS, which is inherently disordered protein, usually in monomeric conformation, according to some studies can adopt oligomeric or fibrillary forms especially in the presence of certain pathological conditions: the A53T mutation, for example, seems accelerate the formation of fibrils *in vitro* (Conway, Harper, & Lansbury, 1998). The destabilization

of the physiological conformation would result in cytotoxicity, although the exact mechanism of action is not entirely clear yet.

aS, like all proteins, is subject to turn over by the main cellular degradation systems, that participate to the overall quality control system of proteins and are responsible for the removal of unnecessary misfolded polypeptides (Villar-Piqué, Lopes da Fonseca, & Outeiro, 2016). The reduction of aS degradation is widely recognized as one of the factors involved in PD the pathogenesis (Lehtonen, Sonninen, Wojciechowski, Goldsteins, & Koistinaho, 2019). On the other hand, pathogenic aS itself can compromise these degradation systems, exacerbating the its own accumulation and, thus, triggering a vicious circle (Martinez-Vicente & Vila, 2013) with neurotoxic potential (Xilouri, Brekk, & Stefanis, 2013).

Although the discovery of levodopa has revolutionized PD treatment, the therapies available today are aimed solely at alleviating symptoms and are not able to block or delay neurodegeneration (Dauer & Przedborski, 2003).

The main obstacle to the development of effective disease therapy is the lack of the understanding of the molecular mechanisms underlying PD pathogenesis. For these reasons and in view of the progressive ageing of the population, studies aimed at answering the questions remaining on this neurodegenerative disease pathogenesis/progression are highly needed.

The aim of this project was to develop an innovative strategy to address the role played in this context by the aggregation of aS, generating a potential tool achieve a specific degradation of aS aggregates.

Two important studies have contributed in particular to the design of this experimental project. The first one, conducted by Tofaris and collaborators (Tofaris et al., 2011), showed that aS, associated with lipid membranes, can be degraded by the endo-lysosomal system and that NEDD4, an ubiquitin ligase, involved in the ubiquitination of associated with membrane proteins, can ubiquitinate aS through the endo-lysosomal pathway.

In addition, those Authors demonstrated that NEDD4 is overexpressed in brain regions of Lewy pathology and they identified in its encoding sequence a single nucleotide polymorphism associated with an increased risk of PD onset (Tofaris et al., 2011).

The second study is conducted by Portnoff and collaborators (Portnoff et al., 2014), showing that it is possible, taking advantage of the modular characteristics of a specific ubiquitin ligase (CHIP), to redirect it towards the degradation of a specific target. In particular, those Authors have successfully developed a chimera that combines CHIP enzyme activity with a specific intrabodies (scFvs) directed towards the target protein of interest. At the same time, the new chimera loses the ability to recognize its physiological target (Portnoff et al., 2014).

Based on these studies, we decided to develop and validate new recombinant ubiquitin ligases based on NEDD4 and direct towards aS.

NEDD4 has a modular structure consisting of an N-terminal C2 domain that determines the specificity of binding to the substrate, four WW domains involved in protein-protein interactions, and an HECT (homologous to E6-AP carboxyl terminus) catalytic domain at the C-terminal (Scheffner & Kumar, 2014).More importantly working on the budding of the human immunodeficiency virus (HIV), Göttlinger and collaborators had previously demonstrated that the ubiquitination activity of NEDD4 can be directed towards a protein of interest by fusing its HECT domain with heterologous proteins able to bind that selected target (Weiss et al., 2010).

Therefore, we decided to fuse the NEDD4 catalytic domain to a series of scFvs specifically recognize human aS. The developed "*ubiquibodies*" should promote the ubiquitination and the consequent degradation of the aggregated forms of aS through the endo-lysosomal pathway.

In particular, three previously characterized scFvs were selected (Lynch, Zhou, & Messer, 2008): NAC32, directed against the non-amyloid (NAC domain) component of the human aS, d5e which binds exclusively the oligomeric form of aS and d10, which is panspecific. These scFvs were fused with a GSGSG spacer peptide, which

provides greater flexibility to the enzyme, followed by the catalytic domain of NEDD4, HECT, and the hemagglutinin epitope sequence (HA).

For each ubiquibody, a control was developed in which the HECT catalytic domain is made inactive due to a known inactivating mutation ($C_{867}S$) within the enzyme active site (Davies et al., 2014).

All chimeras and their controls were cloned into a third-generation self-inactivating (SIN) lentiviral vectors (LV). The choice to use LV is justified by the fact that those vectors show high efficiency of gene transfer *in vivo* and a stable and long-lasting expression of the transgenes in multiple target tissues, including the brain tissue (Dull et al., 1998). The generated lentiviral vectors expressing ubiquibodies, were transfected into human kidney embryonic 293T cells to test their expression and function.

Results confirmed that each ubiquibody is expressed in a single structurally stable protein in the cellular environment. Next, in order to validate the vectors from a functional point of view, we set up appropriate cellular models, characterized by the presence of aS aggregates.

To this end, as the overexpression of wild type aS (Kalia & Lang, 2015) (Dehay et al., 2015) or its mutation at the alanine 53 to threonine (A53T) leads to the protein aggregation (Villar-Piqué et al., 2016) (Chaudhuri & Paul, 2006). First of all, we generated lentiviral vectors expressing both forms of the protein under the transcriptional control of a strong promoter (the human cytomegalovirus promoter). To facilitate the localization within the cell environment, the sequence of the epitope FLAG or the reporter green fluorescent protein (GFP) were added in frame to aS.

Next, recombinant lentiviral particles were generated. Of note, we selected as an envelope the glycoprotein G of the vesicular stomatitis virus (VSV) that gives to the recombinant particles a wide tropism and, in particular, enable them to efficiently transduce neurons and brain derived cells, including neural stem cells (NSCs), which are relevant to this project. We were able to obtain recombinant lentiviral vectors expressing ubiquibodies and aS, capable of transducing different cell types of our

interest, including human induced pluripotent stem cells (hiPSCs) and neural stem cells (NSCs). Also, we demonstrated that in murine dopaminergic cell line, in which aS is overexpressed, the ubiquibodies are not only well expressed but also, by the proximity ligation assay (PLA), they bind aS in a specific manner.

Next, we tested the ability of ubiquibodies to bind and ubiquitinate aS. To this end, immunoprecipitation, co-immunoprecipitation and proximity ligation assay (PLA) experiments were carried out.

From the obtained results, we were able to show that not only ubiquibodies bind aS but also that they lead to its ubiquitination and degradation.

Studies conducted by Oliveira and collaborators have shown that high levels of aS, caused by a triplication of the *SNCA* gene, have a negative impact on hiPSC differentiation and subsequent maturation into NSCs (Oliveira et al., 2015)

Thus, we obtained three lines of hiPSC, one from a healthy donor, and two from PD's patients, one characterized by *SNCA* triplication and the other one bearing A53T aS. These hiPSCs were differentiated into NSC. Next, we confirmed that aS aggregation due to its overexpression or mutation interferes with an NSC differentiation towards mature neurons also under our experimental conditions.

First of all, we observed a statistically significant increase cell mortality in NSCs derived from PD patients and in which aS is overexpressed upon transduction with generated recombinant lentiviral particles. Next, we differentiated NSCs into dopaminergic progenitors (DPs) and, once differentiated, we transduced them with recombinant lentiviral particles expressing ubiquibodies. Cells were then analyzed by cytofluorimetry to evaluate the percentage of cells positive to the FOXA2 that is a typical differentiation marker for DPs.

The results allowed us to conclude that the expression of ubiquibodies rescue the differentiation capacity of aS mutant or overexpressing NSCs.

In conclusion, these results show that, from a structural point of view, NEDD4 is suitable to generate recombinant ubiquitin ligases targeting aS especially in its

aggregated forms, that interfering with aS aggregation has functional consequence in relevant in vitro models.

Sommario

Il morbo di Parkinson (MP) rappresenta il secondo disturbo neurodegenerativo più comune dopo l'Alzheimer (Pankratz & Foroud, 2007) ed è caratterizzato dall'alterazione del sistema motorio (tremore a riposo, rigidità, instabilità posturale) accompagnata da manifestazioni cliniche non motorie (depressione, apatia, disturbi del sonno). Anche se l'invecchiamento rappresenta il fattore di rischio singolo più importante (Schapira & Jenner, 2011), la malattia ha un'eziologia complessa e multifattoriale, risultante dall'interazione di fattori genetici e ambientali. Alcuni studi correlano l'industrializzazione e l'esposizione ad erbicidi (paraquat) e insetticidi (rotenone) con un elevato rischio di sviluppare il MP (Dauer & Przedborski, 2003). Inoltre, ricerche condotte su casi con caratteristiche di familiarità hanno permesso di individuare mutazioni responsabili di alcune forme familiari (Pankratz & Foroud, 2007). I due cambiamenti principali presenti nei pazienti affetti da tale disturbo sono la degenerazione dei neuroni dopaminergici della *Substantia Nigra pars compacta* e la presenza di aggregati proteici, meglio noti come corpi di Lewy (CL), costituiti principalmente dalla proteina cellulare alfa-sinucleina (aS). La scoperta che questi aggregati contengono depositi di aS mal-ripiegata, insieme alla constatazione che mutazioni specifiche a carico del gene *snca* codificante aS sono responsabili di rare forme familiari di MP, sostengono l'ipotesi di un coinvolgimento di aS nella patogenesi della malattia (Dehay et al., 2015). aS è una proteina di 140 amminoacidi (aa) abbondantemente espressa nei neuroni; nonostante la sua funziona fisiologica non sia stata ancora ben chiarita, la sua localizzazione ai terminali presinaptici attribuisce alla proteina un ruolo nella trasmissione sinaptica (Bendor et al., 2013). Sebbene sia stata definita una proteina intrinsecamente disordinata, presente in forma monomerica, secondo alcuni studi, può adottare forme oligomeriche o fibrillari soprattutto in presenza di alcune condizioni patologiche: la mutazione A53T, ad esempio, sembra accelerare la formazione di fibrille *in vitro* (Conway et al., 1998). La destabilizzazione della struttura ad α-elica sarebbe direttamente correlata alla sua tossicità, anche se il meccanismo d'azione non è ancora ben chiaro.

aS, come tutte le proteine, è soggetta a *turn over* da parte dei principali sistemi di degradazione, i quali partecipano al controllo di qualità delle proteine cellulari e sono responsabili della rimozione di polipeptidi non necessari, malripiegati e danneggiati (Villar-Piqué et al., 2016). L'alterazione di questi sistemi, in particolare la riduzione della degradazione aS, è ampiamente riconosciuta come uno dei fattori più implicati nella patogenesi della MP. D'altra parte, l'aS patogena stessa può compromettere questi sistemi, aggravando l'accumulo di aS e innescando così un circolo vizioso (Martinez-Vicente & Vila, 2013) e innescando cosi, il suo potenziale neurotossico (Xilouri et al., 2013).

Anche se la scoperta della levodopa ha rivoluzionato il trattamento della PD, le terapie oggi disponibili mirano esclusivamente ad alleviare i sintomi e non sono invece in grado di bloccare o ritardare la neurodegenerazione (Dauer & Przedborski, 2003).

Il principale ostacolo allo sviluppo di una terapia efficace contro la malattia è la scarsa comprensione dei meccanismi molecolari alla base della patogenesi. Per questi motivi e in considerazione del progressivo invecchiamento della popolazione, la ricerca di risposte alle domande ancora irrisolte sulla patogenesi del morbo di Parkinson è più necessaria.

Lo scopo di questo progetto è finalizzato allo sviluppo di una strategia innovativa che permetta di ampliare le conoscenze sulla patogenesi del morbo di Parkinson, con particolare attenzione al ruolo coperto in questo contesto dall'aggregazione di aS, ed inoltre di sviluppare un nuovo approccio terapeutico per la degradazione di aggregati di aS.

Due importanti studi hanno contribuito in particolare alla concezione di questo progetto sperimentale. Il primo, condotto da Tofaris e collaboratori (Tofaris et al., 2011), ha dimostrato che aS, associata alle membrane lipidiche può essere degradata dal sistema endolisosomiale e che NEDD4, un' ubiquitina ligasi coinvolta nella ubiquitinazione delle proteine associate alla membrana, può ubiquitinare aS ed inviarla cosi, in modo specifico, alla degradazione endolisosomiale. Inoltre, NEDD4

è stato sovraespresso nelle regioni cerebrali che presentavano patologia Lewy e a livello della sua sequenza di codifica è stato identificato un polimorfismo a singolo nucleotide associato a un maggiore rischio di insorgenza della MP (Tofaris et al., 2011).

Il secondo studio è ad opera di Portnoff e collaboratori (Portnoff et al., 2014), i quali hanno dimostrato come sia possibile, sfruttando le caratteristiche modulari dell' ubiquitina ligasi CHIP, indirizzare quest'ultima verso la degradazione di una proteina bersaglio specifica. In particolare, gli autori hanno sviluppato con successo una chimera che combina l'attività enzimatica di CHIP con la specificità di indirizzare gli *intrabodies* (scFv) diretti verso la proteina bersaglio di interesse. Allo stesso tempo, la nuova chimera perde la capacità di riconoscerei suoi obiettivi fisiologici (Portnoff et al., 2014).

Nel complesso, questi dati sono stati fonte di ispirazione per il presente progetto in quanto suggeriscono che l'attivazione di NEDD4 contro aS può essere uno strumento utile per contrastare l'accumulo di proteine. Tuttavia, dal momento che non è facile controllare NEDD4 farmacologicamente, si è deciso di ricorrere a una strategia alternativa.

Pertanto, in questo progetto di ricerca, si è quindi deciso di valutare se ciò che è stato realizzato da Portnoff e collaboratori con l' ubiquitina ligasi CHIP, fosse applicabile all'ubiquitina ligasi NEDD4. Quest' ultima possiede una struttura modulare costituita da un dominio C2 a N-terminale che determina la specificità di legame al substrato, quattro domini WW di interazione proteina-proteina e un dominio catalitico HECT al C-terminale (Scheffner & Kumar, 2014). Inoltre, Göttlinger e collaboratori avevano già dimostrato come sia possibile fondere il dominio HECT di NEDD4 con proteine eterologhe per portare a una specifica ubiquitinazione della proteina Gag strutturale del virus dell'immunodeficienza umana (HIV) (Weiss et al., 2010).

Si è quindi deciso di progettare NEDD4 fondendo il suo dominio catalitico HECT con una serie di scFv in grado di riconoscere specificamente l'aS umana, con l'obiettivo di ottenere chimere chiamate ubiquibodies in grado di promuovere la

ubiquitinazione e la conseguente degradazione forme tossiche e aggregati di aS attraverso la via endolisosomiale.

In particolare, sono stati selezionati tre scFv precedentemente caratterizzati (Messer & Joshi, 2013): NAC32, diretto contro la componente non-amiloide (dominio NAC) dell'aS umano; d5e, che lega esclusivamente la forma oligomerica di aS e d10, che è panspecifico. Tali scFv sono stati fusi con un peptide distanziale GSGSG, che fornisce una maggiore flessibilità all'enzima, seguito dal dominio catalitico di NEDD4, HECT, e dalla sequenza dell' epitopo dell' emoagglutinina (HA).

Per ogni ubiquibody è stato sviluppato il suo relativo controllo costituito in cui il dominio catalitico HECT è reso inattivo a causa di una nota mutazione inattivante (C867S) del sito attivo dell'enzima (Davies et al., 2014).

Tutte le chimere e i relativi controlli sono stati clonati in vettori lentivirali auto-inattivanti di terza generazione. La scelta di utilizzare vettori lentivirali è giustificata dal fatto che essi hanno mostrato un'elevata efficienza nel trasferimento genico *in vivo* e un'espressione stabile e duratura del transgene in più tessuti bersaglio, compreso quello cerebrale (Dull et al., 1998). Gli ubiquibodies infatti, sono sottoposti al controllo trascrizionale di un promotore forte, quello del citomegalovirus umano (CMV), e questo ci permette di avere un efficiente trascrizione e quindi alte concentrazioni di proteine all'interno della cellula.

Pertanto i vettori lentivirali, esprimenti gli ubiquibodies, sono stati trasfettati in cellule embrionali renali umane 293T per valutare la loro espressione e funzionalità.

I risultati hanno quindi confermato che ciascuno degli ubiquibodies è espresso in una singola proteina chimerica strutturalmente stabile all'interno dell'ambiente cellulare, con i pesi molecolari attesi sulla base della somma dei pesi dei singoli moduli introdotti nella fase di clonazione.

Per testare gli ubiquibodies realizzati e per comprendere meglio la MP, è stato necessario avere un modello cellulare adeguato che riproduca i segni tipici della patologia, vale a dire la presenza di aggregati aS.

Con l'obiettivo di rispondere a questa esigenza, sono stati costruiti vettori lentivirali

che esprimono aS, sia in forma WT, poiché la sovraespressione del gene da sola è sufficiente a causare il morbo di Parkinson nei pazienti (Dehay et al., 2015; Kalia & Lang, 2015), sia nella nota forma mutata A53T che è soggetta all'aggregazione (Chaudhuri & Paul, 2006; Villar-Piqué et al., 2016). Per facilitare la localizzazione all' interno delle cellule e seguire il suo corso, è stata fusa ad aS la sequenza dell' epitopo FLAG o la proteina GFP.

Questi nuovi costrutti, come per i precedenti, sono stati clonati all' interno dei vettori lentivirali per esprimere proteine stabili in un ambiente cellulare complesso come i neuroni. Anche questi vettori si sono dimostrati in grado, una volta trasfettati in cellule 293T, di portare all'espressione dei rispettivi transgeni.

Inoltre, tutti vettori lentivirali esprimenti sia le diverse forme di aS, sia gli ubiquibodies sono stati co-trasfettati con i plasmidi di packaging (pMDL, pVSV-G, pRSV-Rev), in cellule 293T al fine di produrre particelle lentivirali ricombinanti pseudotipizzate, con un involucro caratterizzato dalla glicoproteina G del virus della stomatite vescicolare, VSV. Questo involucro dà alle particelle un ampio tropismo e, in particolare, le rende in grado di trasdurre in modo efficiente le cellule di origine nervosa, tra cui le NSC, utili allo scopo del progetto.

Abbiamo voluto testare la capacità degli ubiquibodies di legare e ubiquitinare in maniera specifica aS. Per raggiungere questo obiettivo sono stati fatti degli esperimenti di immunoprecipitazione e co-immunoprecipitazione, utilizzando le particelle virali esprimenti gli ubiquibodies, aS e un plasmide esprimente l' ubiquitina.

Dai risultati ottenuti, abbiamo confermato che non solo gli ubiquibodies legano aS ma anche che portano alla sua ubiquitinazione e degradazione.

A questo punto, prendendo in considerazione tutti i dati ottenuti, abbiamo voluto valutare la capacità delle particelle lentivirali prodotte di trasdurre le cellule umane staminali pluripotenti indotte (hiPSC) e le NSC, ottenute dalle prime. Infatti, hiPSC e NSC che esprimono aS rappresentano un buon modello *in vitro* per lo studio delle strategie per interferire con l'accumulo della proteina stessa.

Recenti studi condotti da Oliveira e collaboratori hanno dimostrato che alti livelli di aS, causati nel loro caso specifico da una triplicazione del gene SNCA, hanno un impatto negativo sul differenziamento delle hiPSC e sulla successiva maturazione verso le NSC (Oliveira et al., 2015).

Per confermare tali dati, tutti gli esperimenti da noi progettati sono stati effettuati su tre linee di hiPSCs, una proveniente da un donatore sano, e due provenienti da pazienti affetti dalla MP, di cui una possiede una triplicazione del gene SNCA e l' altra possiede la mutazione A53T dell' aS. Una volta differenziate e caratterizzate le hiPSCs in NSC, le abbiamo trasdotte con le particelle lentivirali che abbiamo sviluppato e abbiamo valutato se si verificano interferenze con il processo di differenziazione nei neuroni maturi.

I risultati hanno confermato che c'è un incremento della mortalità delle cellule derivanti da pazienti affetti dalla MP e nel controllo positivo sovraesprimente aS.

Partendo da questo dato, abbiamo iniziato a sviluppare un esperimento in cui tutte le linee di NSC sono state differenziate in progenitori dopaminergici (DP) e, una volta differenziate, sono state trasdotte con gli ubiquibodies. Tali cellule quindi sono state sottoposte ad analisi citofluorimetrica per valutare la percentuale di cellule positive al marcatore del differenziamento FOXA2 dei DP.

I risultati ottenuti ci hanno permesso di concludere che si ha una maggiore percentuale di cellule differenziate quando queste sono trattate con gli ubiquibodies WT, rispetto ai loro controlli.

Tuttavia i risultati ottenuti, per quanto buoni, riguardano una bassa percentuale di cellule e pertanto, sono da considerare preliminari. Questo risultato, non è sorprendente, data la difficoltà nota nella manipolazione delle cellule staminali in differenziamento. In futuro saranno pianificati esperimenti per l' ottimizzazione della quantità di particelle lentivirali ricombinanti da utilizzare al fine di ottenere, da una parte, una buona efficienza della trasduzione e dall' altra, non causare citotossicità.

In conclusione, questi risultati mostrano che dal punto di vista strutturale NEDD4 si presta bene all'ingegneria in quanto le chimere si sono dimostrate stabili e funzionali

nell'ambiente cellulare. Ancora più importante, è stato confermato che in queste chimere l'attività catalitica dell'enzima è conservata e soprattutto che è specificamente diretta verso l'aS in forma monomerica e / o oligomerica.

Infine, sarà fondamentale dimostrare che gli ubiquibodies sviluppati sono in grado di ripristinare la vitalità, morfologia e funzionalità dei neuroni dopaminergici, in cui è presente l' accumulo di aS.

Ottimizzeremo tutti questi esperimenti al fine di ottenere un sistema efficiente per passare a modelli *in vivo* appropriati.

1. Introduction

1.1 Parkinson's Disease: main clinical manifestation

Parkinson's Disease (PD) is the second most frequent neurodegenerative disease after Alzheimer's, affecting more than 1% of people over 55 years and more than 3% of over 75 years old (Dehay et al., 2015).

PD is characterized by the death of dopaminergic neurons in the *pars compacta* of the *substantia nigra* of the brain resulting in a deficit of dopamine at the base ganglia (figure 1), in which dopamine is the neurotransmitter that acts as a messenger between the *substantia nigra* and the other parts of the nervous system that control voluntary movements.

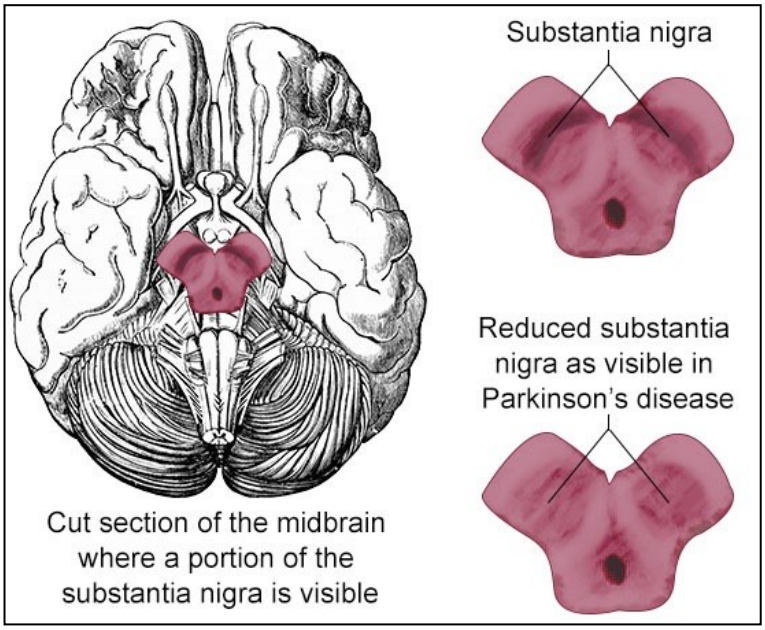


Figure 1**. Degeneration of substantia nigra has been associated with Parkinson's disease.** In this condition, the neurons in the substantia nigra break down, which leads to decrease dopamine levels that is responsible for causing loss of motor skills. Thus, patients face difficulty in moving and maintaining balance.

Its deficiency causes, among other clinical manifestation, motion disorders that compose classic motor symptoms such as resting tremor, trailed step, curved posture, slowness of movements, falls, decreased voice volume, dysphagia. In addition, PD is

also associated with several non-motor symptoms mainly affecting the central nervous system (CNS), such as dementia.

In 1817 the British physician James Parkinson wrote his treatise "*An Essay on the Shaking Palsy*" in which, for the first time, he describes the classic PD motor symptoms including resting tremor, festination, shuffling, curved / flexed posture, slowness of movement, falls, decrease of voice volume, dysphagia and loss of saliva. He also describes the slow progression of the disease over time and identifies some non-motor symptoms such as sleep and bowel disorders (Parkinson, 2002).

Parkinson's, however, did not recognize dementia as a symptom, probably because in the early nineteenth century patients suffering from the disease did not reach the age at which neurocognitive decline typically occurs (Fahn, 2015).

Due to the wide heterogeneity of motor symptoms found in PD patients, the disease has sometimes been classified into two subtypes, based on empirical clinical data:

• tremor-dominant Parkinson's disease, in which the motor symptoms, except for the tremor, are lacking;

• non-dominant tremor Parkinson's disease in which the akinetic syndrome, gait disturbances and postural instability prevail.

Furthermore, there is a subgroup of patients presenting a mixed phenotype with motor symptoms of comparable intensity.

The subtypes differ in the progression and prognosis of the disease and could have different etiology and pathogenesis. In particular the tremor-dominant subtype is considered more benign due to the slower progression of disability and less impairment of general functions compared to the tremor-non-dominant subtype.

Before the onset of typical motor symptoms, in which alterations in smell, constipation, depression and excessive daytime sleepiness are manifested, the pathogenic process responsible for the disease is presumably already underway, involving regions of the peripheral and central nervous system, in addition to the dopaminergic neurons of the *substantia nigra*.

With the progression of the disease a worsening of symptoms is shown, also linked to the long-term therapy which leads, in late stages, to postural instability, falls, dysphagia, language dysfunction, psychosis and in roughly 83% of patients affected for at least 20 years, dementia (Kalia & Lang, 2015).

1.2 Parkinson's disease pathogenesis

PD is a multicentric neurodegenerative disease in which the development of pathological abnormalities stars with changes in the dorsal motor nucleus as well as in the olfactory bulbs nucleus.

In 2002, for the first time, the anatomist Heiko Braak developed a staging system that classifies the disease progression in specific steps (Braak et al., 2003). He also hypothesized that an unknown pathogen (virus or bacterium) could be responsible for the initiation of sporadic PD; once this pathogen enters in the gut, via the nose or gastrointestinal nervous systems, would travels and spread into the CNS. Braak pathogenesis steps, are described as follows and summarized in figure 2:

- Braak stage 1-2. The first sign of the pathology is the loss of olfactory function even before the onset of dopaminergic signs and it may serve to define an at-risk population (Del Tredici, Rüb, de Vos, Bohl, & Braak, 2002).
- Braak stage 3-4. This stage is characterized by cell loss and inclusion formation, named Lewy body, in the *locus coeruleus* of the *substantia nigra pars compacta* (Ponsen et al., 2004).
- Braak stage 5-6. At this stage there is also degeneration in the pedunculopontine nucleus, the dorsal raphe nuclei, and the hypothalamus and the neurodegeneration in those regions already affected (Del Tredici et al., 2002).

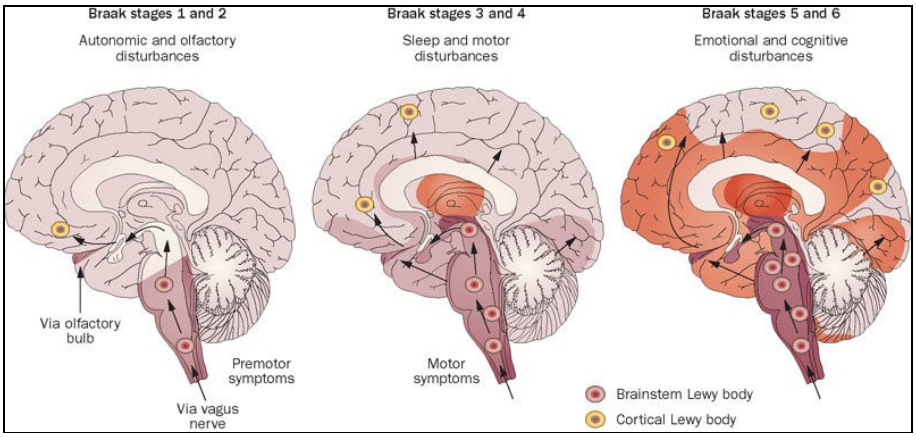


Figure 2. **Illustration of the Braak staging in Parkinson's disease (Petersen, 2017).**

According to Braak, the clinical diagnosis of Parkinson's disease is made only when the patient reaches stage 5-6. The implication is that is very rare to initiate therapy prior to stage 5 and so it is difficult to prevent PD progression before dopaminergic features became severe.

Currently available PD therapies are exclusively aimed at the treatment of its symptoms. In fact, drugs aimed at increasing dopamine intracerebral concentrations, such as levodopa, or those stimulating its receptors remain the hub for the treatment of motor symptoms and significantly reduce both the morbility and the mortality and increase the quality of life (Schapira & Jenner, 2011). Furthermore, long-term pharmacological treatments results in resistance to drug treatment especially during the late phase of the disease (Thanvi & Lo, 2004).

Therefore, an important aspect of PD research is represented by the development of new therapeutic approaches aimed at slowing down or blocking the neurodegenerative process.

Importantly, a treatment effective on non-dopaminergic systems, would prevent the clinical evolution of the non-motor complications that characterize the advanced stages of PD.

1.2.1 PD cellular signs: the Lewy bodies

In 1912, F.J.H. Lewy first described the cytoplasmic protein inclusions which became the hallmark of PD, nowadays known as Lewy bodies (LB).

LB are protein aggregates distinguishable in:

• classics of the brain trunk: spherical neuronal cytoplasmic inclusions with a diameter of 8-30 μm, hyaline eosinophilic nucleus, concentric lamellar bands, and a thinly colored halo. They are granular-filamentous structures composed of 7-20 nm filaments radially arranged, associated with electron-dense granular material and vesicular structures, packed in a dense core;

• cortical: round eosinophilic structures, without halo, structurally unorganized, composed of wide filaments of 7-27 nm without a central nucleus.

The LB's molecular composition was defined thanks to two important discoveries occurred in the same year, the 1997. First of all the A53T missense mutation with the gene (*SNCA*) encoding for the cellular protein alpha-synuclein (aS) was associated to a dominant autosomal form of PD. Secondly it was demonstrated that in idiopathic PD cases, LB and Lewy neurites are highly immunoreactive for aS (Schapira & Jenner, 2011).

Although aS is the major component of LB, immunohistochemical studies have confirmed the presence of more than 90 additional molecules, including tau, parkin, ubiquitin, ubiquitin activating enzyme (E1), ubiquitin conjugating enzyme (E2), ubiquitin-ligase (E3), proteasome subunit, ubiquitin-proteasome related proteins, molecular chaperones, LRRK2, PINK1 (Houlden & Singleton, 2012).

Furthermore, LBs are not located only to the brain but they may also be found in the spinal cord and peripheral nervous system.

Finally, it is important to mention that the main protein of LB, aS, can give rise to various types of aggregates such as small point or filamentous structures, presynaptic deposits and oligomers consisting of 2-100 monomers of aS. These structures,

especially the oligomeric forms of aS, can be toxic to the neurons and would thus be involved in neurodegeneration (Perrett, Alexopoulou, & Tofaris, 2015).
Based on these observations, the currently hypothesis is that LB would be formed as the results of detoxification process untaken by the cells towards aS aggregates, suggesting an important role of this protein in the pathogenesis of the disease (Dexter & Jenner, 2013; Wakabayashi, Tanji, Mori, & Takahashi, 2007). This aspect along with additional information on aS are discussed later on the Introduction (paragraph 1.4).

1.3 Risk Factors

PD is still seen as a complex multifactorial disease resulting from the combination of environmental and genetic factors, both of which play a role in the development of the disease.
Among the various risk factors, aging process, is the predominant one; the prevalence and incidence of the disease, in fact, increase almost exponentially with age and show a peak after the age of 80. This effect is therefore explained as a result of increasing deterioration and malfunctioning of physiological and biochemical cellular processes that make dopaminergic neurons more vulnerable to damage.
The environment also seems to play a role in the development of PD. Various risk factors are described, such as industrialization, vegetable toxins, bacterial and viral infections, exposure to herbicides (paraquat), insecticides (rotenone), organic solvents, carbon monoxide and disulfide (Pankratz & Foroud, 2007).
While the environmental factors described above are modifiable, the environmental exposure change over time and not all factors have been identified yet (Chen, 2018). However, several studies indicate that there is an increasing trend in PD incidence over the past few decades, that indicate a crucial role of environmental factors (Rodolfo Savica et al., 2017).

It has been also suggested that toxic factors, as pesticides, pollution and viruses and) may initiate PD pathogenesis at the olfactory bulb or at gut enteric environment. From these anatomical sites, directly interacting with the environment, these factors spread to the CNS via the olfactory or vagus nerve (Chen & Ritz, 2018).

Interestingly it has been demonstrated that misfolded aS, present in the environment as air pollution, can spread in the CNS via the olfactory system (Rey et al., 2016). The prion-like propagation theory for PD suggests that this up-taken misfolded aS would lead aggregation of natively unfolded aS that will spread over long distances through the axons, starting from the olfactory regions (Beach et al., 2009; Braak et al., 2003) and several months later reaching the CNS. This would be the so called prodromal phase of PD, mainly characterized by nonmotor signs. Prodromal PD would be the ideal stage at which to apply neuroprotective and disease-modifying strategies.

1.3.1 The Gut microbiome and PD

Up to 80% of Parkinson's disease patients report gastrointestinal problems, like dysphagia, in many cases preceding the development of motor symptoms (R. Savica et al., 2009). In addition, changes in composition of the gut microflora, and subsequently in the genomes of the microbial commensal community, defined as *microbiome*, have also been identified in PD patients, suggesting that, these modifications could be used as biomarker of disease progression (Miraglia & Colla, 2019).

The gut–brain axis is a two-way communication system between the gastrointestinal tract and the CNS that uses biochemical signals, like neural, endocrine and immunological signals.

The microbial commensal community, known as microbiota, can send several signals directly to the CNS or indirectly, via the enteric nervous system (ENS) through the synthesis of neurotransmitters or neurochemical-like precursors.

The altered microbial composition, called microbial dysbiosis, have been observed in human PD patients, correlating to a change in the relative abundance of bacterial genera or species, rather than to the appearance or disappearance of a specific microbial population. Several studies reported a decrease in the level of genus *Faecalibacterium*, *Coprococcus* and *Blautia*, *Prevotella* and other genus of the *Prevotellaceae* family in PD patients compared to healthy controls and an increase of bacteria genera like *Lactobacillus*, *Bifidobacterium* and the family of *Verrucomicrobiaceae* (S. Hasegawa et al., 2015; Hill-Burns et al., 2017; Petrov et al., 2017; Unger et al., 2016)

In the early stages of PD, there is an increase of the intestinal permeability due to a defective gut barrier function, that allows the translocation of microorganisms and microbial products, like lipopolysaccharide (LPS) or short chain fatty acids (SCFA) and also aS aggregate which, might initiate inflammation and oxidative stress, thereby leading to synucleinopathy in the enteric nervous system (ENS) (figure 3) (Forsyth et al., 2011).

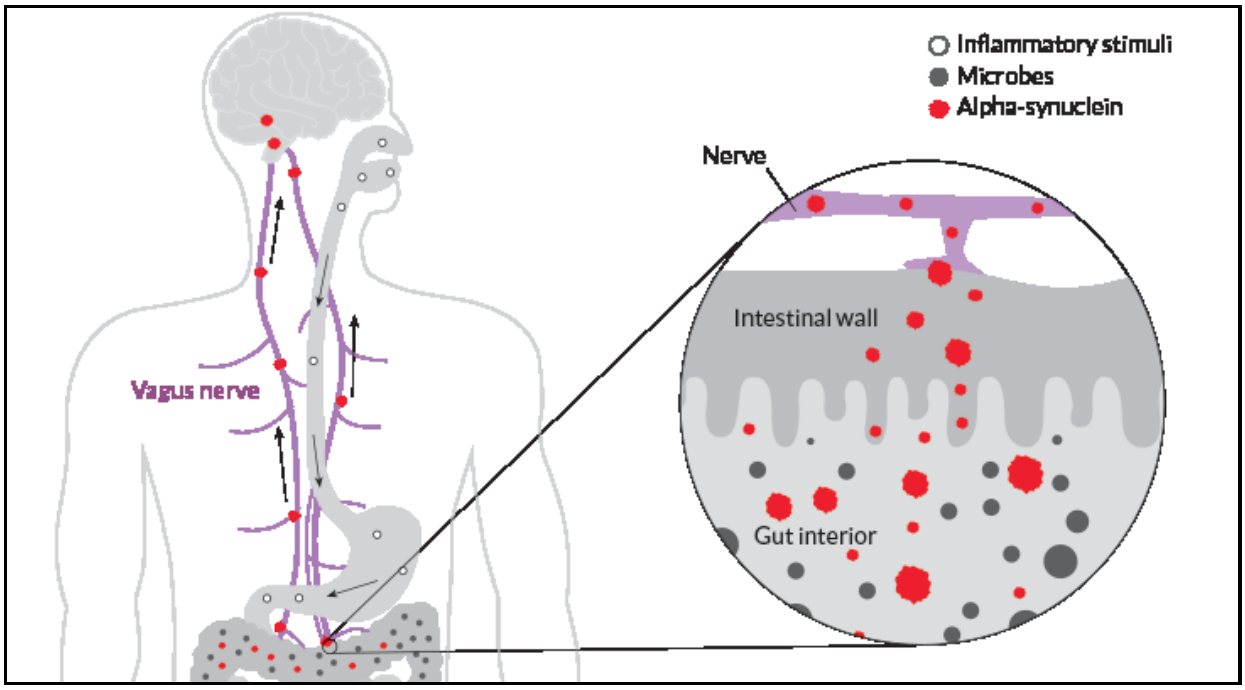


Figure 3. **Alteration of intestinal permeability allows to the aS to pass through the intestinal wall and travel along the vagus nerve, from the lining of the gut to the brain (Breil L., 2018).**

Moreover, in a model of mice overexpressing aS, gut microbiota and SCFA were demonstrated to be required for motor deficits, microglia activation, and aS pathology (Sampson et al., 2016). These findings support the hypothesis that alterations in the human microbiome represent a risk factor for PD.

1.3.3 Genetic mutation in PD

For many years the etiology of PD has been associated exclusively with environmental factors, not considering a possible genetic contribution. However, researches on familiar cases have led to the discovery genetic mutations responsible for rare family forms (Dehay et al., 2015).

Linkage analysis and genome-wide association studies revealed the presence of 17 dominant and recessive autosomal genetic mutations and about 14 genes associated with PD (Kalia & Lang, 2015) that are summarized in Table 1.

Locus	**Gene' symbol**	**Genic product**	**Inherited mutation**
PARK1/PARK4	*SNCA*	α-synuclein	DAM
PARK2	*PARK2*	Parkin	RAM
PARK6	*PINK1*	Pten-induced kinase 1	RAM
PARK7	*PARK7*	DJ 1	RAM
PARK8	*LRRK2*	Leucine-rich repeat kinase 2	DAM
PARK9	*ATP13A2*	ATPase type 13A2	RAM
PARK 14	*PLA2G6*	Phospholipase A2, group VI	RAM
PARK 15	*FBXO7*	F-box protein 7	RAM
PARK 17	*VPS35*	Vacuolar protein sorting 35 homolog	DAM
	PANK2	Pantothenate kinase 2	RAM

Table 1. **Gene locus associated at PD (Houlden & Singleton, 2012). RAM = recessive autosomal mutation; DAM = dominant autosomal mutation**.

Particularly interesting is the so-called PD Type 1 (PARK1), associated with mutations in the sequence of the *SNCA* gene encoding for alpha-synuclein (aS).

It has also demonstrated that duplication or triplication of *SNCA* gene is tightly related with familiar PD (Guhathakurta et al., 2017) and, the triplication of aS is more severe than the duplication and lead to a rapidly progression of the disease, ssuggesting a dose-dependent relationship between disease severity and *SNCA* gene dosage (Dehay et al., 2015).

As mentioned above, the first missense mutation, Ala53Thr in exon 4 of the *SNCA* gene encoding for aS in which there is a substitution of the Alanine with a Threonine, was discovered in 1996 (Polymeropoulos et al., 1996). Since then, four other missense mutations have been identified. A30P and E46K missense mutations were found in German and Spanish kindreds, respectively (Krüger et al., 1998; Zarranz et al., 2004).

The A53T is the most common mutation for familiar PD with an apparently aggressive clinical course. Recently, Papadimitriou and collaborators performed a prospective 2-year longitudinal follow-up study involving A53T symptomatic and asymptomatic carriers, noting prominent motor and nonmotor decline that included olfactory, autonomic, and cognitive dysfunction, with a disease penetrance of ∼90% (Papadimitriou et al., 2016).

Despite these mutations are known, the mechanism through which the disease manifests itself is still unclear. Probably these pathogenic mutations, responsible for roughly 5% of PD cases, are involved in a gain of toxic function of aS resulting in aberrant aggregation, which not only prevents normal protein function, but also causes cellular damage with subsequent neuronal death (Dauer & Przedborski, 2003; Dehay et al., 2015).

1.4 Alpha-Synuclein

The alpha-synuclein (aS) is a protein consisting of 140 amino acids, specifically situated in the presynaptic terminals and, in smaller amounts, in cellular, dendritic or extra-synaptic sites along the neuron axon.

A protein named "*synuclein*" was initially identified in the synaptic vesicles of the electric organ of the marine elasmobranchs *Torpedo californica*. Its particularly localization, suggested an involvement in synaptic transmission (Maroteaux, Campanelli, & Scheller, 1988).

Next, from the study of amyloid plaques recovered in Alzheimer's disease (AD), two unknown peptides that appeared to act as a trigger for the aggregation of Amyloid beta peptides (Aβ) were identified, they were named non-Aβ components (NAC) of the AD plaques (Murphy, LeVine, & III, 2010).

It was also discovered that these proteins derive from a common 140 amino acid (aa) precursor (NACP), mainly located in the brain.

Afterwards, in the human brain, a 140 aa protein corresponding to NACP and with high homology to the Torpedo synuclein protein was identified and it was called "α-synuclein" (aS) (Bendor et al., 2013; Emamzadeh, 2016).

Human aS is coded by the *SNCA* gene and belongs to a small family consisting of 3 proteins: α-, β- and γ-synuclein, encoded by different genes.

The aS, can be divided in three domains (figure 4):

• the N-terminal domain, consist in 11 amino acids repeating unit that contains a conserved core of 6 residues, which thanks to the α-helix structure is capable of binding lipids at the cell membranes;

• the central domain known as NAC component, strongly hydrophobic, which allows the bond between different aS molecules leading to fibril aggregation.

• the C-terminal domain, rich in proline that contributes to the overall negative charge of the protein.

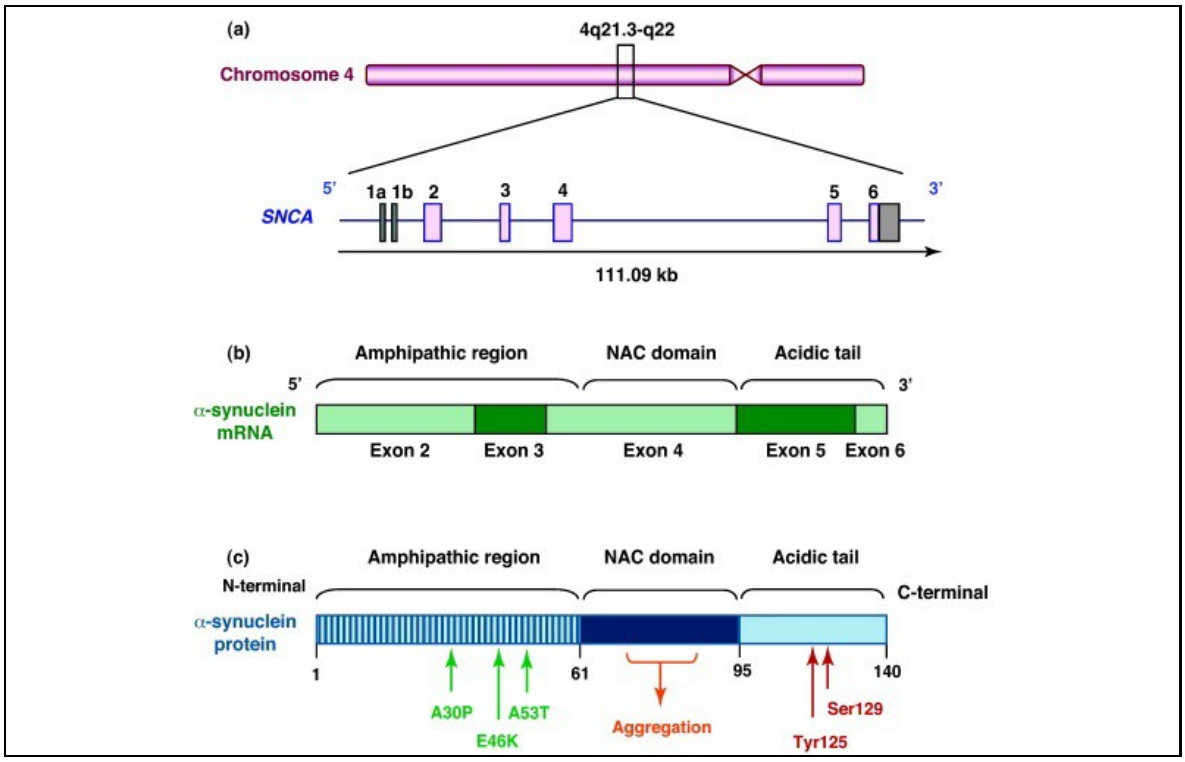


Figure 4**. Schematic representation of human aS depicting**. **(a)** *SNCA* gene structure, **(b)** mRNA, and **(c)** protein domains (Venda, Cragg, Buchman, & Wade-Martins, 2010).

The intrinsically disordered proteins have a primary structure, rich in Proline and charged residues, without long hydrophobic region that contribute to prevent their aggregation. In the case of aS, its aggregation prone region NAC domain is partially protected by the positive and negative charges of the N- and C-terminal domains which, through hydrophobic and electrostatic interactions, prevent its aggregation. However, mutations, changes in environment or post-translational conditions lead to the eversion of the NAC domain, causing misfolding or aggregation (Villar-Piqué et al., 2016).

The native conformation of aS is still unknown. although traditionally it is considered an intrinsically disordered protein, lacking a stable secondary and or tertiary structure. In some studies, aS is described as a disordered monomeric form protein (Fauvet et al., 2012), while in others it is defined as a tetramer rich in alpha structures (Mor et al., 2016). Overall, these studies suggest that, due to its high conformational plasticity, it may be present in different forms and, for this reason, it is also called

"chameleon-protein" (Plotegher et al., 2014).

The physiological function of aS is not completely known. This aspect represents an important limit for the full understanding of its role in the pathogenesis of PD.

Literature data suggest a role of aS in the suppression of apoptosis in dopaminergic neurons by a downregulation of the protein kinase C, a kinase sensitive to the oxidative stress and capable of triggering apoptosis in dopaminergic neurons (Bendor et al., 2013). In this way aS would act as a neuroprotective factor towards dopaminergic neurons.

However, the most accredited theory, also confirmed by the presynaptic localization of aS, gives to the protein a role in synaptic transmission. aS is also defined as a chaperone whose function would be to ensure the maintenance of the structure of the proteins complex, known as SNARE (Soluble N-ethylmaleimide sensitive factor attachment protein receptor) complex, that has to mediate the tethering, docking and fusion between the presynaptic membrane and the vesicles. As it has been reported that the interaction between aS and synaptobrevine 2 inhibits the formation of the complex, any aS dysfunction may lead to neurological damage (Burré et al., 2010).

Another function is attributed to aS, as an antioxidant for dopaminergic neurons, which are particularly vulnerable to oxidative stress that may results from dopamine metabolism (Schulz-Schaeffer, 2015).

Cellular levels of dopamine and its productions are modulated by aS which acts as a down-regulator of the activity of tyrosine hydroxylase (TH). Therefore, the reduction of expression or the aggregation of aS results in an increase of the synthesis of dopamine and, in oxidative stress, as a consequence of its metabolism. By contrast, the over-expression of aS or its mutation, increase the inhibitory effect on TH followed by the reduction of dopamine synthesis and release (Schulz-Schaeffer, 2015).

As mentioned above, the ability of aS to modulate vesicular traffic is due to its structure: the repetitions of 11 amino acids, able to fold in alpha helix, allow aS to selectively bind the curved membrane to modulate vesicle trafficking, to interact with

lipid membranes and to reduce both the quantity and speed of vesicular recycling, from synapses to presynaptic area and thus ensuring homeostasis.
In addition, aS appears to be involved in neuronal differentiation, regulation of glucose levels, modulation of calmodulin activity and maintenance of polyunsaturated fatty acid levels (Schulz-Schaeffer, 2015).

According to a study performed in embryonic neuronal dopaminergic cells, the conversion of the physiological α-helix structure in β-sheets in a single aS can lead to a misfolding of additional aS molecules resulting in aggregate formation (Emamzadeh, 2016).
The presence of highly soluble proteins that undergo a gradual conversion into soluble polymeric filaments and fibrils mirrors what is known to occur in prion-associated disease and, in general, in condition characterized by the accumulation of misfolded proteins, as in AD (Xue et al., 2009). Another common aspect to all these pathologies is the long latency period that precedes the onset of symptoms, in which misfolding, aggregation and progressive cellular impairment occur. The long duration of the prodromal phase is needed for the high-energy barrier that must be overcome before misfolding occurs (Chaudhuri & Paul, 2006).
In the case of PD, the formation of aggregates in which the protein is found in monomeric, dimeric and oligomeric forms, give rise to LB (see above paragraph 1.2.1 of the Introduction), followed by cellular toxicity and neurodegeneration.
Indeed, it has been suggested that aS insoluble oligomers constitute the most relevant toxic species as: 1) they can cause alterations of membrane permeability by acting analogously to pore-forming toxins, with whom they display structural similarities (Plotegher et al.,2014); 2) they act as prion-like proteins, causing PD progression (Plotegher, Greggio, Bisaglia, & Bubacco, 2014).
As far as its physiological cellular turnover is concerned, after being ubiquitinated, aS enters in multiple pathways of degradation, including the endo-lysosomal pathway (ELP) (Tofaris et al., 2011).

Indeed, while initially the ubiquitin-proteasome system (UPS) was the only degradation pathway to be taken into consideration (Martinez-Vicente & Vila, 2013), it is well recognized now that also lysosomes are involved, through multiple pathways such as macro-autophagy, chaperone-mediated autophagy and endocytosis (Figure 5).

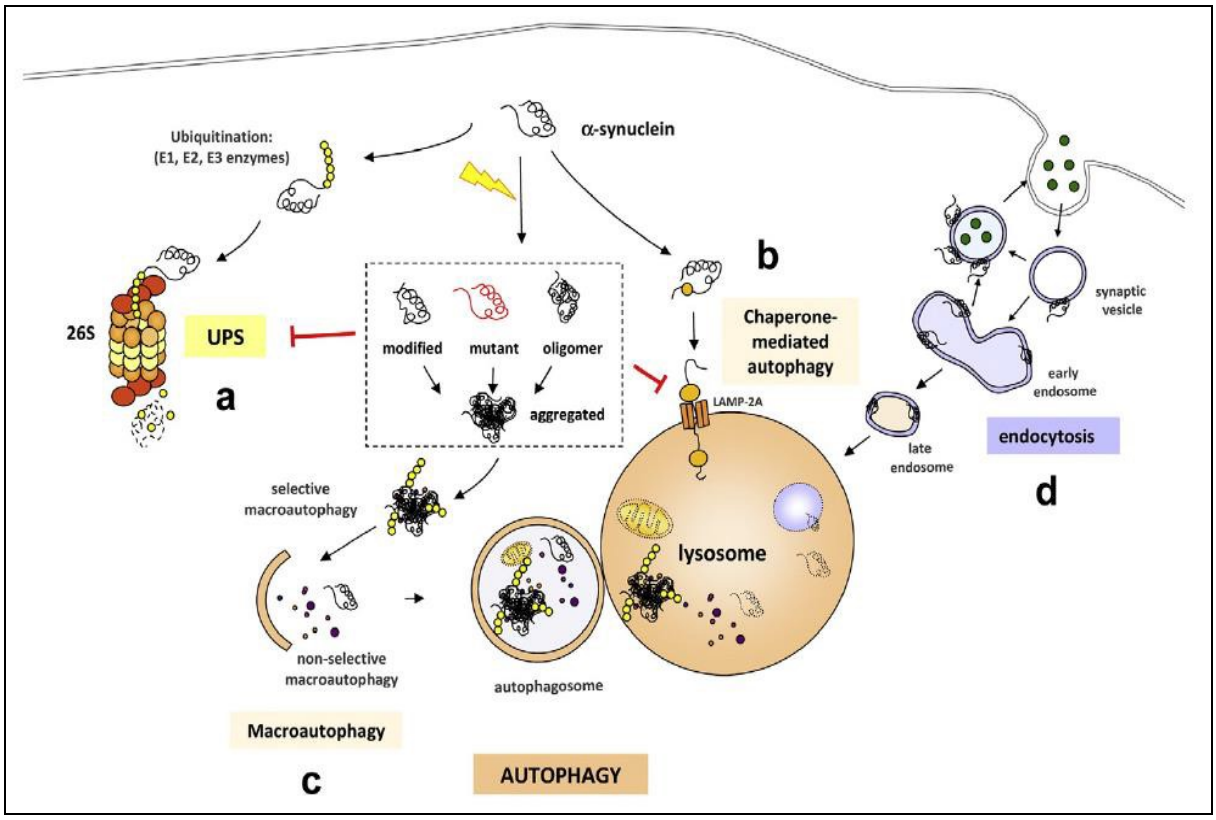


Figure 5**. aS degradation pathways**. (a) aS is degraded by the ubiquitin-proteasome system (UPS), after being tagged with a polyubiquitin chain. (b) aS can also be translocated inside the lysosomes to be degraded through the chaperone-mediated autophagy (CMA) pathway. (c) Selective macroautophagy is able to eliminate aS insoluble aggregates. (d) Lysosomes can also eliminate membrane-associated aS, such as the one present in synaptic vesicles, through endocytosis (Martinez-Vicente & Vila, 2013).

The signals through which aS is sorted between different pathways are not yet fully understood yet, but some factors may contribute: 1) Folding state (poorly folded, non-folded, properly folded), 2) localization, 3) post-translational modifications, 4) solubility, 5) oligomeric state.

Importantly, dysfunction of proteolytic systems causes accumulation of aS and, at the same time, contributes to the formation of pathological species of aS, which interfere with the functioning of such systems, thus inhibiting aS degradation itself and triggering a wrong cycle which result in neurotoxicity (Martinez-Vicente & Vila, 2013; Scheffner & Kumar, 2014).

1.5 The Ubiquitin Ligase NEDD4 and its role in aS turn-over and PD

Most cell proteins are targeted for degradation by conjugation with ubiquitin (Ub) chains. Protein ubiquitination involves an enzymatic cascade, starting with the Ub-activating enzyme/E1, followed by the Ub-conjugating enzyme/E2 and by the Ub-ligase/E3, which form an isopeptide bond between the carboxyl terminus of Ub and the alpha-amino group of a lysine residue on the target protein. NEDD4 (Neuronal precursor cell-Expressed Developmentally Down-regulated gene 4) is the main E3 targeting aS for degradation; it is an HECT (homologous to E6-AP carboxyl terminus) ubiquitin-ligase E3 that has a role in the endo-lysosomal pathway (ELP) by promoting the degradation of membrane-associated proteins (figure 6). It is composed by:

- An HECT domain of approximately 350 amino acids, at the C-terminal region, which has high homology with the E6AP (E6-associated protein, a polypeptide that interacts with HPV E6 protein) and represents the protein catalytic domain. It mediates the interaction with E2 and possesses a preserved Cysteine residue to which the ubiquitin is bound, forming an intermediate thioester prior to its transfer to the substrate. The HECT domain assumes a bilobar structure whose C-terminal lobe has the Cysteine catalytic residue, while the N-terminal represents the binding domain for E2. The two lobes are joined together by a flexible hinge region that probably facilitates the assumption of the correct positioning and

which reflects differing specificities for one of the over 30 different E2s (Jentsch, 1992).

- Four WW domain, of approximately 35 amino acids each and named for the presence of two conserved tryptophan (W) residues spaced 20–22 amino acids apart. This domain seems to mediate substrate recognition. WW modules are responsible for cellular localization and substrate recognition with affine proline-rich motifs, one of which found to be present in the C- terminal of aS (Tofaris et al., 2011).
- The C2 domain, located within the N-terminal, is a Ca^{2+}-dependent phospholipid binding domain. It consists in 120 amino acids and it is rich in aspartate responsible for the binding of Ca^{2}. Furthermore, this domain contributes to the substrate specificity (Scheffner & Kumar, 2014).

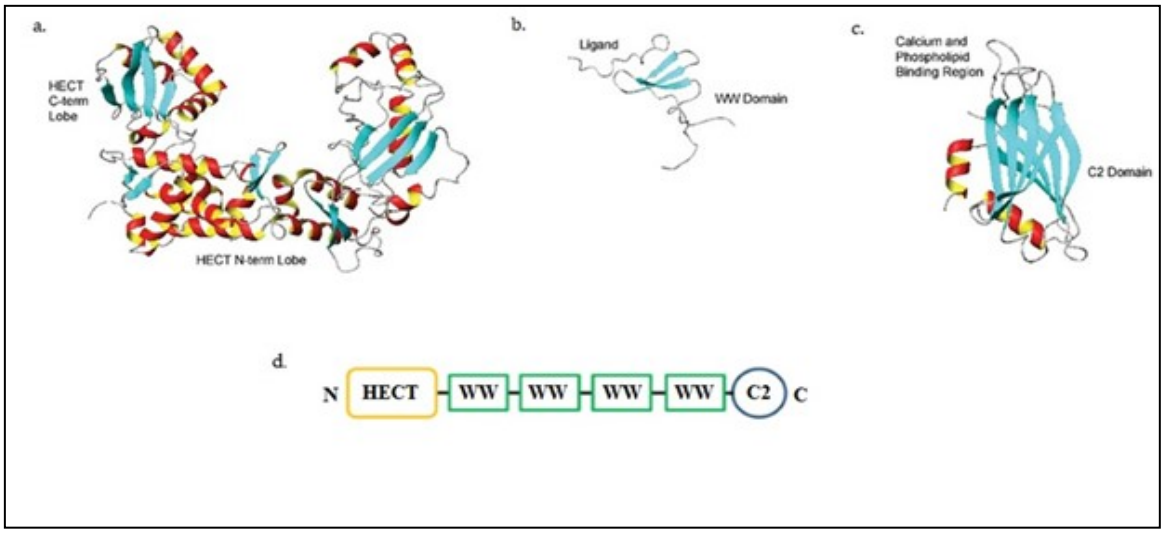


Figure 6. **Molecular and organization of human NEDD4 HECT E3.** (a) The two lobes that compose the HECT domain are shown in the crystal structure. (b) WW domains are formed by a three-stranded anti-parallel β-sheet as shown for the NEDD4 WW domain. A ligand that contains a PPxY motif, forming a polyproline type II helix, binds primarily to one face of the WW domain β-sheet. (c) The loop regions of the C2 domain, found at one end of the β-sandwich fold, form the binding site for calcium and phospholipids. (d) Human NEDD4 ligase domain architecture (Ingham, Gish, & Pawson, 2004).

NEDD4 is involved in several ubiquitin-mediated processes (Hicke, 2001): 1) it targets proteins for degradation trough the 26S proteasome; 2) it is responsible of plasma membrane protein monoubiquitination that mediates their internalization; 3) it

helps sorting proteins in limiting membrane of endocytic vesicles to the multivesicular body, leading to their destruction at the lysosome. Finally, NEDD4 mediates ubiquitination also occurs at the trans-Golgi network when newly synthesized proteins need to be targeted for degradation to the lysosome (Ingham et al., 2004).

The involvement of NEDD4 in the degradation process of aS through ELP has been demonstrated by studies conducted by Tofaris and coworkers (Tofaris et al., 2011). Those authors tested the ability of several E3s of ubiquitinating aS and demonstrated this activity only for NEDD4. Furthermore, they showed that NEDD4 induces aS degradation mainly through the ELP and upon the recruitment of the Endosomal Sorting Complex required for transport factors.

Further supporting NEDD4 involvement in aS physiological turn over ad particularly relevant, are the results found in yeast. Indeed, it has been reported that, Rsp5p, the NEDD4 yeast orthologue, catalyzes ubiquitination and degradation of aS, but also that, by preventing the formation of aS inclusions, it plays a neuroprotective role preventing the formation of its inclusions and therefore its toxicity (Tofaris et al., 2011). These observations suggest that NEDD4 activity towards aS might represent a cellular protective response aimed at reducing the formation of proteins aggregates, by stimulating protein removal aggregates (Portnoff et al., 2014). In agreement with this hypothesis, genome studies conducted in humans, have led to the discovery of an SNP (Single Nucleotide Polymorphism) within NEDD4 encoding sequence that would increase the risk of PD (Srinivasan et al., 2009).

It also has been demonstrated that NEDD4 is protective against human α-synuclein toxicity in evolutionarily distant model systems (yeast, *Drosophila*, rat) (Perrett et al., 2015) strongly suggest that activation of this conserved ubiquitination pathway should be considered as a target for neuroprotective therapies. Finally, in a large screening for small molecules with neuroprotective activity, as NAB2, are able to activate NEDD4 and protect neurons against aS toxicity in distant evolutionary

models like yeast and cortical neuronal model of aS toxicity, showed that NEDD4 overexpression is protective in the A53T iPSc-derived neuronal model (Chung et al., 2013).

1.6 Chimeric Ubiquitin Ligases: The Ubiquibodies

It has been demonstrated that the ubiquitin ligase NEDD4 might be a target for the development of innovative therapeutic interventions against the neurodegenerative disease. However, since E3 activation cannot be easily obtained pharmacologically and despite promising results with compound screens (Chung et al., 2013), data supporting this strategy are still limited. E3s are usually the main determinants of substrate specificity in the ubiquitin proteolytic pathways. Their conformational flexibility, along with the fact that "consensus" motifs are not required for ubiquitin conjugation, support the possibility of engineering Ub-ligases to specifically target proteins of interest. In this context, chimeras have been generated called "ubiquibodies" that combine the activity of E3 ubiquitin ligases with scFv intrabodies, which can be used to target any protein for degradation, while losing their ability to modify their natural target.

Portnoff and collaborators were the first Authors to show that is possible to generate chimeric Ub-ligases where the catalytic domain is fused with a single chain antibody conferring to the enzyme the substrate specificity. ScFvs are genetically engineered antibodies, containing a complete or partial antigen binding site (Fv). ScFvs are formed by the VH and VL antibody variable regions connected by a short flexible linker (Figure 7).

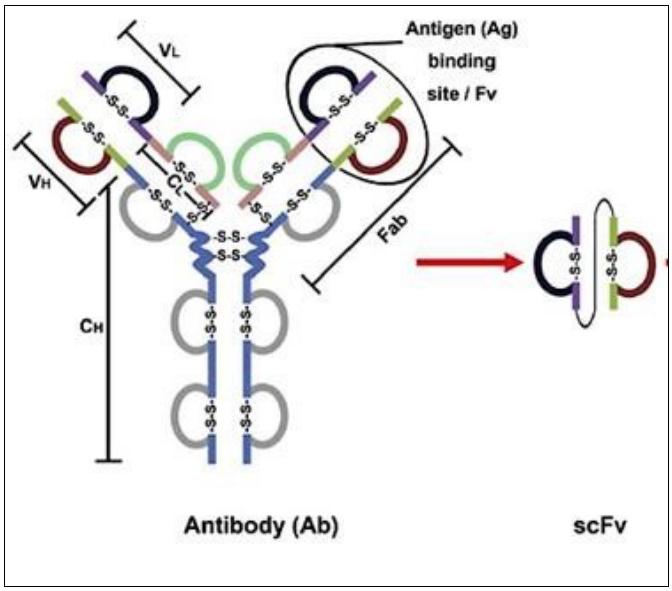


Figure 7. **Schematic representation of an antibody (Ab) and an intrabody (scFv)** (Zhou & Przedborski, 2009)**.**

In particular, Portnoff and colleagues have adopted the C-terminal U-box of the E3 CHIP (carboxyl terminus of Hsc70-interacting protein fused in frame to a scFv that specifically binds β-galactosidase. Those Authors were able to show that this developed chimera is able to bind and ubiquitinate the target β-galactosidase, confirming that the chimera also maintains the E3 ligase activity of the CHIP ubiquitin ligase. Also, Authors demonstrated that the ubiquitination is possible however, only in the presence of all the components of the complex, indicating that the activity of the chimera is dependent on it, and was not instead realized by the scFv alone or by the isolated catalytic domain.

The resulting chimeras, named *ubiquibodies*, are therefore highly specific for the target and do not recognize the substrate of the wild type E3. In addition, a single ubiquibody molecule can eliminate numerous copies of the target protein.

Therefore, ubiquibodies are good candidates as a simple, reproducible and customizable technique to selectively accelerate the turnover of specific proteins as well as isoforms in somatic cells.

1.6.1 scFvs and Parkinson's disease

Following the successes reported by intrabodies in various disease like AIDS and cancer, this technology has also been applied in the study of neurodegenerative diseases in order to prevent abnormal protein aggregation (Zhou & Przedborski, 2009).

In particular, the goal is to block the self-aggregation of aS, without interfering with its physiological implication. To this end, several scFvs versus aS have been developed and reported in Table 2 (Zhou & Przedborski, 2009).

Name	Source library	Epitope (aa)	Binding to α-syn aggregates	Affinity (K_D, M)	*In vitro* effects	Intracellular effects
F8[54]	Phage	101–111/ 27–37	?	10^{-8}	Inhibit aggregation	Unstable expression
D10[25]	Phage	*	Yes	10^{-6}	?	Inhibit α-syn aggregation and rescue impaired adhesion
6E[55]	Phage	*	Yes	?	?	?
3[57]	Phage	71–85	Yes	?	?	?
14[57]	Phage	106–120	Yes	?	?	?
15[57]	Phage	117–131	Yes	?	?	?
D5[56]	Phage	*	Yes	?	Inhibit aggregation	?
NAC3[26]	Yeast	61–78	?	?	?	No significant protection
NAC24[26]	Yeast	61–78	?	?	?	Unstable expression and no significant protection
NAC32[26]	Yeast	53–87	?	?	?	Inhibit α-syn aggregation and rescue cytotoxicity

?: not reported.
*: The scFvs D10, 6E, and D5 recognize α-syn conformational epitopes, although the amino acid sequences that constitute the epitopes are not identified.

Table2. **scFvs against aS. Zhou & Przedborski, 2009.**

Current available anti-aS scFvs are selected from three phage libraries and one yeast library (Table 2).

- The ScFvs F8, d10, 6E and d5e are selected from two synthetic libraries that contain synthesized variable regions of scFvs (Zhou, et al.,2004).

- The scFvs NAC3, NAC24 and NAC32 are selected from a yeast library that is composed of scFv gene segments from the mRNA pool of 58 human adult spleens and lymph nodes (Lynch et al., 2008).
- The scFvs 3,14 and 15are from a phage library generated using the mRNAs from human peripheral blood lymphocytes of a large (N > 100) pool of donors (Maguire-Zeiss et al., 2006).

The identified sequences in the NAC region (aa 68–76 or aa 71–82) of aS, which are required for its aggregation, are the most promising candidates in this aspect. To this end, the scFv NAC32 is selected against a peptide containing the amino acids 53–87 of aS. When tested in mammalian cells as an intrabody, it inhibited aS aggregation and reduced the cytotoxicity caused by mutant aS (Zhou & Przedborski, 2009). Another kind of useful ScFvs is that one against the epitope necessary for aggregation formation; this kind scFv is also expected to block aS self-aggregation. In this case, d5e and d10 recognize this conformational epitope, also d10, that bind the monomeric forms of aS, prevent the formation of high molecular weight of aS species and restore cellular adhesion lost following the overexpression of aS; indeed, d5e, that recognizes the oligomeric form, it gives protection against toxicity induced by aS.

1.7 Lentiviral vector as the tool for an efficient delivery of the ubiquibodies

The *Retroviridae* family of viruses was first investigated as vectors for mammalian gene transfer over 30 years ago, and this technology continues to develop. Lentiviral vectors (LVs) are successfully employed for gene therapy application (Cartier et al., 2009; Cavazzana-Calvo et al., 2010) and they are involved in 9% of all the gene therapy clinical trials worldwide and in 19% of those for monogenic diseases (http://www.abedia.com/wiley/index.html – update 2018). LVs are a suitable tool used in therapeutic genes, because they exploit only the first stage of the viral

replicative cycle to transfer and integrate the therapeutic transgene within the chromatin of the target cells (Borsotti, Borroni, & Follenzi, 2016) The surface design of LVs has been constantly improved during the last decade (Lévy, Verhoeyen, & Cosset, 2015). Moreover, LVs are optimal vehicles for gene delivery as they are able to transduce dividing and non-dividing cells. The current generations of LVs are based on HIV type 1 (HIV-1) (Dull et al., 1998). Their properties, like allowing stable long-term transgene expression through integration properties into the target cell genome, carrying genetic information into a broad range of cell types, efficiently integrating their genomes into target cell chromosomes, resulting in permanent DNA delivery, make lentiviral vectors useful in a range of scenarios, such as studying the biological effects of transgenes in dividing and not dividing cells, on pre-clinical disease models, the generation of transgenic animal strains, and the transfer of therapeutic sequences to treat human disease.

Currently, the last improved design of LVs is represented by the third generation packaging construct that separates the gag and pol genes (structural and enzymatic viral proteins) from the rev gene (regulatory protein), increasing the platform biosafety and the transduction efficiency, combined with the improved self-inactivating (SIN) transfer vector, that prevents the mobilization by replication-competent virus and assigns transgene expression control completely to the internal promoter (Dull et al., 1998)

The first efficient lentiviral expression system was derived from the human immunodeficiency virus type 1 (HIV-1) and its genome differs from the original HIV genome in many characteristics (Figure 8): insertion of 5′ CMV promoter drives efficiently transcription of the transgene; insertion of RRE (Rev-responsive element) allows efficient nuclear export of the RNA; the LTRs mediate integration of the dsDNA into the host cell's genome as a provirus; the insertion of woodchuck hepatitis virus post-transcriptional regulatory element (WPRE) increases the stability of the mRNA (Lois, Hong, Pease, Brown, & Baltimore, 2002; Zennou et al., 2001;

Zufferey, Donello, Trono, & Hope, 1999); deletion of the U3 region (ΔU3), that due to the deletion TATA box and binding sites for transcription factors, lacks any viral transcriptional activity; the accessory and regulatory proteins, like env, tat, rev, vpr, vpu, vif and nef, have been deleted. Moreover gag-pol, rev and VSV-G envelope proteins, necessary for production of viral particles, are expressed from three helper vectors provided *in trans* (Dull et al., 1998; Naldini et al., 1996).

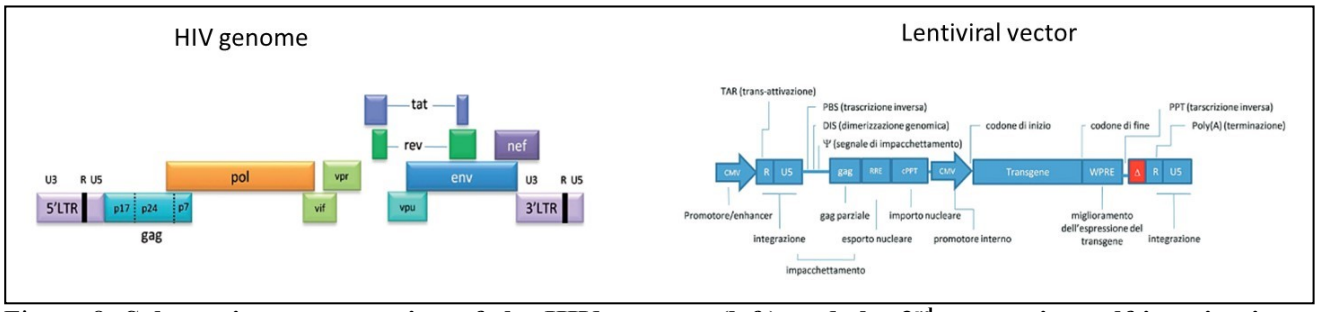


Figure 8. **Schematic representation of the HIV genome (left) and the 3rd generation self-inactivation (SIN) lentiviral vector.**

1.8 In vitro experimental models used for the study of Parkinson's disease

The development of new therapeutic strategy to understand the pathogenesis of the disease and to test an intervention that could have beneficial effect, requires the use of experimental models that mimic as much as possible the *in vivo* environment and that itself develop pathology quickly and reliably (Falkenburger et al., 2016).

In fact, the study of the disease in patients is limited by its heterogeneity, by its slow progression, by technical obstacles and of course by ethical considerations.

The choice of model will depend on the aspect of the disease we want to study and the kind of therapy we want to develop.

Cellular models are more favored than animal models because: 1) is obtained a more rapid progression of the pathology, 2) are less expensive, 3) genetic and pharmacological manipulation is easier, 4) have few ethical and regulatory problems. All these characteristics translate into the possibility of carrying out tests in a shorter period.

The neuroblastoma cell line SH-SY5Y or the mouse immortalized neuronal progenitor cell line MN9D and the pheochromocytoma cell line PC12 are used (T. Hasegawa et al., 2004; Rick et al., 2006). They produce endogenously aS and release catecholamines and can develop neuron-like properties including neurite-like processes upon specific differentiation protocols. They are easier to maintain than primary neurons, but differentiation can be difficult.

Furthermore, they are excellent models for studies with pharmacological interventions and need genetic manipulations (over-expression, siRNA) and often require viral transduction (Kim et al., 2015) or the stable integration of inducible constructs (Ejlerskov et al., 2013).

However, all results obtained from cultured cells subsequently be validated in animal models, as many important steps in the pathogenesis of PD require the interaction of different cell types.

The cellular models currently used for the pathology study reproduce two of the main characteristics present in the brain of the affected patients: the loss of dopaminergic neurons in the SNC and the presence of protein aggregates consisting mainly of aS.

1.8.1 Cellular models for the induction of alpha-synuclein (aS) aggregates

In humans, Parkinson's disease (PD) can be caused by mutations in the *SNCA* gene, which encodes the aS protein, or by its duplication / triplication. Consequently, the overexpression of aS or of its specific mutated forms can be used in appropriate cell lines to validate the therapeutic strategies. In order to generate aS aggregates, is crucial to express high level of this protein. For example, in human embryonic cells 293T the transfection with vectors expressing aS under the transcriptional control of the strong human cytomegalovirus (CMV) promoter is efficient, unlike other weak promoters who are not always able to induce aggregates. Even in primary neurons, transduction with viral vectors containing strong promoters is often necessary to achieve sufficiently high concentrations of aS within the cell (Falkenburger et al.,

2016). Furthermore, to detect aS and its aggregates, the simplest way is to fuse in frame the transgene with a fluorescent reporter protein, such as the "enhanced green fluorescent protein" (EGFP). With this construct, most of the cells show a uniform distribution of fluorescence, and it is possible to count the labeled proteins with different techniques like immunofluorescence, cytofluorometry or Western Blot.

Human iinduced pluripotent stem cells (hiPSC) are a unique and important tool for defining the mechanisms of PD, for the development of early diagnosis and new therapeutic agents (Oliveira et al., 2015). In fact, it is practically impossible to obtain brain cells from Parkinson's patients, especially in the early stages of the disease, when the patient has no symptoms. The cellular reprogramming gives access to a large number of neurons of the particular type affected in the PD. HiPSCs derived from adult specialized cells of patients with PD and are used to produce neurons in the laboratory (Weiss et al., 2010). HiPSC derived from an PD patient which bearing the triplication of *SNCA* gene, is a good model for assessing the impact of overexpression on maturation and neuronal differentiation. Moreover, in a study conducted by Oliveira and collaborators (Oliveira et al., 2015) the effect of the increase of aS expression in two clones of hiPSC derived from a patient with triplication in the *SNCA* gene was analyzed, the two clones and appropriate controls were differentiated into neurons in order to evaluate different aspects: the ability to differentiate, cell survival, neuritic growth and electrophysiological properties. Neural progenitors (NSC) with *SNCA* triplication showed increased levels of aS than controls.

Interestingly the increased expression of aS was accompanied by an increase in the autophagy flow: the over-regulation of autophagy could therefore act as a compensatory cellular mechanism in order to eliminate the monomeric aS accumulated.

However, this could lead to non-specific degradation of essential proteins and organelles, including mitochondria, thus contributing to the development of the disease. All these results allow us to speculate that the dosage of aS is an important

modulator of the neuronal development efficiency of progenitor cells (Oliveira et al., 2015). In addition, over-expressing hiPSCs are a good *in vitro* model for validating strategies to interfere with effects specifically related to the accumulation of this protein.

2. Aim of The Study

The main pathological features of Parkinson's disease (PD) are the death of dopaminergic neurons and the diffuse accumulation of aS aggregates in neurons. Here we propose to exploit chimeric ubiquitin (Ub) ligases specifically targeting aS to prove the protective role of aS degradation pathway towards the development of a novel therapeutic strategy.

We have developed lentiviral vectors encoding well characterized human scFv intrabodies fused to the catalytic HECT domain of NEDD4, in order to obtain enzymes that specifically ubiquitinate aS either in its monomeric or oligomeric form. The aim of my PhD project was to test these vectors for their ability to i) promote aS specific degradation; ii) reverse the formation of aS aggregates; iii) rescue their toxicity in different *in vitro* settings, including neuronal models obtained from human induced pluripotent stem cell lines derived from PD patients.

3. Materials and Methods

Materials

3.1 Cell Lines

In this experimental work the following cell lines were employed:

293T (ATCC CRL-3216 ™) cell line, kindly provided by Dr. D. Baltimore (Rockfeller University, New York), is a highly transfectable derivative of human embryonic kidney (HEK) 293 cells, with stellar morphology. 293T cells constitutively express the large T antigen of the simian virus 40 (SV40), thus enabling efficient replication of vectors carrying the SV40 region of replication.

SH-SY5Y (ATCC® CRL-2266™) cells are a thrice cloned sub-line of the epithelial neuroblastoma cell line SK-N-SH (ATCC HTB-11) which was established in 1970 from a metastatic bone tumor. These cells are both floating and adherent and grow as cluster of neuroblastic cells.

MN9DwtsynIRESgfp (MN9Dsyn) cells derives from MN9D cells obtained from mouse embryonic mesencephalon (Choi et al., 1991). MN9Dsyn are immortalized dopaminergic-like cells that harbor an integrated transgene affording doxycycline [(DOX; 2.0 μg/mL media); Sigma-Aldrich Co., St. Louis, MO, USA] regulated human wildtype aS expression and separately, thanks to an internal ribosome entry site (IRES), green fluorescent protein (GFP) expression (Feng, Federoff, Vicini, & Maguire-Zeiss, 2010).

The different cell lines were grown in Dulbecco's Modified Eagle's Medium Growth Media (DMEM) supplemented with either 10% v/v Foetal Bovine Serum (FBS) (293T and SH-SY5Y) or 5% v/v FBS (MN9Dsyn) inactivated at 56 °C for 30 minutes.

Three lines of human induced pluripotent stem cells (hiPSCs) were also adopted with the following characteristics:

- **Ep.11** derived from human fibroblastoid cell line BJ cell (ATCC® CRL-

2522™) as previously described (Trevisan et al., 2015).

- **hiPSC-49 (**ND50040 RUCDR Infinite biologics®) derived from a Parkinson patient carrying the aS encoding gene *SNCA* triplication.
- **hiPSC-52 (**ND50050 RUCDR Infinite biologics®) derived from a Parkinson patient bearing the A53T mutation within the amminoacid sequence of aS.

HiPSCs were seeded on a specific substrate (Geltrex™ Matrix LDEV-Free) and were grown in mTeSR™ medium with 20% (v/v) of its supplement (Stem Cell™).

Neural Staminal Cells (NSCs), derived from hiPSCs as described in paragraph 4.9 of the Results section of this thesis, were plated on Geltrex™ Matrix LDEV-Free coated dishes and cultured with PSC Neural Induction Medium (Gibco™ Life Technologies™). Culture of cells in this neural induction medium generates homogenous cultures of NSCs (more than 95% of the cells).

Dopaminergic progenitors (DPs), derived from NSCs as described in paragraph 4.9 of the Results section of this thesis, were plated for 7 days on Poly-ornithine/ laminin (P4638-1G Sigma; L2020-1MG Sigma) coated culture dishes in DMEM/F12 medium (11330057, Life Technologies™) plus 1% v/v of N2 supplement (17502048, Life Technologies™), 200 ng/ml of Sonic Hedgehog (SHH;1591950025, Provitro) and 100 ng/ml of Fibroblast Growth Factor 8 (FGF8; 1376950025, Provitro)

Dopaminergic Neurons (DNs), derived from DPs as described in paragraph 4.9 of the Results section of this thesis, were plated on Poly-ornithine/laminin coated dishes, in DMEM/F12 medium, supplemented with 1% v/v of N2 supplement, 20 ng/ml of Brain Derived Neurotrophic Factor (BDNF; 78005, StemCell), 0.2 mM of Ascorbic Acid (AA; A5960-25G, Sigma), 20 ng/ml of Glial cell-Derived Neurotrophic Factor (GDNF; 01-A0460-0010, ORF genetics), 0.5 mM of N^6,2′-O-Dibutyryladenosine 3′,5′-cyclic monophosphate (dbcAMP; D0627-25MG, Sigma), 1 ng/ml of Transforming Growth Factor beta3 (TGFb3; 1938950002, Provitro). At day 7, the medium was swapped to Neurobasal medium supplemented with 2% v/v of B27 (17504044, Life Technologies™), 1% v/v of N2 and BDNF, AA, GDNF, dbcAMP,

TGFb3 (the last six reagents at the same concentrations as above). The differentiating DN were further kept up to 35 days in the same medium which was renewed every 3-4 days.

All cells were maintained in incubator (Heraeus BDD 6220) at a constant temperature of 37 °C in a humidified atmosphere with 5% (v/v) of CO_2 and subjected to periodic controls to exclude contaminations.

3.2 Plasmids

pcDNA3.1+®: this plasmid contains the human cytomegalovirus promoter (hCMV) that ensures high levels of expression in mammalian cells, neomycin and ampicillin antibiotic resistance, the promoter and origin of replication of Simian virus 40 (SV40) and the polyadenylation signal from the Bovine Growth hormone (BGH) (Invitrogen®).

pCR2.1topo: this plasmid is provided by the commercial kit "TA cloning" (Invitrogen) in a linearized form with a single deoxytidine (dTTP) protruding at the 3' end, to the which the Topoisomerase I of the *Vaccinia* virus is covalently bound. The plasmid contains the lac promoter, presents genes for resistance to ampicillin and kanamycin, the origin of bacterial replication pUC, the promoters recognized by the T7 phage RNA polymerase and the binding sites for primers T3, T7 and M13.

PBJ5UbHA: previously described (Strack et al., 2000) and kindly provided by H. Göttlinger (Massachusetts University), this plasmid contains the ubiquitin encoding sequence under the transcriptional control of the SRα promoter and fused in frame with the immunodominant epitope HA. SRα is a strong promoter composed of the SV40 early promoter fused to the RU5 sequences from the HTLV-1 LTR, thus ensuring a high expression of the transgene of interest, in the specific case HA-ubiquitin.

pBJ5Nedd4WT and pBJ5Nedd4C/S: previously described (Strack et al., 2000) and kindly provided by H. Göttlinger (Massachusetts University), these plasmid contains the Nedd4 ubiquitin ligase encoding sequence either WT (pBJ5Nedd4WT) or bearing the inactivating mutation $C_{867}S$ (pBJ5Nedd4C/S), under the transcriptional control of the SRα promoter.in WT or mutated $C_{867}S$ (C/S).

pEGFP-aSWT and pEGFP-aSA53T (Addgene #40822 and #40823): these constructs were obtained from Addgene and contain either the wild-type (pEGFP-aSWT) or the A53T mutant form (of the human aS) encoding sequence fused in frame with the enhanced green fluorescent protein EGFP under the transcriptional control of the HCMV promoter.

pcDNAaSWT3'F and pcDNAaSA53T3'F: these plasmids contain either the wild-type (pcDNAaSWT3'F) or the A53T mutant form (pcDNAaSA53T3'F) of the aS encoding sequence fused in frame with Flag tag, under the transcriptional control of the HCMV promoter. These constructs were generated in this thesis work extracting the aS encoding sequence from two plasmids pTOPOasynWT/pTOPOasynA53T3'F already available in the laboratory and inserting it within the poly-cloning site of the pcDNA3.1 by enzymatic restriction.

3.3 Lentiviral constructs

pptCMVEGFPWPRE: this plasmid contains the entire sequence of a third generation *Self-Inactivating* (SIN) lentiviral vector (LV), based on the human immunodeficiency virus type 1 (HIV-1) and characterized by, at the 5' Long terminal repeat (LTR), the Rous Sarcoma Virus (RSV) U3 region in place of the one of the HIV-1, along with a partially deleted 3'-LTR U3 region. This vector is further characterized by human Cytomegalovirus (hCMV) promoter, which allows the expression of EGFP, PBS (Primer Binding Site) and the *cis*-acting cPPT (central polypurine tract) sequences that enhances vector transduction efficiency, a part of the

gag region, the RRE (Rev Responsive Element) sequence and the WPRE (Woodchuck Hepatitis C Virus Post-transcriptional Regulatory Element) sequence that increases transgene expression in target cells. The presence of the SV40 origin of replication allows the efficient replication of this plasmid in cells constitutively expressing the SV40 T-antigen, as the 293T cells (see above, paragraph 3.1).

pptCMVNEDD4HAWTWPRE/pptCMVNEDD4HAC/SWPRE: these vectors were generated in this thesis work starting from the pptCMV-EGFP WPRE and additional appropriate plasmids as explained later (see paragraph 4.1.1 of the Results section). These lentiviral constructs express the NEDD4 ubiquitin ligase either WT (pptCMVNEDD4HAWTWPRE) or bearing the inactivating mutation $C_{867}S$ (pptCMVNEDD4HAC/SWPRE) both enriched with an HA epitope at the 3' end, under the transcriptional control of the HCMV promoter.

pptCMVHECTHAWTWPRE/pptCMVHECTHAC/SWPRE: these vectors were generated in this thesis work starting from the pptCMV-EGFP WPRE and additional appropriate plasmids as explained later (see paragraph 4.1.2 of the Results section). These lentiviral constructs express the NEDD4 ligase catalytic domain either WT (pptCMVHECTHAWTWPRE) or bearing the inactivating mutation $C_{867}S$ (pptCMVHECTHAC/SWPRE) both enriched with an HA epitope at the 3' end, under the transcriptional control of the HCMV promoter.

pptCMVNac32HECTHAWTWPRE/pptCMVNac32HECTHAC/SWPRE: these vectors were generated in this thesis work starting from the pptCMVEGFPWPRE and additional appropriate plasmids as explained later (see paragraph 4.1.3 of the Results section). These lentiviral constructs encodes for the single chain fragment variable (scFv) Nac32 (see paragraph 1.6.1 of the Introduction) fused in frame with the HECT catalytic region of the NEDD4 ligase, respectively in the WT (pptCMVNac32HECTHAWTWPRE) or bearing the inactivating mutation $C_{867}S$

(pptCMVNac32HECTHAC/SWPRE) enriched with an HA epitope at the 3' end, under the transcriptional control of the HCMV promoter

pptCMVd10HECTHA-WT-WPRE/pptCMVd10HECTHAC/SWPRE: these vectors were generated in this thesis work starting from the pptCMVEGFPWPRE and additional appropriate plasmids as explained later (see paragraph 4.1.4 of the Results section). These lentiviral constructs encodes for the single chain fragment variable (scFv) d10 (see paragraph 1.6.1 of the Introduction) fused in frame with the HECT catalytic region of the Nedd4 ligase, respectively in the WT (pptCMVd10HECTHAWTWPRE) or bearing the inactivating mutation $C_{867}S$ (pptCMVd10HECTHAC/SWPRE) enriched with an HA epitope at the 3' end, under the transcriptional control of the HCMV promoter

pptCMVd5eHECTHAWTWPRE/pptCMVd5eHECTHAC/SWPRE: these vectors were generated in this thesis work starting from the pptCMVEGFPWPRE and additional appropriate plasmids as explained later (see paragraph 4.1.5 of the Results section). These lentiviral constructs encodes for the scFv d5e (see paragraph 1.6.1 of the Introduction) fused in frame with the HECT catalytic region of the NEDD4 ligase, respectively in the WT (pptCMVd5eHECTHAWTWPRE) or bearing the inactivating mutation C867S (pptCMVd5eHECTHAC/SWPRE) enriched with an HA epitope at the 3' end, under the transcriptional control of the HCMV promoter

pptCMVaSWT3'FWPRE/pptCMVaSA53T3'FWPRE: these vectors were generated in this thesis work starting from the pptCMVEGFPWPRE and additional appropriate plasmids as explained later (see paragraph 4.1.6 of the Results section). These lentiviral constructs encode for either the wild-type (pptCMVaSWT3'FWPRE) or the A53T mutant form (pptCMVaSA53T3'FWPRE) of the aS encoding sequence fused in frame with Flag tag, under the transcriptional control of the HCMV promoter

pptCMVGFPasynWTWPRE/pptCMVGFPasynA53TWPRE: these vectors were generated in this thesis work starting from the pptCMVEGFPWPRE and additional appropriate plasmids as explained later (see paragraph 4.1.7 of the Results section).

These lentiviral constructs encode for either the wild-type (pptCMVGFPaSWTWPRE) or the A53T mutant form (pptCMVGFPaSA53TWPRE) of the aS encoding sequence fused in frame with EGFP encoding sequence, under the transcriptional control of the HCMV promoter.

3.4 Packaging constructs

pMDL: this plasmid contains the *gag, pol* regions of the HIV-1 genome under the transcriptional control of the HCMV promoter. The RRE sequence and the polyadenylation signal from the human β-globin gene are also present.

pVSVG, this plasmid encodes the Vesicular Stomatitis Virus envelope glycoprotein G (VSV-G) gene under the transcriptional control of the HCMV promoter along with the polyadenylation signal from the human β-globin gene.

pRSV-Rev: this plasmid encodes for the HIV-1 REV protein under the transcriptional control of the Rous Sarcoma Virus (RSV) promoter along with and the polyadenylation signal from the human β-globin gene.

All the packaging plasmids were kindly provided by Prof. Naldini (San Raffaele Milano, Italy).

3.5 Bacterial strain

In this experimental work the *E. coli* strain DH5α (chromosomal genotype: fhuA2 lac(del)U169 phoA glnV44 Φ80' lacZ(del)M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17) was employed for cloning and DNA plasmid preparation.

3.6 Oligonucleotides

In this experimental work, the following oligonucleotides were employed (Table 2):

Name	Sequence 5'-3'	Use
bACT Fw	CCATCGAGCA CGGCA	RT- PCR
bACT Rv	CAGGATGGCATGGGG	RT- PCR
BGH	TAGAAGGCACAGTCGAGGC	Cloning; sequencing;
CMV Fw	CGCAAATGGGCGGTAGGCGTG	Cloning; sequencing;
d5e Fw	TTGACTACTGGGGCCAGGGA	Sequencing
d5e Rv	AGGATGGAGACTGGGTCATCT	Sequencing
d10 Fw	AAGGTACCCTGGTCACCGTCT	Sequencing
d10 Rv	TGTCTCCTACAGATGCAGACA	Sequencing
Foxa2 Fw	CTGGTCGTTTGTTGTGGCTG	RT-PCR
Foxa2 Rv	GGAGGAGTAGCCCTCGG	RT-PCR
GAPDH Fw	ATGCTTGACAATT	RT-PCR
GAPDH Rv	TGCTC ACCTTGCCC	RT-PCR
HECTFw	CGCGGATCCGCCACCATGTTTGAAATGAAACTTC GCCGAGCAACT	Cloning; sequencing
Ki-HECT Fw	CGCGGATCCCTTTGAAATGAAACT	Cloning; sequencing
hSynFW	GCCACCATGGATGTATTCATGAAAG	Cloning; sequencing;
hSyn3'FlagRv	AGCGGCCGCTTACTTATCGTCGTCATCCTTGTAAT CGGCTTCAGGTTCGTAGTC	Cloning; sequencing;
Lmx1B Fw	CTTCAACCAGCCTCAGCGACT	RT- PCR
Lmx1B Rv	TCAGGAGGCGAAGTAGGAAC	RT- PCR
Map2 Fw	GACTGCAGCTCTGCCTTTAG	RT- PCR
Map2 Rv	AAGTAAATCTTCCTCCACTGTGAC	RT- PCR
M13F	GTAAAACGACGGCCAG	Cloning; sequencing;
M13R	CAGGAAACAGCTATGAC	Cloning; sequencing;
Nac32 Fw	TTCTAGGATCCGGTGGCGGT	Sequencing

NaC32 Rv	AGGGTGGCTCTTTCCCCTGGA	Sequencing
Nanog Fw	TCCAACATCCTGAACCTCAG	RT- PCR
Nanog Rv	GACTGGATGTTCTGGGTCTG	RT- PCR
Nestin Fw	CTCCAGCTTTCAGGACCCCAA	RT- PCR
Nestin Rv	GTCTCAAGGGTAGCAGGCAA	RT- PCR
Nedd4Fw	CGCGGATCCGCCACCATGGCAACTTGCGCGGTGG AGGTGT	Cloning; sequencing;
Nedd4HARv	GACGTCGACCTAAGCGTAATCTGGGACGTCGTAT GGGTAATCAACTCCATCAAAGCCCTGGGTGTT	Cloning; sequencing;
Oct4 Fw	GTGGAGGAAGCTGACAACAA	RT- PCR
Oct4 Rv	CAGGTTTTCTTTCCCTAGCT	RT- PCR
Pax6 Fw	TCTTTGCTTGGGAAATCCG	RT- PCR
Pax Rv	CTGCCCGTTCAACATCCTTAG	RT- PCR
Sox1 Fw	CAACCAGGACCGGGTCAAAC	RT- PCR
Sox1 Rv	CCTCGGACATGACCTTCCAC	RT- PCR
TH Fw	TCATCACCTGGTCACCAAGTT	RT- PCR
TH Rv	GGTCGCCGTGCCTGTACT	RT- PCR
Tubulin III Fw	GGCCAAGTTCTGGGAAGTCAT	RT- PCR
Tubulin III Rv	CTGAGGCACGGTACTTGTACTTGTGA	RT- PCR
T7	TAATACGACTCACTATAGGG	Cloning; sequencing;
WPRE Rev	CATAGCGTAAAAGGAGCAACA	Cloning; sequencing;

Table 3. **Oligonucleotides employed in the experimental protocols.**

Methods

3.7 Chemical transformation of bacteria

Plasmids or ligations were transformed into chemically competent bacteria *E.coli* strain DH5α (BiolabsInc). Briefly, 250 ng of plasmids or the ligation mix were adding at the appropriate volume of competent bacteria (50 µl of bacteria). After 30 minutes on ice, an incubation of 2 minutes at 37℃ was performed followed by 1 minute on ice. Next, after resuspension of bacteria in 150 µl of Luria-Bertani (LB) medium (for 1 L: 10g Bacto-tryptone, 5g Bacto-Yeast extract, 10g NaCl) without antibiotics followed by 1 hour of incubation at 37℃ with shaking at 300 rpm, the bacteria were plated on LB agar plates with the appropriate antibiotic for selection (usually 0.1 mg/ml ampicillin) and incubated at 37℃ overnight.

3.8 Mini preparation of plasmid DNAs

Bacteria were grown overnight in 5 ml of LB medium with appropriate antibiotics at 37℃ (plasmid DNA) for mini DNA preparation. The next day, the bacteria cultures were centrifuged at 4500 rpm for 10 minutes at 4℃ and the bacterial pellet was resuspended in 300 µl of solution 1 (50 mM Tris-HCl pH 8.0, 10 mM EDTA and RNAse with final concentration 100 µg/ml) and transferred in tube. 300 µl of solution 2 (200 mM NaOH, 1% w/v SDS) were added, mixed by turning gently for five times and incubated at room temperature for 5 minutes. 300 µl of cold solution 3 (3 M Potassium Acetate pH 5.5) were added, the tube inverted for five times and incubated on ice for 20 minutes. After a centrifugation at 13000 rpm for 20 minutes at 4℃, 800 µl of supernatant were transferred in a new tube and 450 µl of isopropanol were added. The plasmid DNA was precipitated by centrifugation at 13000 rpm for 20 minutes at 4℃ and the DNA pellet was washed with 500 µl of 70 % v/v ethanol and centrifuged at 13000 rpm for 10 minutes at room temperature. The pellet was dried at room temperature and suspended in 30 µl of Tris-EDTA buffer pH 8 (10 mM TrisHCl pH 8, 1 mM EDTA pH 8).

3.9 Maxi preparation of plasmid DNAs

QIAGEN Plasmid MAXI kit" (QIAGEN), was used for maxi preparation of plasmid DNA, following the manufacturer's instructions. Briefly, 5 ml of LB medium supplemented with 0.1 mg/ml ampicillin were inoculated with the bacteria and incubated overnight at 37℃. The next day, bacteria were resuspended and inoculated overnight in 500 ml of LB medium supplemented with 0.1 mg/ml ampicillin at 37 °C. The next day, following a centrifugation at 4000 rpm for 15 minutes at 4℃, the bacteria pellet was resuspended in 10 ml of RNase A-resuspension solution along with 10 ml lysis buffer. Five minutes later, 10 ml of neutralization buffer were added, and the tube was inverted and the solution was incubated for 15 on ice. Following a centrifugation at 14000 rpm for 30 minutes, the supernatant was transferred into a column pre-treated with the column preparation solution. Next, the column was washed with 60 ml of washing. Finally, 15 ml of elution solution were added to the column along with 10 ml of isopropanol to precipitate the DNA and a centrifugation was performed 13000 rpm for 10 minute. Tris-EDTA buffer pH 8 (10 mM TrisHCl pH 8, 1 mM EDTA pH 8) was used to elute the purified plasmid DNA.

3.10 Sequencing

In order to verify the correct sequence of all generated recombinant constructs, Sanger sequencing was performed using the Big Dye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) according to the manufacturer's instructions. Each sequencing reaction included 200-250 ng of plasmid DNA as template, 1 µl of 1 µM primer, 1 µl of 5X buffer (200mM Tris-HCl, pH 9, 5 mM $MgCl_2$), 1 µl of Big Dye® mix, containing, dNTPs and dideoxynucleoside triphosphates (ddNTPs) for DNA chain termination, and diethyl-pyrocarbonate (DEPC) water to a final volume of 10 µl. Cycling parameters of sequencing PCR reactions were as follows:

1 cycle: 95 °C for 10 minutes

35 cycles: 95 °C for 10 seconds
50 °C for 10 seconds
62 °C for 4 minutes

1 cycle : 72 °C per 7 minutes

Products of sequencing reactions were purified by precipitation first in 50 µl of ethanol 96% (v/v) and 2 µl of sodium acetate (3 M, pH 5.2) and, secondly, in 150 µl of ethanol 70% (v/v). Samples were then resuspended in 15 µl of formamide and denatured at 95 °C for 4 minutes. Capillary electrophoresis of DNA extension fragments was finally performed in the automated sequencer ABIPRISM 3100 Genetic Analyzer (Applied Biosystems). The primers employed for sequencing are reported in Table 3.

3.11 Cloning methods

In order to generate the vectors reported at the paragraph 4.1of the Results sections of this thesis, the following molecular biology protocols were adopted:

a) Enzymatic restriction

The enzymatic restriction was performed in a total volume of 20 µl usually containing 200 ng of DNA, 1x enzymatic buffer and 1U/ µg of endonuclease (New England Biolabs, 20,000 units/ml). The restriction was carried on for 1 hour at 37 °C and the endonucleases were inactivated by heat (72 °C for 5 minutes) according to the manufactures' instructions. The resulting size bands were verified in electrophoresis agarose gel.

b) DNA Polymerase I Large fragment (Klenow) reaction

The Klenow (New England BioLabs®, Inc.) is used for blunting ends by 3’ overhang removal and fill-in of 3' recessed (5' overhang) ends. The reaction was performed using T4 DNA Ligase Buffer supplemented with 33 μM each dNTP along with the Klenow (1U/μg). The reaction was incubated for 15 minutes at 25°C and EDTA 10 mM and heating for 20 minutes at 75°C was used to block the reaction

c) Calf Intestinal alkaline Phosphatase (CIP) reaction

The CIP (New England BioLabs®, Inc.) and to prevent re-circularization and re-ligation of linearized cloning DNA by non-specifically catalysing the dephosphorylation of 5′ and 3′ ends of DNA and RNA phosphomonoesters. The reaction was performed using CutSmart® Buffer (New England BioLabs®, Inc.) supplemented along with the CIP (1U/μg). The reaction was incubated at 37°C for 30 minutes and heating at 56°C for 15 minutes was used to block the reaction.

d) Ligation

The ligation was performed in a total volume of 30 μl usually containing 100 ng of the digested vector, 150 ng of digested insert, 1x ligation buffer and 50 units of T4 ligase (Fermentas, 50 units/μl). Ligations were carried over night at 16°C.

e) PCR

Each PCR reaction was performed in a thermal cycler (Master Cycler Personal; Eppendorf) using the following thermal profile: 95 °C for 5 minutes, 95 °C for 30 seconds (denaturation phase), X °C (depending on the specific primer pairs) for 30 seconds (annealing phase), 72 °C for 30 seconds (extension phase) and 72 °C for 10 minutes (inactivation phase). The size bands were verified in electrophoresis agarose gel.

f) DNA extraction from agarose gel

The correct fragments, resulting after PCR reaction or enzymatic digestion, were purified from agarose gel using Qiagen Gel Extraction Kit, following the manufactures' instructions.

g) DNA quantification

Plasmid DNA quantification was performed by spectrophotometer reading (NanoDrop®ND-1000), the absorbance was red at a wavelength of 260 nm and the DNA concentration was calculated using the Lambert-Beer law. To consider a good quality of the sample, the A260 / A280 ratio has to be 1.8 - 2.0 values.

3.12 Production, concentration and titration of recombinant lentiviral particles

In order to produce recombinant lentiviral particles, 293T cells (11×10^6) were seeded in T_{150} tissue culture flask 24 hours before transfection by a slightly modified calcium phosphate method. Specifically, cells were co-transfected with 20 µg of the appropriate gene transfer vector, 10 µg of each packaging plasmid (pMDL, pVSVG and pRSV-Rev, previously diluted in TE 1:10 (TE: Tris-HCl 1 mM pH 8, EDTA 0.5 mM pH 8) up to a final volume of 1800 µL. Subsequently, 200 µL of 2.5 M $CaCl_2$ was added to the DNA and then 2 mL of 2X HPB buffer pH 7.1 (5M NaCl, 0.5 M HEPES pH 7.1, 0.15 M $Na_2HPO4\ 7H_2O$) were added dropwise into the precipitate. Finally, the mixture was added dropwise to the cell culture, that was incubated 3 to 4 hours at 37 °C. Next, cells were gently washed twice with 10 mL of phosphate buffer saline (PBS: 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4) and fresh growth medium was replaced. The culture supernatants were collected on day 2 post-transfection, filtered with a 0.45-µm-pore-size membrane (Millipore), concentrated 50 times by ultracentrifugation (27000 rpm, 2 hours, 4 °C in a Beckman SW28 rotor) or using the VivaSpin column (Sartorius VS2042) and stored at -80 °C until use.

The infectious titre of lentiviral particles expressing WT and A53T GFP-aS was determined by flow cytometry analysis of the EGFP expression, after transduction of 293T cells with different amount of lentiviral particles.

Briefly, 5×10^5 cells were seeded per well in 6-well plates in 2 mL of DMEM medium supplemented with FBS 10% v/v, 24 hours prior to transduction. The next day, medium was replaced with 0.5 mL of DMEM medium supplemented with FBS 10% v/v, containing serial dilutions of the lentiviral stocks. 0.5 mL of fresh culture medium was added to the cells approximately 8 hours later. Three days after transduction, cells were detached from the tissue culture dish and transferred into tubes, washed twice with PBS and by centrifugation at 1200 rpm at 4 °C for 7 minutes and resuspended in 500 µL of cold PBS to evaluate the EGFP expression by flow cytometry. Samples were acquired on FACSCalibur (Berton Dickinson) and the data analysis was performed with Flowing software®.

The infectious titre was expressed as transducing units/mL (TU/mL) and was calculated according to the following formula:

$$\frac{\left(\frac{\%\ \text{eGFP positive cells}}{100}\right) \text{X transduced cell number X diluition factor}}{\text{Lentiviral particle diluition volume (ml)}}$$

3.13 Transduction of different cell types with recombinant lentiviral particles

In order to transduce the recombinant lentiviral particles, cells were seeded ($2.7\text{X}10^4$ cells/cm^2) in the appropriate medium and were incubate at 37 °C for 16 hours. Next, recombinant lentiviral particles were added onto cells, in which previously the medium was halved. After 2- 8 hours post-transduction complete medium was added to the appropriate final volume. The analysis of proteins expressed from lentiviral particles were evaluated 48 hours post-transduction.

3.14 Protein extraction, quantification and Western Blotting analysis

For protein analysis, cells, were harvest from the plate and centrifugate for 1100 rpm at 4 °C for 5 minutes. Next, 300 µl radio-immunoprecipitation (RIPA) buffer 1X (PBS 1X, 1% v/v NP40, 0.5% v/v Sodium Deoxycholate, 0.05% v/v Sodium Dodecyl Sulphate) were added along with protease inhibitors (Complete, Roche). Samples were kept in ice for 30 minutes and after, centrifuged at 13000 rpm for 30 minutes at 4 °C and the supernatants were collected and stored at -20 °C. Proteins were quantified by colorimetric bicinchoninic acid (BCA) assay (Pierce BCA Protein Assay Kit, ThermoFisher), following the manufacturer's instructions. Samples were mixed with 2 x SDS sample buffer (0.5 M Tris-HCl pH 6.8, 10% SDS, 10% v/v glycerol, 10% v/v β-mercaptoethanol, 1% v/v bromophenol blue) and incubated at 100 °C for 5 min. Twenty µg of the whole cell lysate of each sample was loaded on a 10-15 % SDS-PAGE gel, depending on the size of the protein of interest. As a standard, *Page Ruler Prestained Protein Ladder* (Thermo Scientific) was loaded.

SDS-PAGE gels were prepared, run and blotted as described in Molecular Cloning (Sambrook & Russell, 2001), by adopting the appropriate buffers. For Western Blotting the gels were blotted into nitrocellulose membrane (N+ bond, Amersham) and incubated in blocking solution [5% w/v nonfat dry milk (NFDM), 0.1% v/v Tween in Tris Buffered Saline (TBST) 1X (for 1L of TBS 10X: 24g Tris Base and 88g NaCl in water)], 1 hour at room temperature. The primary antibody was applied over night at 4 °C rotating. Blots were washed twice for 10 min with TBST and incubated with the secondary antibody for 1 hour at room temperature while rotating. Finally, the membrane was developed by chemiluminescence method (ECL, Sigma), following the manufacturers' instructions and images were captured using Hyper Film MP (GE Healthcare, Italy). The following primary antibodies diluted in 5% NFDM/TBS-T were used in this experimental work:

- Anti-HA (Covance, MMS-101P): mouse affinity purified anti-HA antibody against the epitope HA, dilution 1:1000.

- Anti-tub (Sigma, T8328): mouse affinity purified anti-tubulin used as loading control, dilution 1:1000
- Anti-aS (Abcam, ab138501): rabbit affinity purified antibody against the aS, dilution 1:10000.
- Anti-aS (Neomarkers, MS-1572-PABX) mouse affinity purified antibody against the aS, dilution 1:10000.
- Anti-Flag (Sigma, F1804) mouse affinity purified anti- flag antibody against the epitope flag, dilution 1:1000.

The α-Rabbit and a-Mouse IgG HRP (GE Healthcare) secondary antibodies, diluted 1:5000 in 5% NFDM/TBS-T were employed for 1 hour at room temperature.

3.15 Immunofluorescence

To investigate intracellular localization of the proteins of interest, immunofluorescence was performed. Cells were transduced according the transduction protocol (as reported in paragraph 3.13 of the Methods). Cells were fixed in paraformaldehyde 4% for 20 minutes and permeabilized with Triton X-100 0.1% in PBS for 15 minutes. Cells were then incubated overnight with the following primary antibodies:

- anti-Pax6 (Sigma, HPA030775) 1:200
- anti-Nestin (Sigma, N5413) 1:200
- anti-Lmx1A (Abcam, ab139726) 1:200
- anti-Foxa2 (Abcam, ab108422) 1:200
- anti- TH (Abcam, ab75875) 1:200

All the antibodies were diluted in PBS. The following day cells were incubated with the secondary antibody anti-Rabbit (Alexa Fluor 568, Thermo Fisher) or anti-Mouse (FITCH, Santa Cruz Biotechnology) 1:500 for 1 hour at room temperature (RT). Nuclear staining was performed with DRAQ5 (Cell Signaling, cat. #4084S), diluted

1:1250 in PBS for 15 minutes at RT for confocal microscope observation. Microscope observation was performed on Leica Epifluorescence DC100 or on a Leica TCS confocal microscope (Leica Microsystems AG, Wetzlar, Germany).

3.16 FACS analysis

In order to analyse the amount of the expression of a specific protein in the cellular environment, cells were transduced with recombinant lentiviral particles (as reported in the paragraph 4.7 of the Method). The cells were collected and pelleted at 1200 rpm for 10 minutes at 4 °C then, after washings two times in cold PBS, resuspended in 500 µl of PBS and analyzed at the flow cytometer (FACSCALIBUR; Beckton & Dickinson). For the analysis of the EGFP protein, it does not require the presence of any other substrate or cofactor, since it absorbs at a wavelength of 395 nm in the visible and has its maximum emission peak at the wavelength of 508 nm in the channel of the FL-1 (green). For the analysis of FOXA2, cells were fixed in paraformaldehyde 4% v/v at 37 °C for 10 minutes and permeabilized with Methanol 90% v/v at -20 °C over night. Next, cells were rinse with PBS and incubated with anti-Foxa2 (Abcam, ab108422) 1:250 primary antibody for 1 hour at room temperature and then cells were incubated with anti-Rabbit (Alexa Fluor 568, Thermo Fisher) 1:500 secondary antibody for 1 hour at room temperature.

3.17 Extraction of total RNA

The total RNA was extracted from neural stem cells (NSCs) using high affinity columns consisting of a silica gel (RNeasy Mini Kit Qiagen), following the manufacturer's instruction. These columns allow to purify the RNAs of a length greater than 200 bases directly from a cell lysate. The experimental procedure is based on the cell lysis of cells in the presence of a highly denaturing buffer containing guanidine isothiocyanate, which inactivates cellular RNase, and β-mercaptoethanol, able to reduce disulphide bridges. After the addition of 70% ethanol

(v/v), cell lysates were transferred to the column, that was centrifuged a 13000 rpm for 15 seconds, followed by washings with appropriate buffers. Finally, the RNA was eluted in 30 µl of water supplemented with diethylpyrocarbonate (DEPC). Total RNA extracted was incubated at 37 °C for 30 minutes with 30 U of DNase - RNase free (Roche), in order to digest any genomic cellular DNA remained in the eluate. Next, an incubation at 95 °C for 15 minutes was carried out to inactivate the DNase followed by 2 minutes on ice. Total RNA was quantified by reading the optical density at the spectrophotometer (NanoDrop ND-1000) and stored at -80 °C.

3.18 Reverse Transcriptase (RT)-PCR

The retrotranscription of the extracted RNA was performed by preparing the following mix:

Total extracted RNA	2 µg
Buffer 10X without $MgCl_2$ (Applied Biosystem)	5 µl
$MgCl_2$ 50 mM	11 µl
dNTPs 2.5 mM	10 µl
RNase IN 20U/µl	1.5 µl
MuLv (50 Units/µl) (Applied Biosystem)	1.5 µl
Random primers (50µM, Invitrogen)	2.5 µl
H_2O diethylpyrocarbonate (DEPC)	To 50 µl

The retrotranscription reaction it was carried out in the thermal cycler (Master Cycler Personal; Eppendorf) by adopting the following program: 25 °C for 10 minutes (annealing phase), 42 °C for 60 minutes (extension phase) and 95 °C for 5 minutes (inactivation phase).

The cDNA obtained by reverse transcription was employed for amplification by PCR end point by preparing the following mix:

cDNA	10 µl
Buffer 10X with $MgCl_2$ (Applied Biosystem)	5 µl
TaqGold (5 U/ µl, (Applied Biosystem)	0.5 µl
dNTPs 0.625 mM	5 µl
Primers 10 µM (forward and reverse)	2.5 µl
H_2O diethylpyrocarbonate (DEPC)	To 50 µl

The sequence of the primers used to evaluate the expression of tubulin III, Nestin, Pax6, Lmx1B mRNAs are reported in the table 3 of this section thesis.

The amplification was performed in the thermal cycler (Master Cycler Personal; Eppendorf) using the following program: 95 °C for 5 minutes, 95 °C for 30 seconds (annealing phase), 50 °C for 30 seconds (extension phase) and 72 °C for 30 seconds (inactivation phase) and the size bands were verified in electrophoresis agarose gel.

3.19 Immunoprecipitation and co-immunoprecipitation

Immunoprecipitation and co-immunoprecipitation of Flag-tagged proteins were performed by FLAG® Immunoprecipitation Kit (Sigma FLAGIPT1), following the manufacturer's instruction. Briefly, 293T cells (1.2 $x10^6$/well) were grown in 6-well tissue culture plates and transduced/transfected as requested by the specific experiment. After the appropriate time post-transduction/transfection, cells were lysed with a buffer (50 mM Tris HCl, pH 7.4, 150 mM NaCl, 1mM EDTA, 1% Triton X-100). Next, columns containing the ANTI-FLAG®-M2 affinity gel, an agarose resin coated with a highly specific anti-Flag monoclonal antibody were adopted. After formation of the antigen-antibody complex and appropriate washing

(washing buffer 10X: 0.5M Tris HCl, pH.4, 1.5 M NaCl), Flag-tagger protein dissociation from the immune complex was achieved by using the sample buffer (2X: 125 mM Tris HCl, pH 6.8, 4% SDS, 20% (v/v) glycerol, 0.004% (w/v) bromophenol blue).

3.20 Proximity Ligation Assay (PLA)

MN9Dsyn cells (4 x 10^4 cells/plate) were grown on polyethyleneimine-coated (PEI) glass coverslips in 24-well plate (Nunclon™). The next day, cells were transduced with the appropriate quantities of lentiviral particles expressing the ubiquibodies and after 24 hours, were treated in the absence or presence of doxycycline (DOX). After 24 hours, cells were fixed in paraformaldehyde (PFA) 4% for 20 minutes and permeabilized with Triton X-100 0.1% in PBS for 15 minutes and were processed according to Duolink® manufacturer's protocol. Briefly, cells were incubated with blocking solution at 37°C in a humidity chamber for 60 minutes. Later, cells were incubated with the primary antibodies overnight at 4°C. The next day, cells were washed twice for 5 min with buffer A, followed by incubation with the PLA probes (secondary antibodies against two different species bound to two oligonucleotides: anti-mouse MINUS and anti-rabbit PLUS) in antibody diluent for 60 min at 37°C. After two washes of 5 min with buffer A, the ligation step was performed with ligase for 30 min at 37°C. In the ligation step, the two oligonucleotides in the PLA probes are hybridized to the circularization oligonucleotides. The cells were washed with buffer A twice for 2 min before incubation for 100 min with amplification stock solution at 37°C. The amplification stock solution contains polymerase for the rolling circle amplification step and oligonucleotides labelled with fluorophores, which will bind to the product of the rolling circle amplification and thus allow detection. Finally, after two washes of 10 min with buffer B the cover slips were washed with PBS and mounted with Duolink® in situ mounting medium containing DAPI. For every antibody, a negative control experiment was performed where only one

antibody was incubated with the PLA probes. The experiments were performed three times. Primary antibodies utilized were human synuclein (syn211, Neomarkers, MS-1572-PABX)1:1000 diluted, anti HA (Covance, PRB-101P) 1:250 diluted, and glutamine synthetase (Abcam, ab-16802) 1:250 diluted. The data were analyzed using GraphPad Prism. One-way ANOVA followed by Bonferroni post hoc test was performed to compare the PLA puncta in soma/processes of negative control and samples. Data were means ± standard deviation of three independent experiments in triplicates, unless otherwise stated. Differences were considered significant when $p < 0.05$.

4. Results

4.1 Generation of lentiviral vectors expressing chimeric NEDD4-based recombinant ubiquitin ligases

The first goal of this project was to generate chimeric ubiquitin ligases, herein named ubiquibodies, consisting of well-characterized single chain fragment variables (scFvs) directed towards human aS, fused in frame to the catalytic domain HECT of NEDD4. The ectopic expression of those recombinant proteins should result into ubiquitination and subsequent degradation of the aggregated forms of aS. In particular, three different types of scFv were selected, each with a well characterized affinity for a different conformation of aS:

- Nac32, which specifically binds aS in monomeric form (Lynch et al., 2008).
- d5E, which binds the oligomeric form of aS (Zhou et al., 2004).
- d10, pan-specific, which binds both forms of aS with equal affinity (Zhou et al., 2004).

Recombinant proteins were expressed in the context of lentiviral vectors (LVs) due to the specific feature of these delivery systems that are particularly appropriate to achieve a long lasting and efficient expression of transgenes in the central nervous system (CNS). To this end the pptCMVEGFPWPRE, whose main characteristics are described at the paragraph 3.3 of the Materials, was selected.

4.1.1 Lentiviral vector expressing WT and C/S NEDD4

First of all, in order to develop appropriate tools to be adopted in the following experiments to control for ubiquibodies specificity, we developed LVs expressing the full length sequence encoding NEDD4 (NCBI Ref.Seq. NM_006154.3), both in the wild type (WT) and bearing a functional inactivating mutation within the HECT domain (C/S) fused in frame to the HA epitope and under the transcriptional control

of the HCMV promoter. The main steps of the adopted cloning strategy are reported in figure 9.

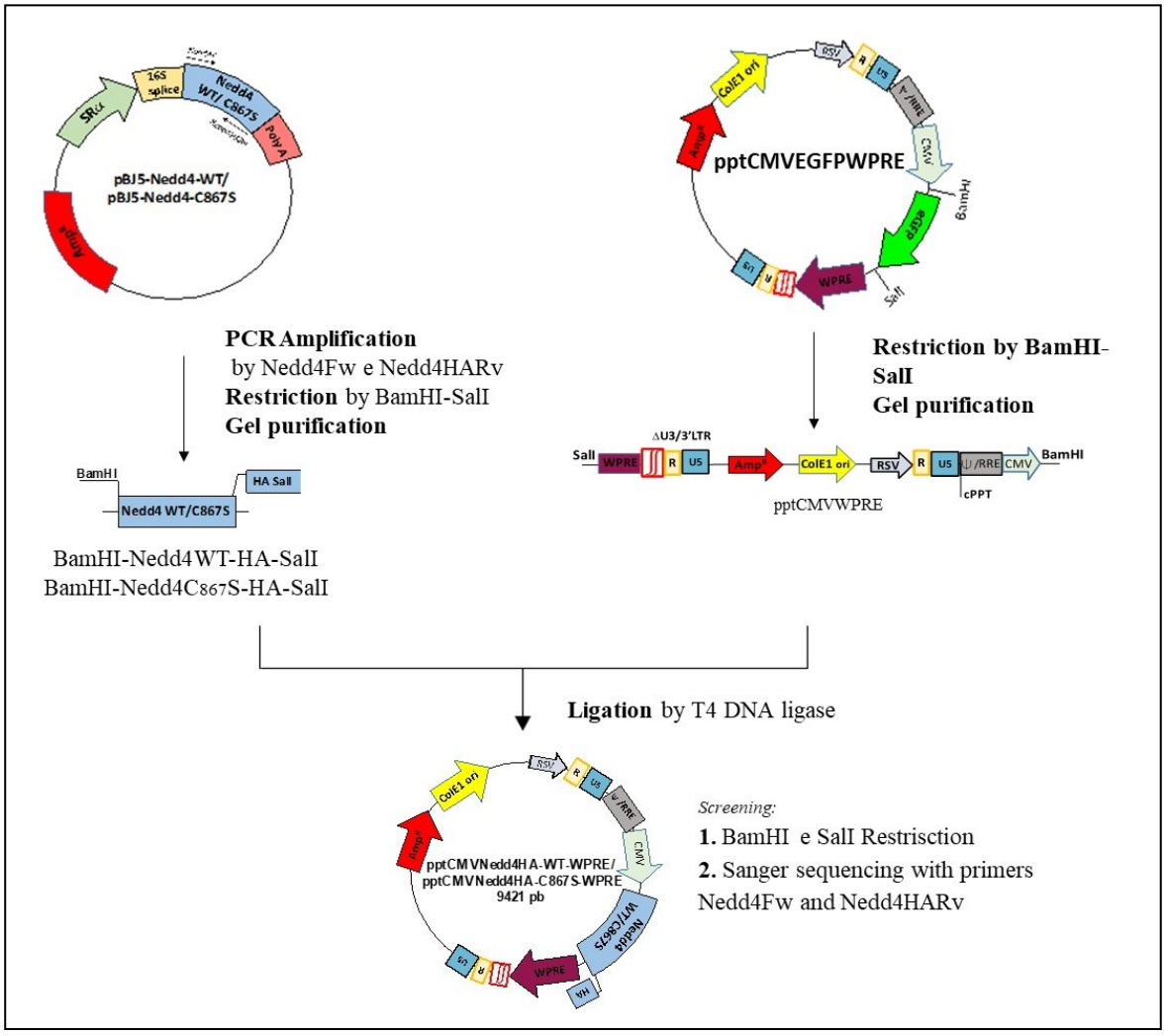


Figure 9. **Schematic representation of the main steps of the cloning strategy adopted to obtain the LVs expressing the NEDD4WT-HA / NEDD4C/S-HA sequences (pptCMVNEDD4WTHAWPRE/pptCMVNEDD4C/SHAWPRE)**

Briefly, we PCR amplified the appropriate sequences using the plasmids pBJ5-Nedd4-WT and pBJ5-Nedd4-C/S (see paragraph 3.2 of the Materials), already available in the laboratory, as a template and Nedd4Fw and Nedd4HARv as primers (see paragraph 3.11 of the Methods). The adopted oligonucleotides introduce BamHI and SalI restriction sites at 5' and 3' terminal ends of the PCR product, respectively.

Furthermore, the 5’ primer also introduced and optimized Kozak sequence, immediately upstream of the ATG start codon, while the 3’ primer introduces an HA tag. The PCR products BamHI-Nedd4WT-HA-SalI and BamHI-Nedd4C/S-HA-SalI, were analyzed in agarose gel in order to evaluate their correct molecular weight and subsequently subjected to enzymatic restriction by BamHI and SalI endonucleases. In parallel, the pptCMVEGFPWPRE was also digested with the same restriction enzymes, thus removing the EGFP encoding sequence (see figure 9). Upon ligation and bacterial transformation, DNA extracted from bacterial colonies was analysed both by enzymatic digestion and by sequencing using the Nedd4Fw and NEDD4HARv primers (table 3).

4.1.2 Lentiviral vectors expressing WT and C/S HECT

As an additional control, we developed LV expressing the isolated catalytic domain of NEDD4, HECT, both in WT and C/S form. The main steps of the adopted cloning strategy are reported in figure 10.

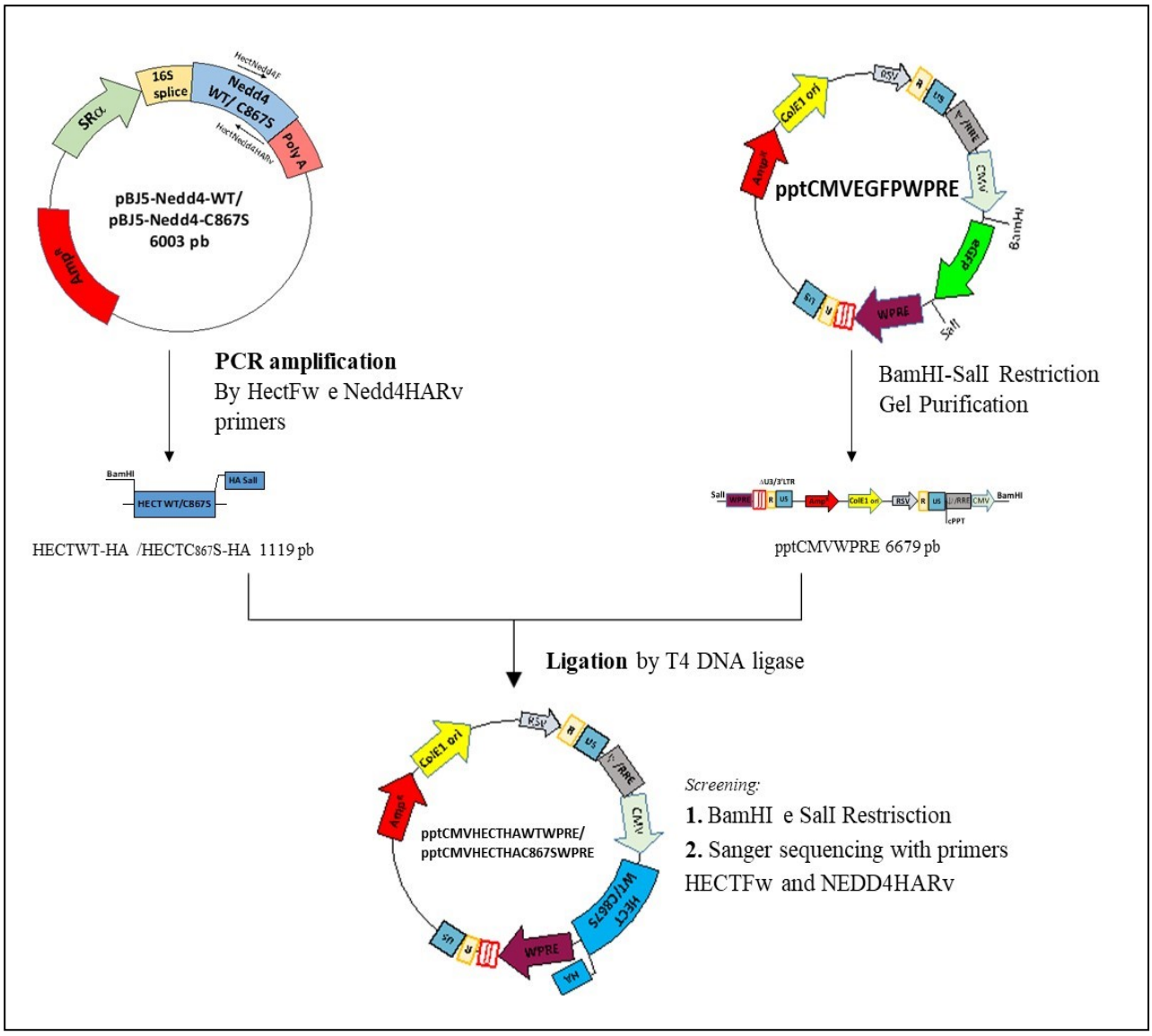


Figure 10. **Schematic representation of the main steps of the cloning strategy adopted to obtain the LVs expressing the HECTWT-HA / HECTC/S-HA sequences (pptCMVHECTWTHAWPRE/pptCMVHECTC/SHAWPRE)**

The appropriate sequences were amplified by using, as a template, the pBJ5-Nedd4-WT and pBJ5-Nedd4-C/S constricts , and by adopting the oligonucleotides HECTFw and Nedd4HARv (Table 3) as primers. Those oligonucleotides not only amplify the coding sequences of interest, but they also introduce in the PCR product the recognition sequence of BamHI and SalI at 5 ' and 3' terminal, respectively. Furthermore, an optimized Kozak sequence (at the 5' end) and the HA tag (at the 3'

end) are also introduced. The PCR products were analyzed in agarose gel to verify their molecular weight and the possible presence of amplification contaminants. Next, the gel-purified amplicons were subjected to enzymatic restriction by BamHI and SalI and ligate along with the pptCMVEGFPWPRE vector cut with the same restriction enzyme. DNA extracted from bacterial colonies was checked by enzymatic restriction and sequencing, by using HECTFw and NEDD4HARv oligonucleotides (table 3).

4.1.3 Lentiviral vector expressing WT and C/S ubiquibodies

In order to develop the LV expressing ubiquibodies, first of all we designed synthetic genes constituted by the Nac32, d10 and d5e scFv sequence, fused in frame with the GSGSG-S peptide linker. The scFv amminoacid sequences were obtained by different groups that have characterized them (Lynch et al., 2008; Zhou et al., 2004) and were converted into nucleotide sequences taking into account the human codon usage to optimize their expression. The linker peptide was added as it has been previously demonstrated that its presence allows the optimal flexibility of the scFv along with the activity of the protein fused to it (Reddy Chichili et al.,2013). The synthetic genes were generated by the Bio-Fab research Company (Rome, Italy), following our indications and inserted within the poly-linker of the prokaryotic plasmid pBluescriptSK+. Starting from those constructs, we generated a fusion between the scFv-GSGSG-S and the WT and C/S NEDD4 HECT domain, to be cloned within the pptCMVEGFPWPRE LV. To this end, particular attention was dedicated to the frame that needs to be maintained between the scFv-GSGSG-S encoding sequence and the WT and C/S HECT domain ones. The main steps of the adopted cloning strategy are reported in figure 11.

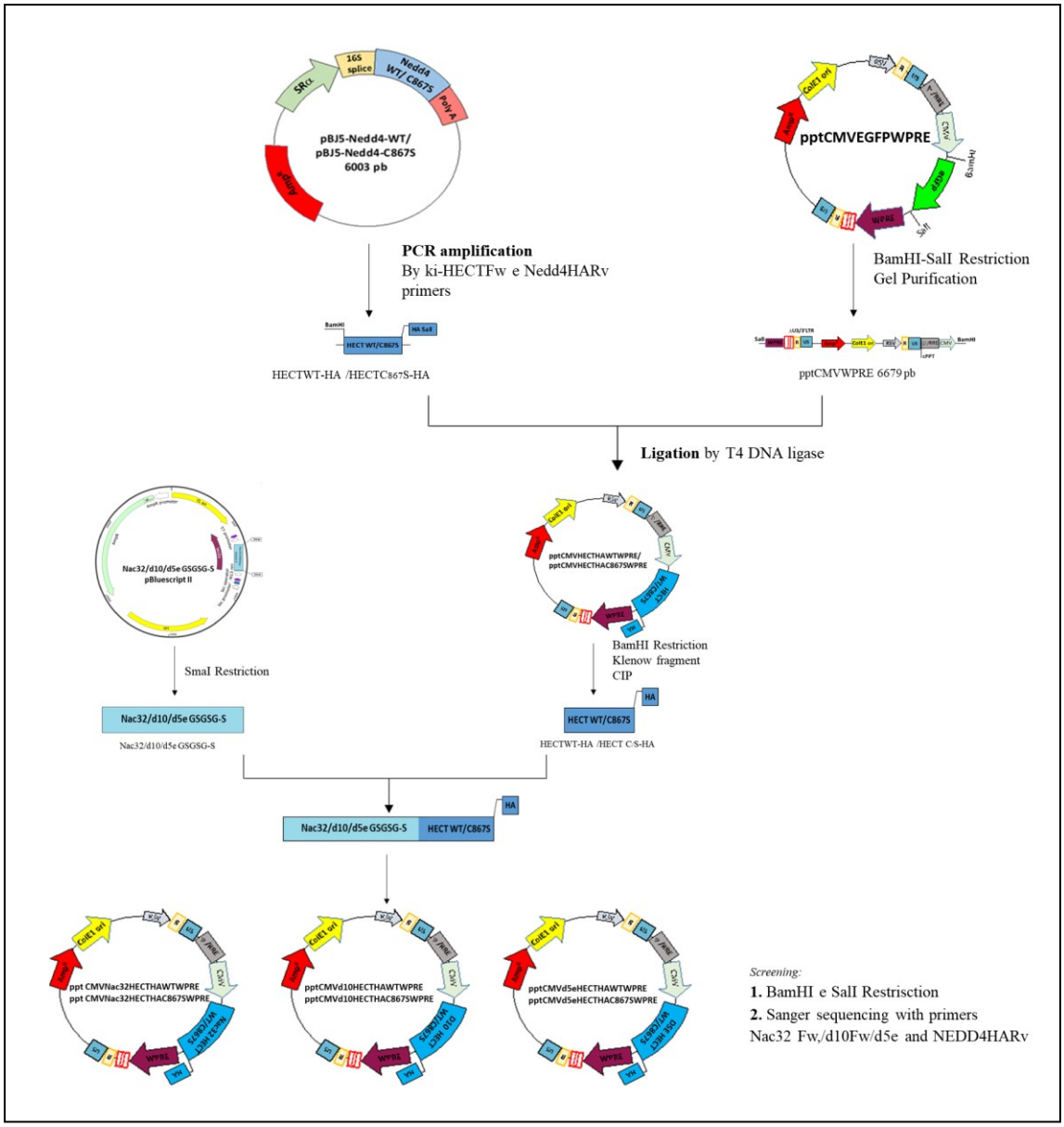


Figure 11. **Schematic representation of the main steps of the cloning strategy adopted to obtain the LVs expressing the Nac32/d10/d5eHECTWT-HA / Nac32/d10/d5eHECTC/S-HA sequences (pptCMVNac32HECTWTHAWPRE/ pptCMVNac32HECTC/SHAWPRE ; pptCMVd10HECTWTHAWPRE/ pptCMVd10HECTC/SHAWPRE; pptCMVd5eHECTWTHAWPRE/ pptCMVd5eHECTC/SHAWPRE)**.

Firstly, we cloned the WT and C/S HECT domain in the LV pptCMVEGFPWPRE. In this case, the adopted oligonucleotides in addition to the BamHI and SalI restriction sites introduces at the 5' of the amplified sequence the appropriate number of extra-nucleotides to maintain the frame at the end of the cloning procedure. To this

end, the HECT domain in the WT and C/S version was amplified from the pBJ5-Nedd4-WT and pBJ5-Nedd4-C/S plasmids by adopting ki-HECTFw and Nedd4HARv as primers (table 3). Once introduced within the pptCMVEGFPWPRE vector in place of the EGFP encoding sequence, following the same strategy as in the previous cloning, the correct plasmids were cut with BamHI and subjected to fill-in and dephosphorylation in order to create not sticky blunt ends.

As the same time, the scFvs-GSGSG-S, were excised from the plasmid BlueScriptKS+ by cutting with SmaI and a ligation was performed with the blunt end vectors. In this case, DNA extracted by bacterial colonies was carefully checked not only for the presence of the insert, but also for its correct orientation. The selected clones were also controlled by sequencing by adopting the Nac32Fw, d10Fw, d5eFw and NEDD4HARv (table 3).

All the developed LV expressing the ubiquibodies are reported in figure 12.

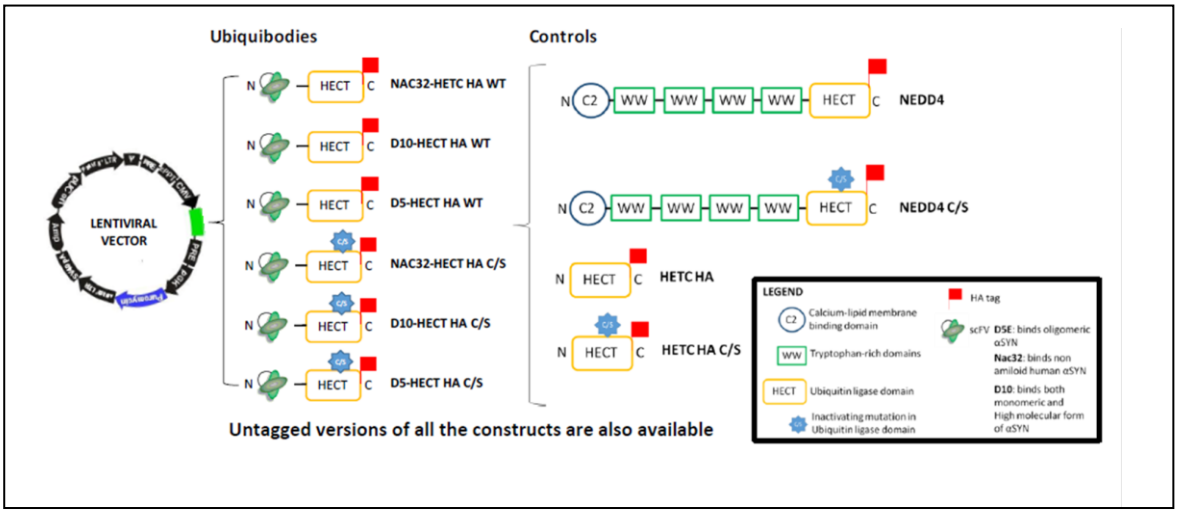


Figure 12. **Schematic representation of the developed ubiquibodies along with the map of the third-generations self-inactivating lentiviral vector employed for the cloning are reported.**

4.2 Developed recombinant LV efficiently express ubiquibodies in 293T cells

As a next step, we evaluated weather the developed LVs efficiently express ubiquibodies and whether the recombinant proteins were stable and of the expected size. To this end all the generated constructs were transfected in human embryonic

kidney 293T cells. Forty-eight hours post transfection cells were lysed and extracted proteins were run in an SDS-gel followed by Western Blotting. As shown in figure 16 all the constructs lead to the expression of a protein of the expected size:

- 100 kDa for NEDD4HAWT and NEDD4HAC/S;
- 71 kDa for Nac32HECTHAWT and Nac32HECTHAC/S;
- 69 kDa for d5eHECTHAWT and d5eHECTHAC/S;
- 68.5 kDa for d10HECTHAWT and d10HECTHAC/S.

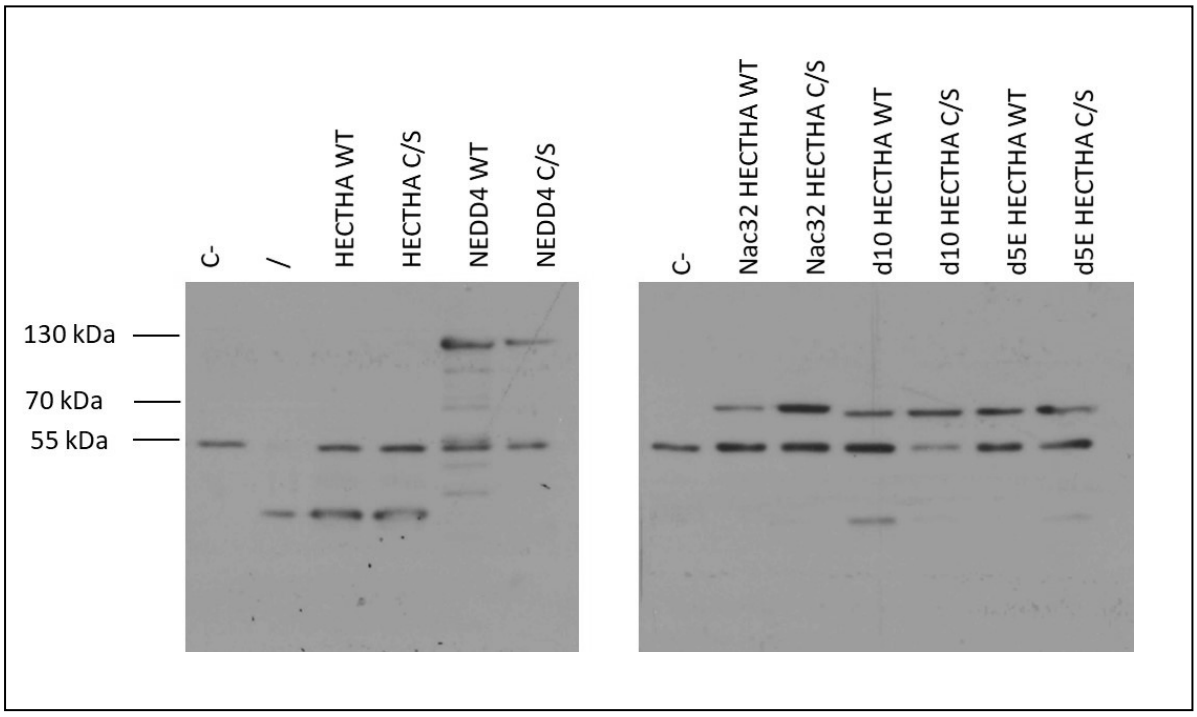


Figure 16. **The developed LV efficiently express ubiquibodies and their controls.** All generated LV (2.5 µg) were transfected in 293T cells by Lipofectamine® 2000 method, following manufacturers' instructions. Forty-eight hours post transfection Western Blot with an HA specific antibody (Ab) and with an anti-Tubulin Ab, as loading control, were performed. C- stands for untransfected cells.

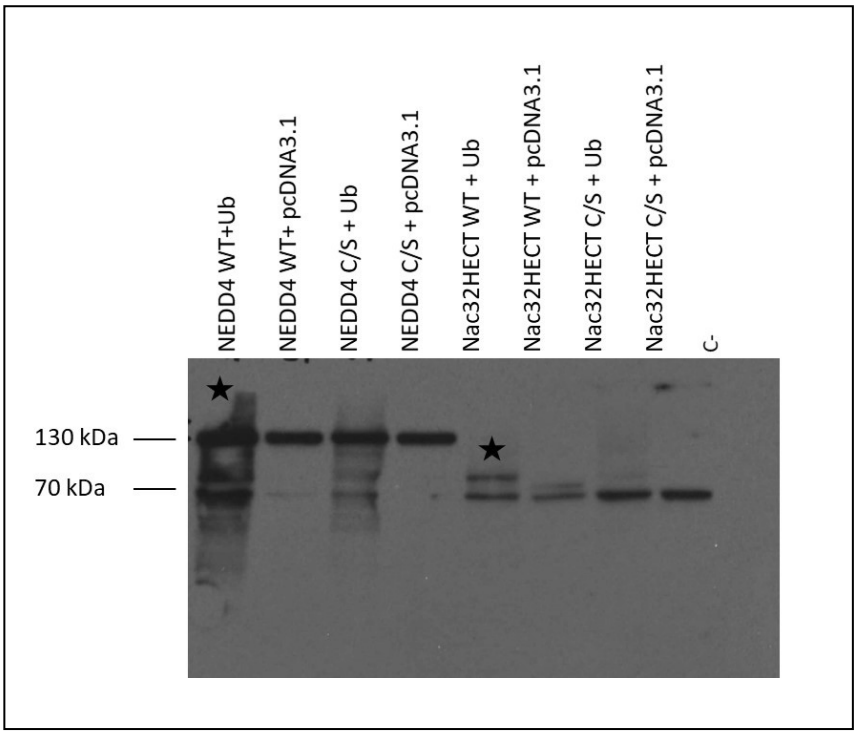


Figure 17. **LV expressing WT ubiquibodies show auto-ubiquitination activity that is reduced by the C/S mutation.** LV expressing WT and C/S ubiquibodies (2.5 μg) were co-transfected by Lipofectamine 2000 along with a construct expressing ubiquitin (Ub) or with the corresponding an empty plasmid (pcDNA3.1). Forty-eight hours post transfection a Western Blot with the anti-HA Ab was performed. The star points to the upper bands due to autoubiquitination activity of the expressed proteins. C- stands for untransfected control.

It is known that NEDD4, as all HECT containing ubiquitin ligases displays an auto-ubiquitinating activity (Bruce et al., 2008).

Thus, as a first indication of the ubiquibodies functionality, we also co-transfected 293T cells with LV expressing full length NEDD4 and LV expressing one of the ubiquibody, Nac32HECT along with a plasmid expressing ubiquitin. Forty-eight hours post-transfection cells were lysed and proteins were run on an SDS-PAGE gel followed by western blotting analysis. As shown in Figure 17, upper bands are clearly visible in the presence of WT NEDD4 when ubiquitin is co-expressed (first lane). This auto-ubiquitination activity is accompanied by lower running bands that represent, as expected, degradation products of the protein. Furthermore, indicating the specificity of what observed in the case of WT NEDD4, there is a clear decrease in the intensity of these bands both when ubiquitin is absent (second lane) and when

the NEDD4 HECT domain is made inactive by the C/S mutation (fourth lane). Importantly, also in the case of WT Nac32HECT (fifth lane), a strong upper band appears in the presence of ubiquitin that is reduced when the catalytic domain bears the C/S modification (seventh lane) or ubiquitin is not co-transfected (sixth lane). Similar results were obtained with the other ubiquibodies (data not shown)
Overall, our results indicate that ubiquibodies are efficiently expressed in 293T cells and they are functional.

4.3 Recombinant lentiviral particles efficiently express ubiquibodies in 239T cells

Based in these findings, we moved toward the production of recombinant lentiviral particles (RLP). To this end, 293T cells were cotransfected with the generated LV along with the packaging plasmids that encode the structural and enzymatic proteins needed to assemble viral particles (pVSVG, pMDL and pREV), as explained in paragraph 3.12 of the Methods. Forty-eight hours later, cell supernatants were harvested and concentrated as reported in paragraph 3.12 of the Methods. Next, the ability of RLP to efficiently express the transgenes was evaluated by Western Blot upon transduction of 293T cells, as explained in paragraph 3.13 of the Methods. Briefly, cells were seeded in a 6-well plate and the following day different volumes of concentrated RLP were added. LV expressing full length NEDD4 either WT or C/S were also tested. Forty-eight hours post transduction, cells were harvested, lysed and a Flag specific Western Blot was performed (figure 18).

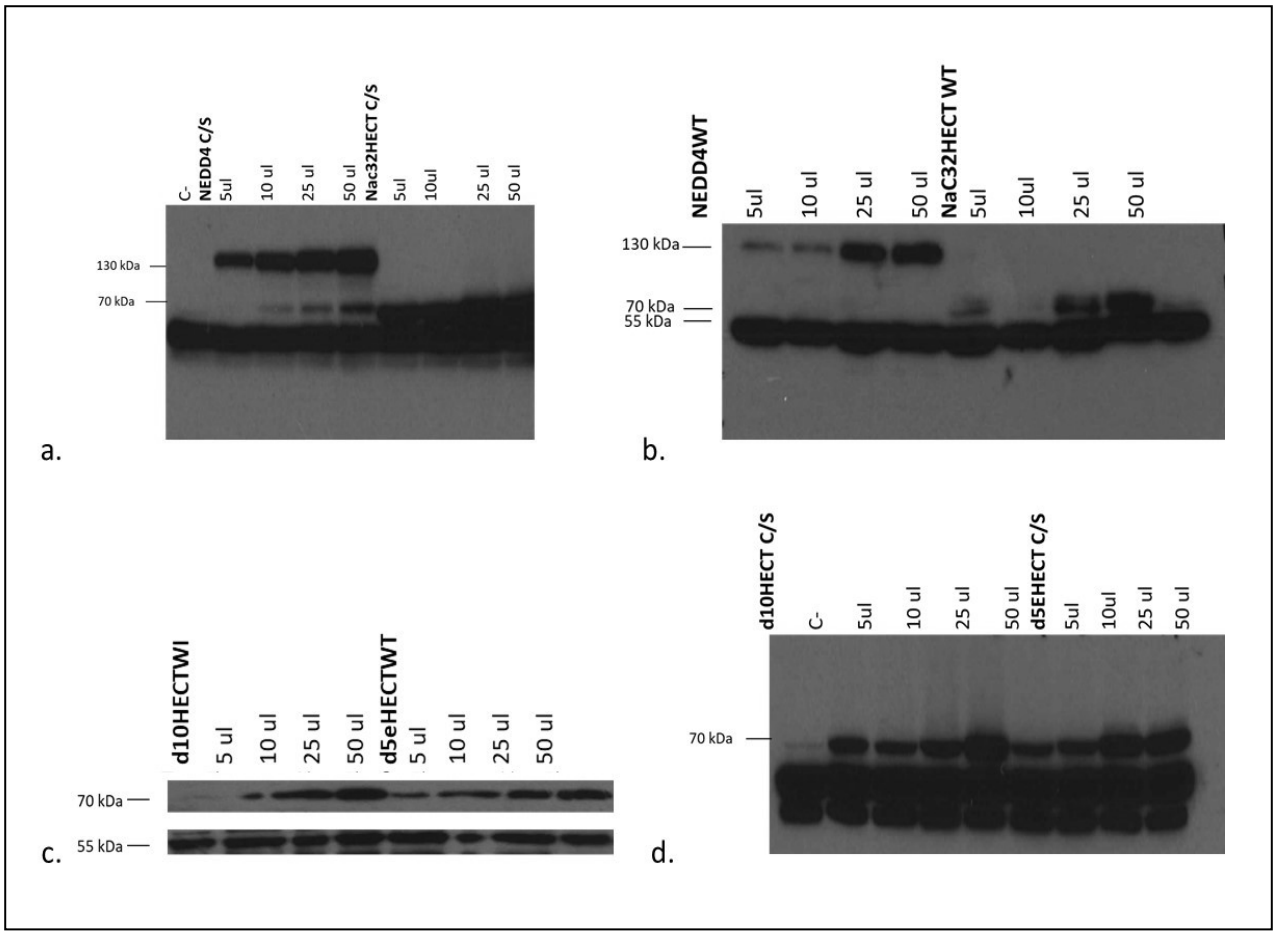


Figure 18. **RLPs lead to an efficient expression of the different ubiquibodies in 293T cells**. Cells were transduced with different volumes (as indicated) of RLPs expressing the following constructs; (a) WT NEDD4 and Nac32HECT; (b) C/S NEDD4 and Nac32HECT; (c) WT d10 and d5eHECT; (d) C/S d10 and d5eHECT. Forty-eight hours post-transduction cells were harvested and proteins were run on an SDS-PAGE gel followed by Western Blot.

As visible in Figure 18, in 293T cells, all chimeric constructs and the relative controls are expressed, giving rise to bands of the expected size.

Overall our data confirm that i) RLP based on the generated vectors can be obtained and are able to transducer target cells; ii) RLP efficiently express ubiquibodies in a single structurally stable chimeric protein in the cellular environment.

4.4 Development of WT and A53T aS expressing vectors for the generation of suitable in vitro system to check the functionality of generated ubiquibodies

Once generated all the LV expressing the ubiquibodies and demonstrated that they express a stable recombinant protein in eukaryotic cells, the following step was to develop suitable *in vitro* system in order to preliminary validate our strategy. It has been reported that the over-expression of aS either WT or in its mutated form A53T per se or fused in frame with reporter proteins, as EGFP, leads to the accumulation of aS mimicking Lewy body (LB) (Dehay et al., 2015) (paragraph 1.2.1 of the introduction). Indeed, eukaryotic cells overexpressing aS are recognized and widely employed in the Parkinson's disease (PD) field to study strategies aimed at interfering with aS aggregation (Oliveira et al., 2015) (paragraph 1.8.1 of the Introduction).

Based on these considerations and taking into account that the most relevant cell type where to investigating the effect of ubiquibodies are dopaminergic neurons (DNs), we generated different constructs expressing WT and A53T aS either alone or fused in frame to EGFP to be adopted in order to obtained over-expression of the protein in different cell types.

4.4.1 Lentiviral vector and pcDNA3.1 expressing WT and A53T aS 3'F

First of all, we cloned WT and the mutated A53T aS fused in frame with a Flag tag in the LV pptCMVEGFPWPRE. The strategy we have adopted is reported in figure 13.

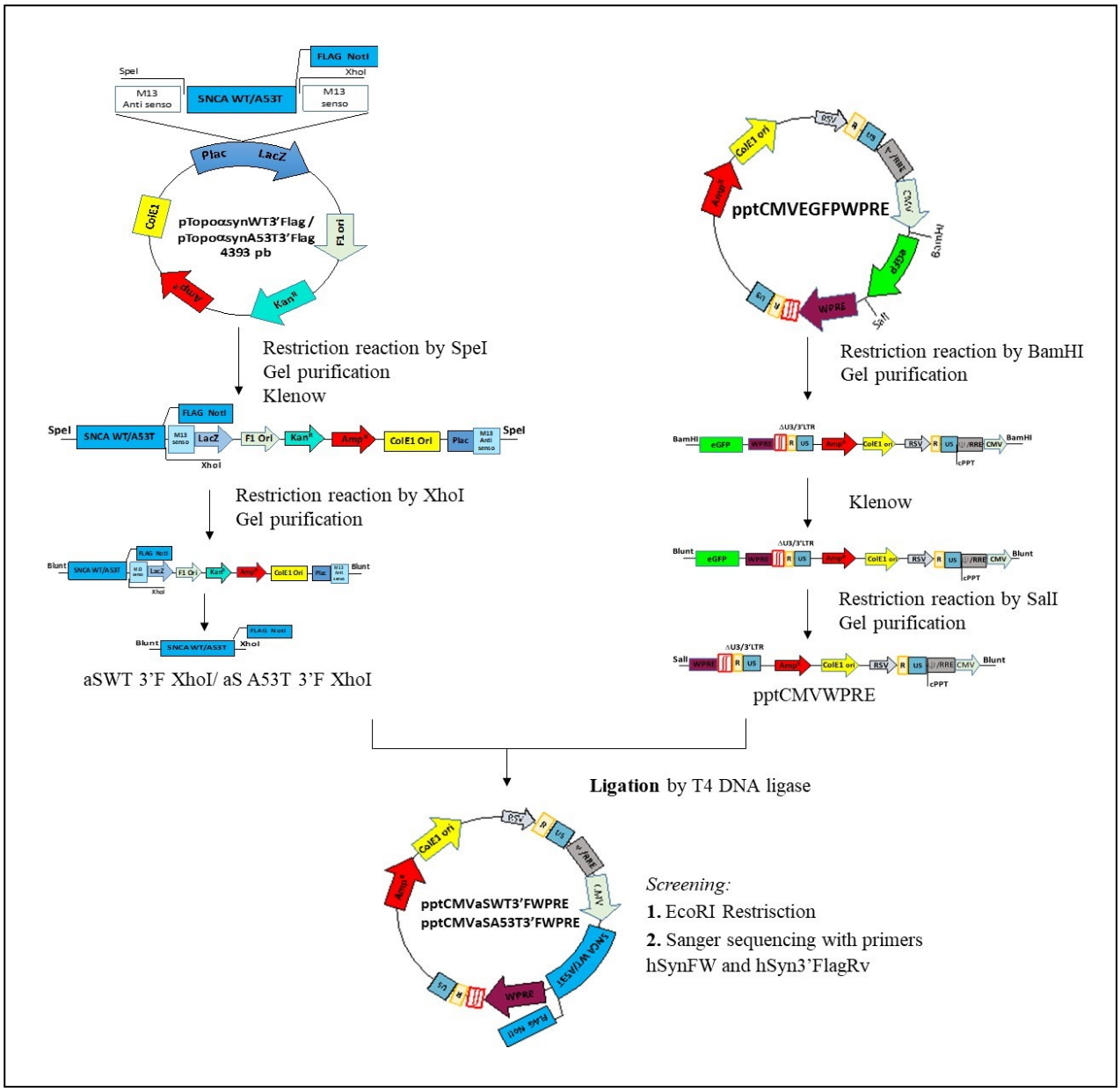


Figure 13. **Schematic representation of the main steps of the cloning strategy adopted to obtain the LVs expressing the aSWT3'F/aSA53T3'F sequences (pptCMVaSWT3'FWPRE/ pptCMVaSA53T3'FWPRE)**.

Briefly, the intermediate plasmids pTopoasynWT3'F and pTopoasynA53T3'F, containing the aS encoding sequence bearing a Flag Tag at the 3' end and already available in the laboratory, were subjected to enzymatic restriction by the SpeI endonuclease, followed by treatment with the Klenow fragment to create blunt ends and additional restriction with XhoI. In parallel, the pptCMVEGFPWPRE vector was

restricted with BamHI, treated with the Klenow fragment in order to obtain blunt ends and then digested with SalI to excise the EGFP reporter gene. After appropriate ligation, to verify the successful cloning and correct orientation of the insert, the plasmids were purified from the colonies and were verified by enzymatic restriction performed with the EcoRI endonuclease. The showing the correct restriction pattern were sequenced using hSynFw and hSyn3'FlagRv primers (table 3).

At the same time, we cloned aS 3'Flag into the eukaryotic expression plasmid pcDNA3.1 plasmid. The cloning strategy is reported in the figure 14.

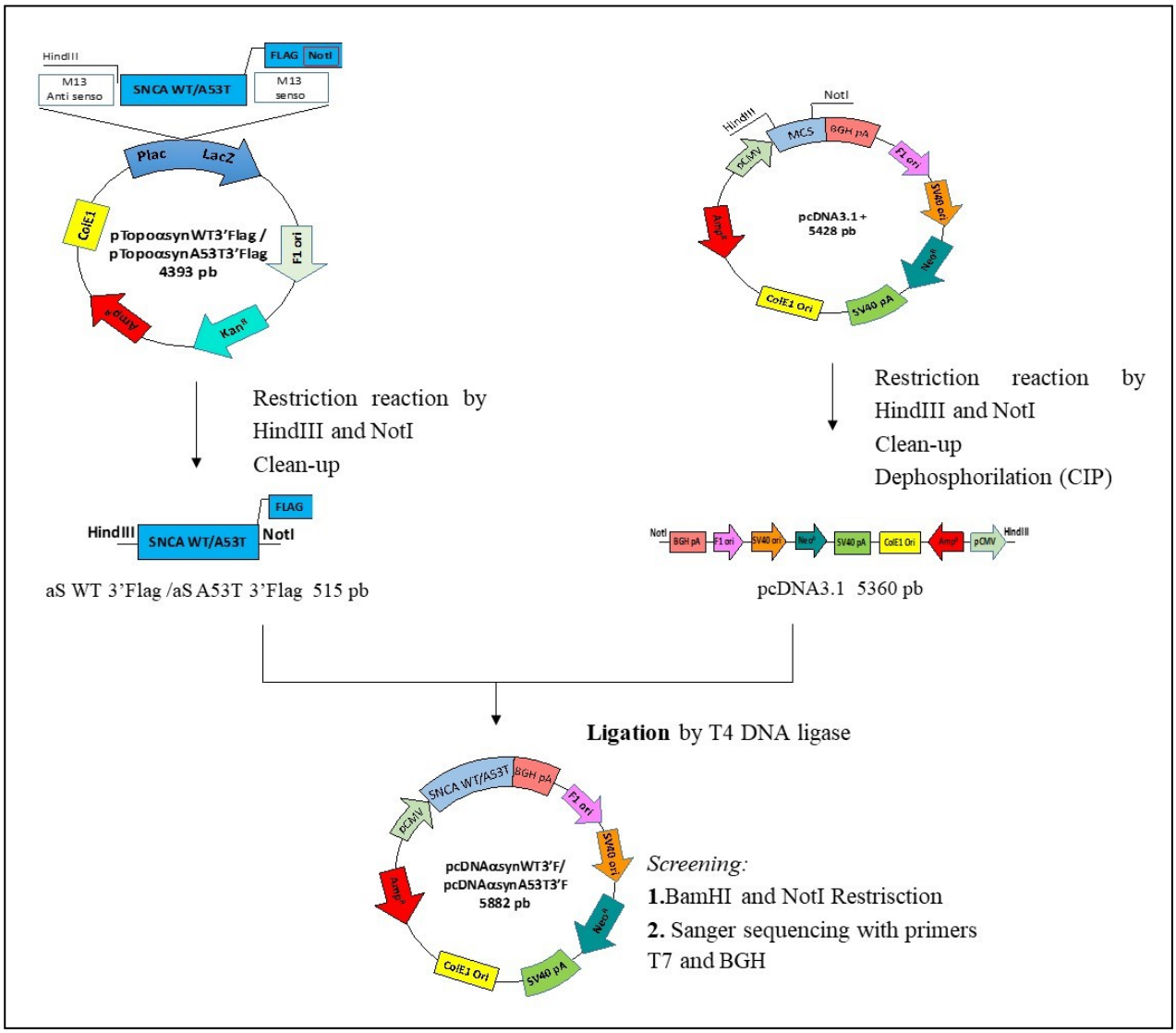


Figure 13. **Schematic representation of the main steps of the cloning strategy adopted to obtain the plasmid expressing the aSWT3'F/aSA53T3'F sequences (pcDNA3.1asynWT3'F/ pcDNA3.1asynA53T3'F)**.

In this case, the intermediate constructs pTopoasynWT3'Flag and pTopoasynA53T3'Flag, were restricted by HindIII and NotI endonucleases.

In parallel, pcDNA3.1 plasmid was digested with the same restriction endonucleases. Upon ligation, to verify the successful cloning and correct orientation of the insert, the plasmids were purified from the colonies and were controlled by enzymatic restriction performed with the BamHI and NheI endonucleases. Finally, the clones which showed the expected band pattern were analyzed by sequencing, using the T7 and BGH primers (table 3).

4.4.2 Lentiviral vector expressing WT and A53T aS fused in frame with EGFP

As it has been shown the overexpression of aS fused in frame with EGFP leads to the formation of aggregates that can be easily visualized, in order to obtain a method to rapidly detect the presence of aS in the cellular environment, we decided to clone aS, fused in frame with the EGFP, in the context of LV. The cloning strategy is represented in figure 15.

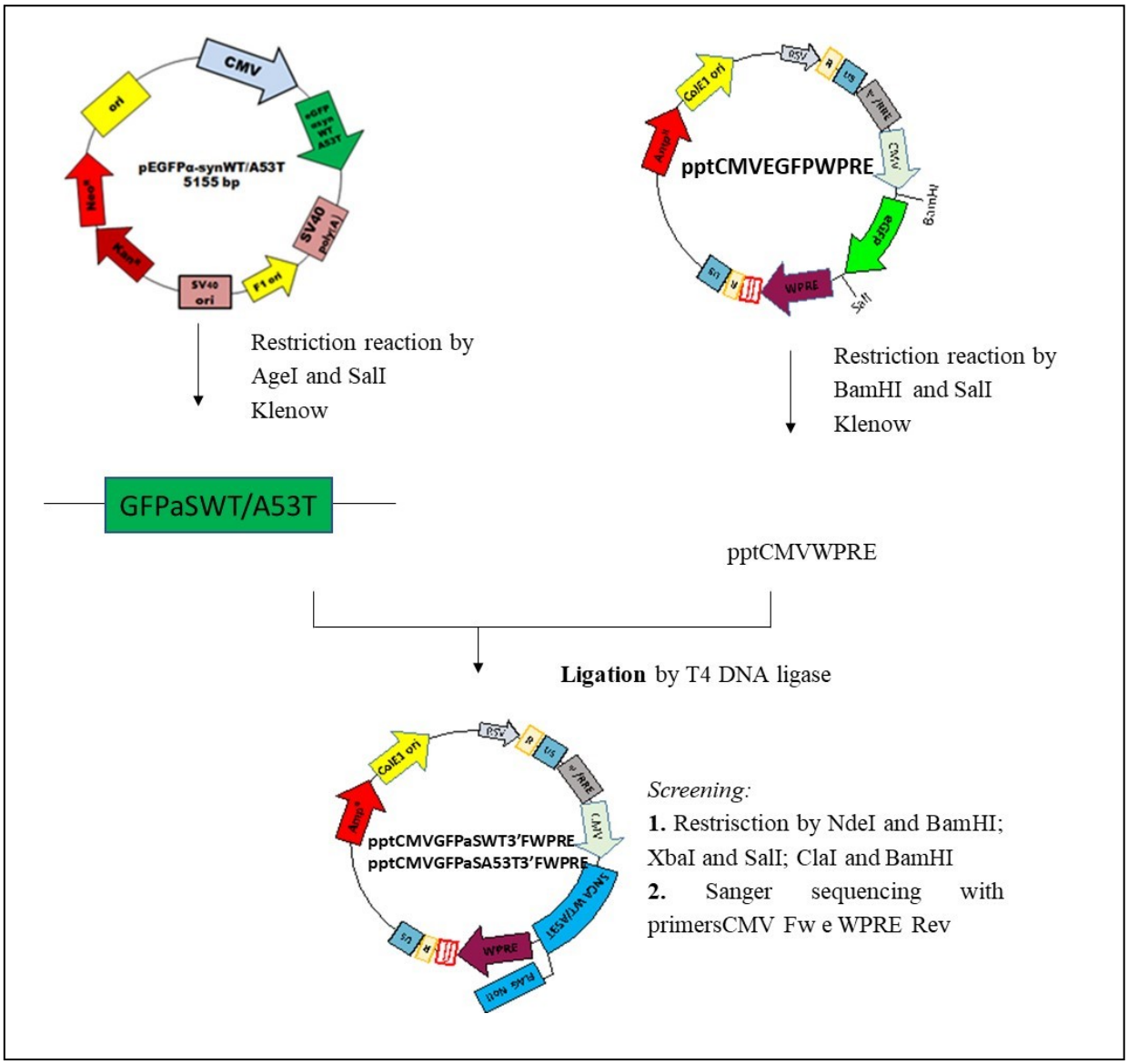


Figure 15. **Schematic representation of the main steps of the cloning strategy adopted to obtain the LV expressing the GFP aS WT/GFP aS A53T sequences (pptCMVGFPaSWT and pptCMVGFPaSA53T).**

To this end, we took advantage of the pEGFPα-synWT and pEGFPα-synA53T plasmids that contains the aS sequence fused in frame with EGFP and with cut them with Age I and SalI restriction. At the same time, the pptCMVEGFPWPRE vector, was subjected to BamHI and SalI endonucleases restriction. Fragments and vectors were subjected to fill-in to create blunt ends and ligated. To verify the successful cloning and correct orientation of the inserts, plasmids purified from the bacterial

colonies and controlled by enzyme restriction. Finally, all the selected clones, were analyzed by sequencing, using CMV Fw e WPRE Rev primers (table 3).

4.5 Recombinant LVs efficiently express aS In 293T cells

As following step, first of all we evaluated the ability of the generated vectors to express aS in 293T cells. LVs and pCDNA3.1 based constructs were transfected in 293T cells and 48 hours later, cells were harvest, lysed and Western Blots were performed (figure 19).

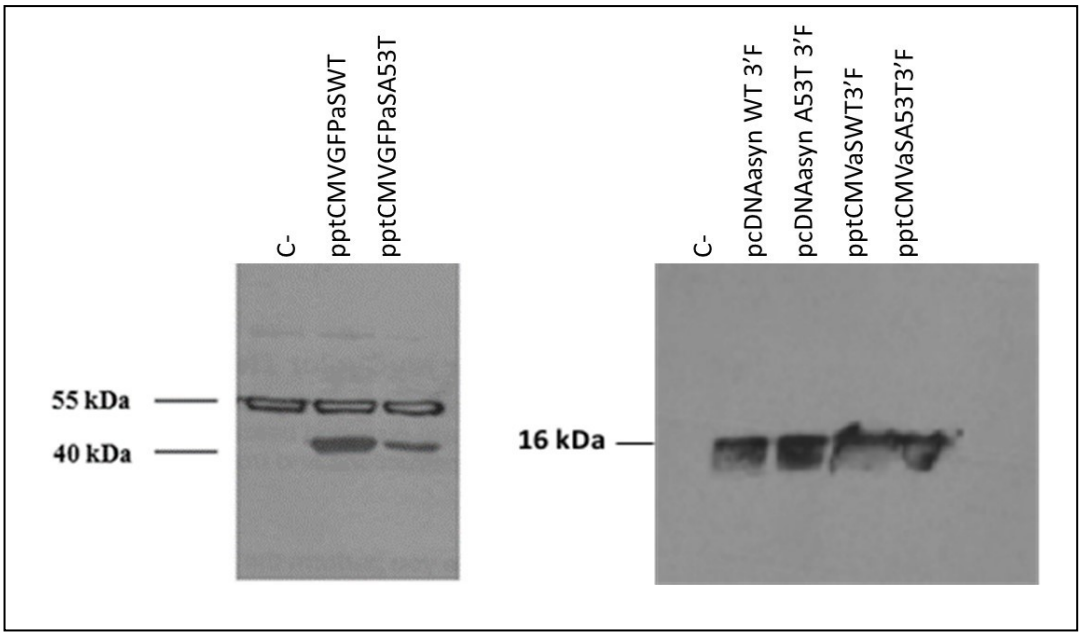


Figure 19. **The developed LVs and pCDNA3.1 based constructs efficiently express aS.** 293T cells were transfected with (2.5 μg) of the indicated plasmids, by Lipofectamine® 2000. 48 hours later, a Western Blot was performed either employing an Ab against aS (left panel) or against Flag-tag (right panel). C- stands for untransfected cells.

As shown in figure 19, aS is well expressed in cell environment, both alone and fused in frame with EGFP.

4.6 Recombinant lentiviral particles efficiently express aS in 293T cells

Next, RLP were produced, by cotransfecting 293T cells with the generated LV along with the packaging plasmids (pVSVG, pMDL and pREV). Forty-eight hours later, cell supernatants were collected and concentrated, as explained in paragraph 3.12 of the Methods. 293T cells were transduced with different volumes of the concentrated RLP, as reported in figure 20. Forty-eight hours post-transduction cells were harvested proteins run on an SDS-PAGE gel and a western blot was performed to check for the expression of the different transgenes.

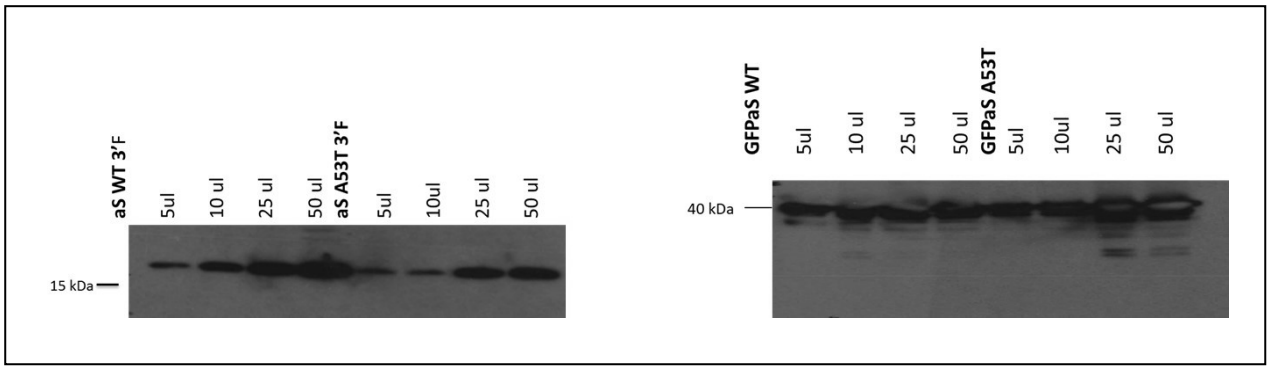


Figure 20**. RLP are able to transducer 293T cells and lead to the expression of aS.** 293T cells were transduced with different volumes of RLP (as reported). Forty-eight hours post-transduction, cells were harvested a western blot was performed on cell lysates either with a Flag specific Ab (left panel) or with an aS one (right panel).

As shown in figure 20, all the RLPs are able to transducer 293T cells and lead to the expression of aS either alone or fused in frame with EGFP.

4.7 The ubiquibodies functionally interact with aS in a specific manner

To be employed to specifically interfere with aS accumulation, the generated chimeric ubiquitin ligases need to recognize aS and lead to its ubiquitination as well as degradation, possibly in a specific manner. Thus, we set up a series of experiments to test these different aspects.

First of all, we assayed the ability of the generated ubiquibodies to bind aS. To this end, we performed a co-immunoprecipitation in 293T cells, following the protocol reported at paragraph 3.19 of the Methods. In particular, we took advantage of the constructs expressing the catalytically inactive forms of ubiquibodies (C/S), as they bind more tightly than the WT forms to aS, as previously reported (Strack et al., 2000). First of all, we transfected LVs expressing C/S ubiquibodies and twenty-four hours later we transduced RLPs expressing WT aS 3'F. Forty-eight hours post-transduction cells were harvested and cells were lysed with a buffer reported on the paragraph 3.19 of the Methods. Next, agarose resin coated with a highly specific anti-Flag monoclonal antibody were adopted and proteins immunoprecipitated run on an SDS-PAGE gel and a western blot was performed to check for the expression of the different transgenes.

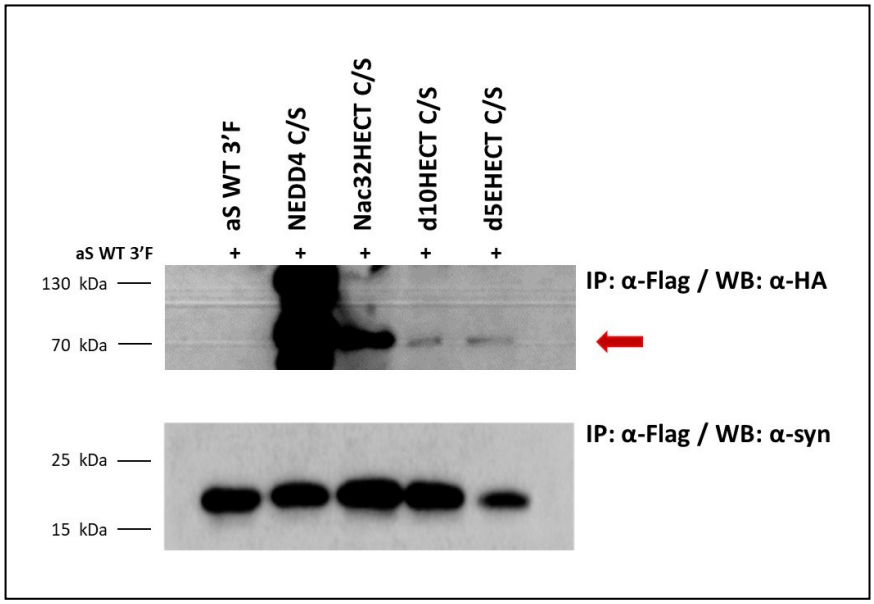


Figure 21. **Evaluation of the ability of the ubiquibodies to bind aS.** 293T cells ($1.5x10^6$) were transfected with (8 µg) LVs expressing the ubiquibodies; twenty-four hours later cells were transduced with RLP expressing aS3'F. Flag co-immunoprecipitation was performed followed by a Western Blot with an anti HA Ab to evaluate the presence of the ubiquibodies (upper panels) or anti-aS to control for the presence of aS in all the samples. The experiment was repeated 3 times with similar results.

As shown figure 21, while we confirm that Nedd4 strongly bind aS, all the generated ubiquibodies co-immunoprecipitate with aS. In particular, Nac32HECT ubiquibody seems to bind more tightly aS as compare the amount expression of aS.

Next, we tested the ability of WT ubiquibodies to ubiquitinate aS. To this end, First of all, we transfected LVs expressing WT ubiquibodies along with the ubiquitin plasmid; twenty-four hours later we transduced RLPs expressing WT aS 3'F. Forty-eight hours post-transduction cells were harvested, and cells were lysed with a buffer reported on the paragraph 3.19 of the Methods. Next, agarose resin coated with a highly specific anti-Flag monoclonal antibody were adopted and proteins immunoprecipitated run on an SDS-PAGE gel and a western blot was performed to check for the expression of the different transgenes (figure 22).

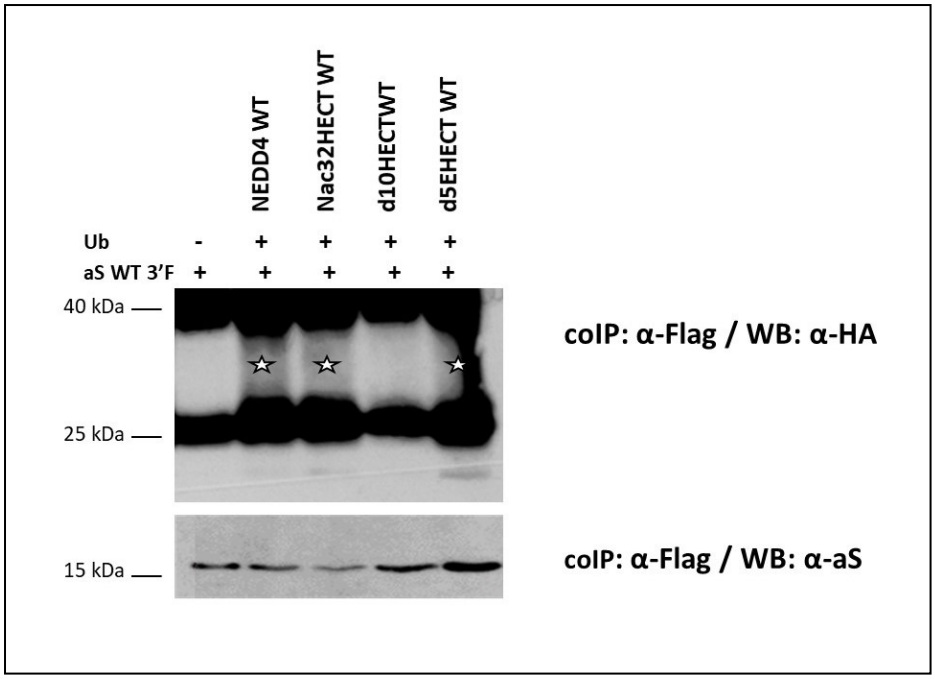
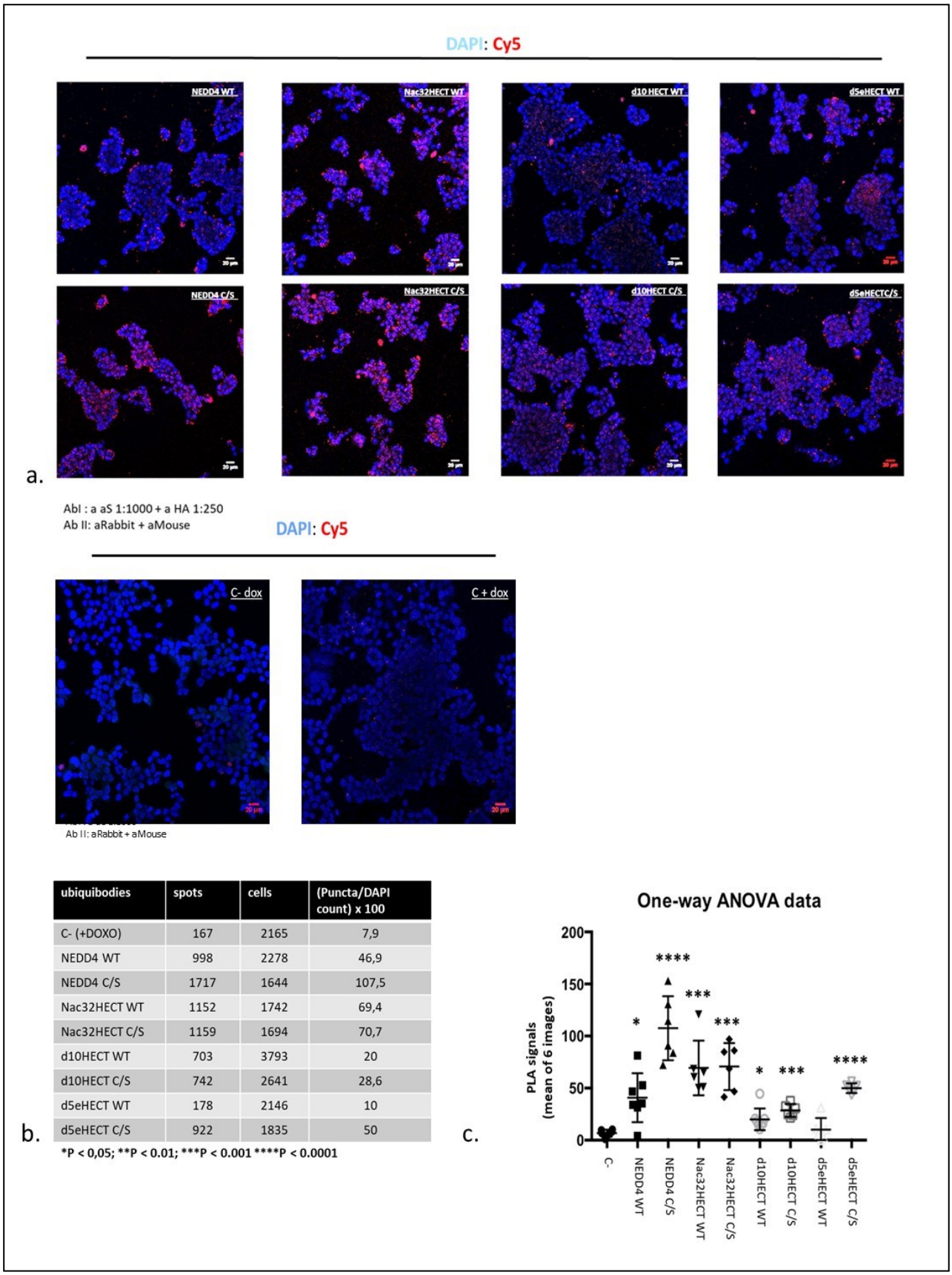


Figure 22. **Evaluation of the ability of the ubiquibodies to ubiquitinate aS.** 293T cells ($1.5x10^6$) were transfected with (8 µg) LVs expressing the ubiquibodies along with the ubiquitin plasmid (2 µg); twenty-four hours later cells were transduced with RLP expressing aS3'F. After a Flag-immunoprecipitation a Western Blot was performed using an anti HA antibody (upper panel) or an anti-aS antibody (lower panel). Star points to the Ab chains. The experiment was repeated 3 times with similar results.

As shown in figure 22, only in the presence of either full length NEDD4 (lane 2) or of the chimeric proteins (lanes 3-5), a smear appears as expected in the case of aS ubiquitination. Furthermore, we could observe that Nac32HECT and d5eHECT ubiquibodies are more efficient than d10HECT, which, interestingly, is based on the scFv binding to the monomeric form of aS.

Based on these results and to move to a more relevant cellular model, we tested the ability of ubiquibodies to interact with aS in a different cell type in a murine dopaminergic-like cell line, MN9Dsyn, which is widely employed as *in vitro* experimental system to study aS biology and therapeutic strategies (Feng et al., 2010). In particular this specific cell line is engineered with a plasmid that allows to express aS and EGFP affording doxycycline as explained in the paragraph 3.1 of the Materials. Furthermore, since co-immunoprecipitation is a strong indication of protein/protein binding, but it is based on cell lysates, we decided to test aS/ubiquibodies interaction with a method that is based on intact cells. Thus, we adopted the proximity ligation assay (PLA). As explained in more details at the paragraph 3.20 of the Methods. PLA allows to display the interaction between aS and the ubiquibodies, by fluorescent spots visible with microscopy. MN9D cells were transduced with RLP expressing WT and C/S ubiquibodies and twenty-four hours later the production of aS were induced with doxycycline (DOX). Twenty-four hours later, cells were fixed and permeabilized and PLA were performed. The images in figure 23 were taken with the confocal microscope.



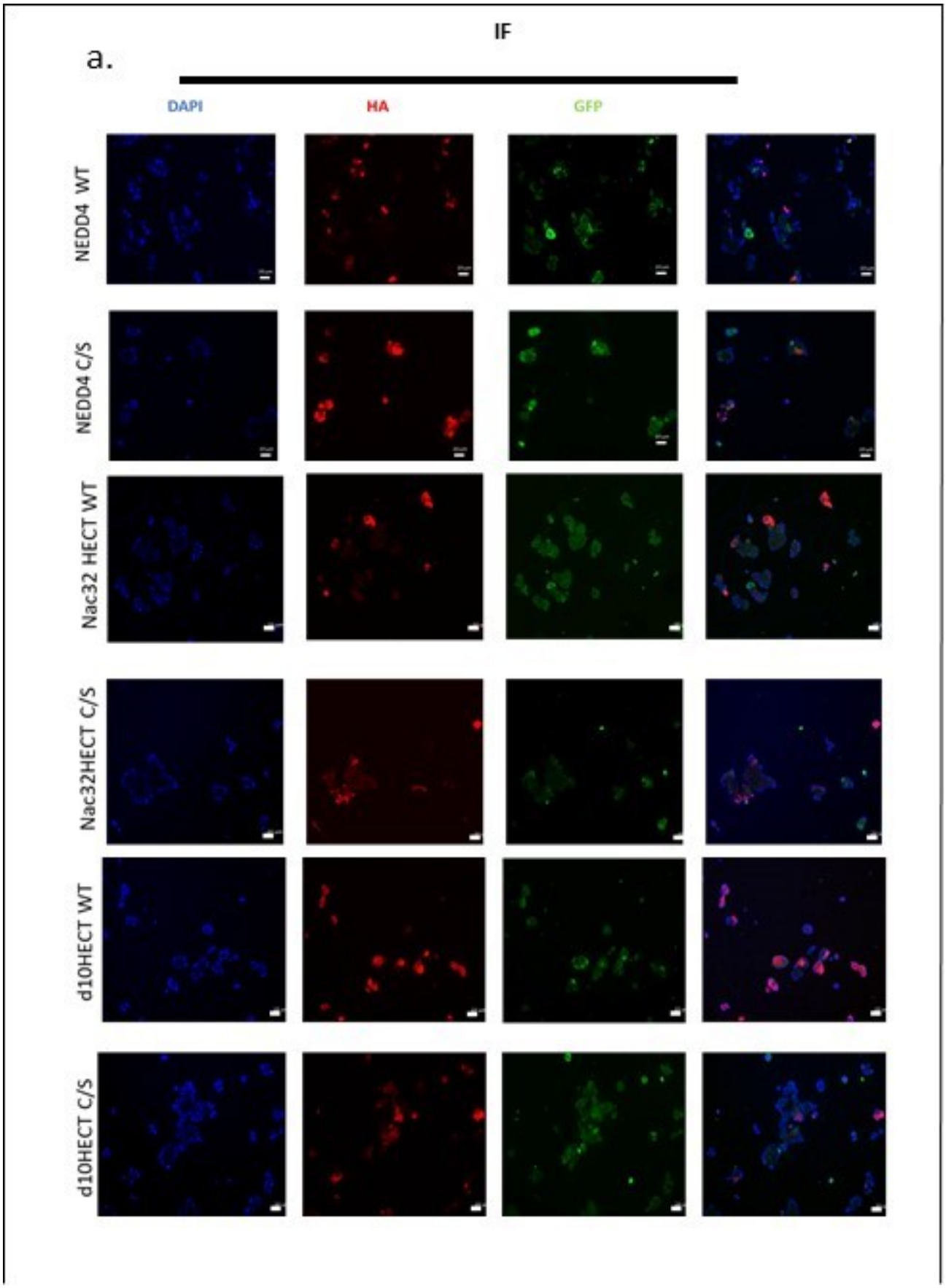
ubiquibodies	spots	cells	(Puncta/DAPI count) x 100
C- (+DOXO)	167	2165	7,9
NEDD4 WT	998	2278	46,9
NEDD4 C/S	1717	1644	107,5
Nac32HECT WT	1152	1742	69,4
Nac32HECT C/S	1159	1694	70,7
d10HECT WT	703	3793	20
d10HECT C/S	742	2641	28,6
d5eHECT WT	178	2146	10
d5eHECT C/S	922	1835	50

*P < 0,05; **P < 0.01; ***P < 0.001 ****P < 0.0001

Figure 23. **Ubiquibodies interact with aS in murine dopaminergic cell line (MN9Dsyn) with PLA**. (a) Cells ($4x10^4$) were transduced with RLP expressing ubiquibodies and 24 hours later DOX induced the expression of aS. Cells were fixed with 4% PFA and staining for anti-HA (monoclonal rabbit antibody) and

aS (monoclonal mouse antibody), followed by PLA probes minus and plus, ligation and amplification. For negative control, cells were incubated only with aS antibody and the two PLA probes. Cells were analyzed with the confocal microscope. In blue: nuclei; in red: PLA spots. The name of ubiquibodies expressed in cells are reported with the white writing. (b) The table represents the total spots counted and the total amount of cells based on dapi: the PLA signals derived is normalized. (c) the ANOVA (GraphPad Prism, Inc) statistical analysis were performed for 6 images per each sample. Scale bar, 20 μm.

As shown in the figures 24 and 25 and as expected (see above), the C/S form of each ubiquibody interacts more tightly with aS with respect to the WT form (figure 23, panel A). Furthermore, based on the statistical analysis (panel C), we demonstrate that Nac32HECT interacts more efficiently than both d10HECT and d5eHECT ubiquibodies. After the optimization of the PLA protocol, we performed the same experiment additional two times, in which we added an additional negative control in which cells were incubated with a primary antibody directed against the endogenous protein Glutamine Synthetase (GS) and aS primary antibodies, as a control. Confirm findings displayed in figure 24. Importantly, data show that aS/ubiquibody interaction is specific compare to the negative control. Finally, in this case, we also checked by immunofluorescence the expression of the ubiquibodies in MN9Dsyn cells and we were able to show that indeed they are well expressed, thus further supporting the specificity and significance of these results (figure 24, panel A).


IF
a.
DAPI
HA
GFP
NEDD4 WT
NEDD4 C/S
Nac32 HECT WT
Nac32HECT C/S
d10HECT WT
d10HECT C/S

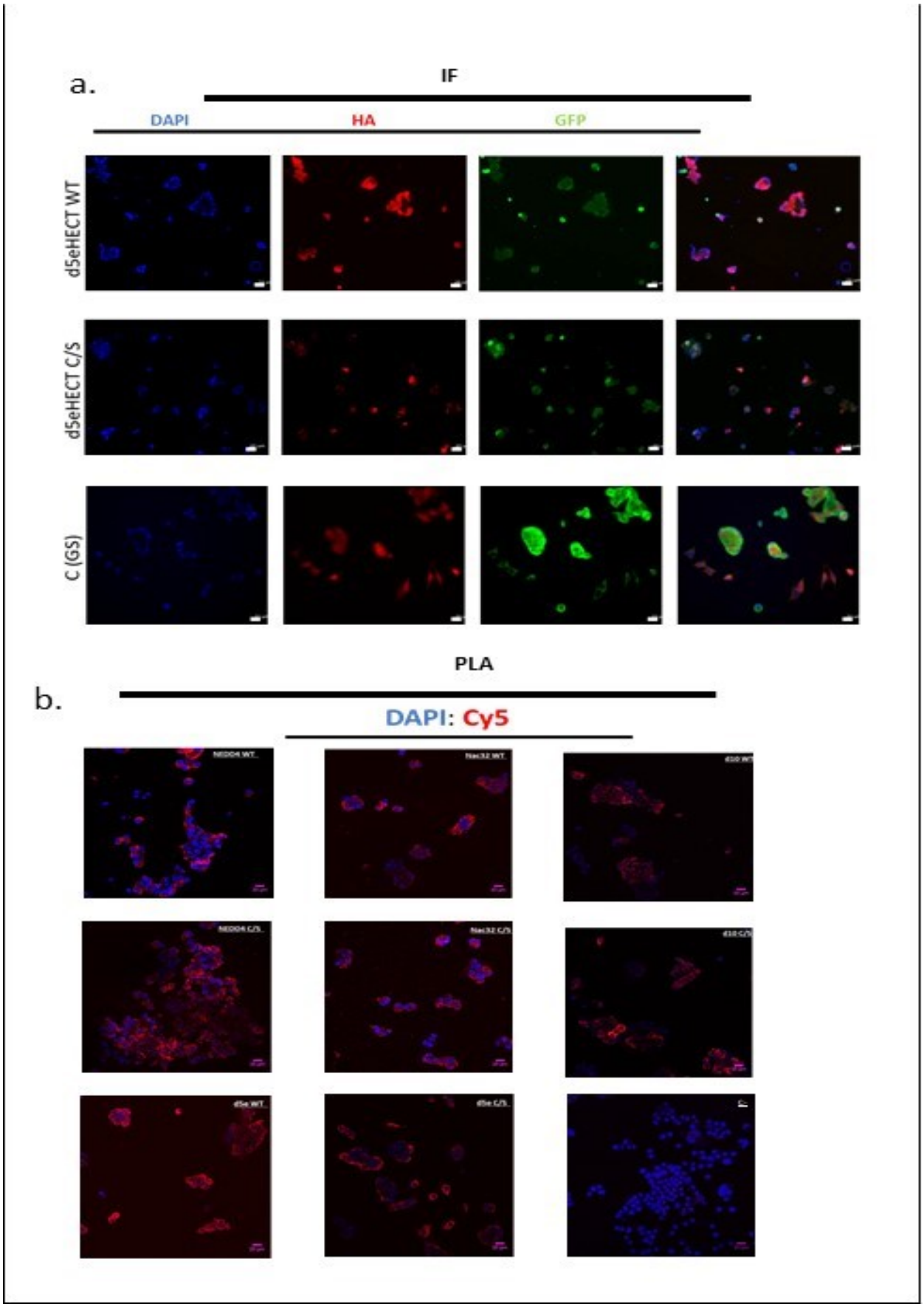

a.
IF
DAPI
HA
GFP
d5eHECT WT
d5eHECT C/S
C (GS)
PLA
b.
DAPI: Cy5

Figure 24. **Ubiquibodies interact with aS in murine dopaminergic cell line (MN9Dsyn) with PLA**. (A-B) Cells ($4x10^4$) were transduced with RLP expressing ubiquibodies and 24 hours later DOX induced the expression of aS. Cells were fixed with 4% PFA and staining for anti-HA (monoclonal rabbit antibody) and aS (monoclonal mouse antibody); in the negative control, cells were incubated with anti-aS (monoclonal mouse antibody) and anti-GS (monoclonal rabbit antibody), followed by incubation of (A; **IF**) fluorescently labelled anti-rabbit IgG antibodies (red fluorescence) for ubiquibodies and GS and GFP for the expression of aS (green fluorescence), (B; **PLA**) PLA probes minus and plus, ligation and amplification. Cells were analyzed with the confocal microscope. In blue: nuclei; in red: PLA spots. The expression of each ubiquibody is reported in each image written on the left side. Scale bar, 20 µm.

Neg control	NEDD4 wt	NEDD4 c/s	nac32 Hect wt	nac32 Hect c/s	d10 Hect wt	d10 Hect c/s	d5e Hect wt	d5e Hect c/s
0.6217949	11.939390	14.70122	23.066670	18.47500	15.80597	15.93000	18.279070	12.272730
0.5485437	21.692310	22.46512	20.336740	19.93333	15.80172	16.58947	24.022220	10.519690
0.4044944	21.300000	16.84564	20.840000	29.78125	16.14189	14.38597	12.671880	12.272730
0.6793478	9.515152	16.81657	30.840910	24.55556	27.54688	23.50000	20.586210	9.100720
0.6217949	16.110000	24.27273	20.375000	19.92135	15.69697	12.65116	20.871800	19.484850
0.679426	16.576920	12.39286	30.522930	12.26786	10.00000	11.79000	12.650790	6.886793
0.041199	16.450000	11.38776	5.463918	22.97959	22.00000	22.00000	5.881356	12.358490
0.8039216	10.250000	11.56410	17.817650	21.34884	12.73656	12.73656	12.674700	14.093020
0.2380952	22.714290	17.53846	17.800000	14.20253	24.28572	24.28572	2.714286	18.511110
0.1606218	8.484848	24.95122	13.464650	18.51899	10.70000	10.70000	16.170210	15.697680

*P < 0,05; **P < 0.01; ***P < 0.001 ****P < 0.0001. All the samples have **** p < 0.0001

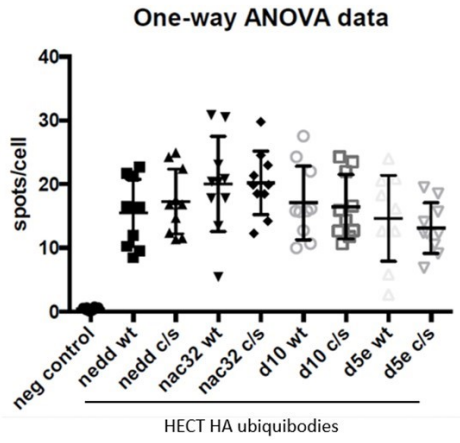


Figure 25. **Statistical analysis using ANOVA test (GraphPad Prism, Inc.)**. Results are expressed as mean ± SD taken in 6 different regions of the cell culture, from two separate experiments (*p < 0.05; **p < 0.01;p <0.001;p<0.0001). All the samples have **** p< 0.0001 respect to the negative control.

Furthermore, using the GS within the negative control we demonstrated that ubiquibodies are specific against aS.

Overall our data indicate that among the generated ubiquibodies Nac32HECT is the one that better interact with aS and thus was adopted in all the following experiments.

Overall our data indicate that Nac32HECT ubiquibody is more specific ubiquibodies against aS respect on the others and is the candidate more relevant for a future therapeutic strategy.

4.8 Nac32HECT ubiquibody affect aS intracellular levels.

Having established the ability of Nac32HECT to bind and ubiquitinate aS, in order to analyzed its effect on aS intracellular amounts, 293T cells were transduced with RLP expressing A53T aS GFP and, 48 hours later, with RLP expressing Nac32HECT and NEDD4, as positive control, either in the WT or in the C/S form. Indeed, we measured the intensity of the fluorescence signal due to the EGFP fused in frame with aS was measured by FACS analysis before and after the treatment with the ubiquibodies (figure 26).

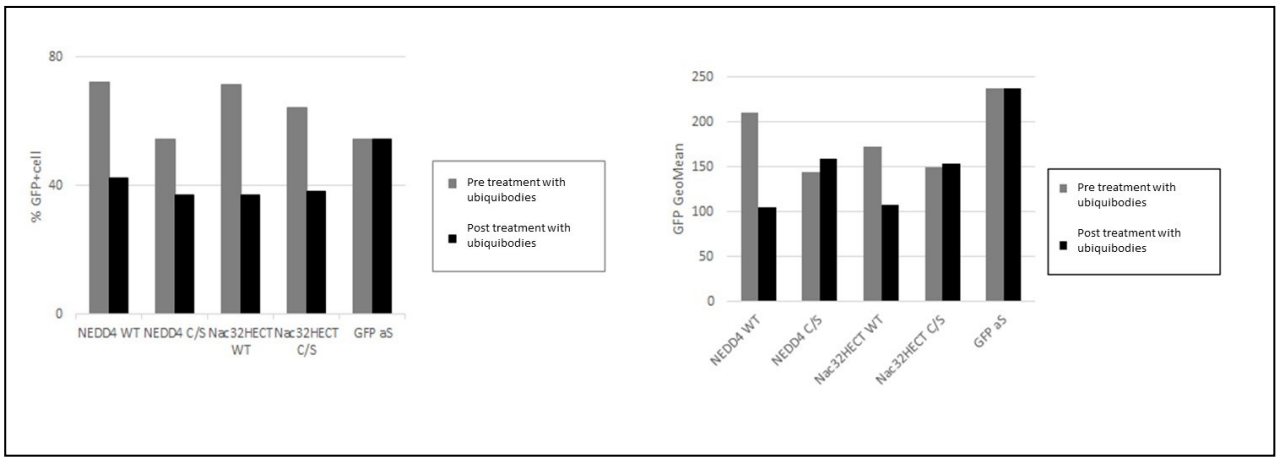


Figure 26. **Evaluation of the expression of GFP fused in frame with aS in 293T by FACS analysis.** Cells were transduced with RLPs expressing GFP aS and the day after, with WT and C/S NEDD4, as positive control, and Nac32HECT. Cells were harvest and green fluorescence derived from EGFP were measured by cytofluorimetry, before (grey histograms) and after (black histograms) treatment with ubiquibodies. We measured the average of green fluorescence positive cells (% GFP^+) and the GFP Geometric Means of green fluorescence (GFP GeoMean). 10,000 events were analyzed for each of three independent experiments Results of a representative experiment are displayed.

Graphs of table 4 highlight two parameters: the average of green fluorescence positive cells (% GFP^+) and the GFP Geometric Means of green fluorescence (GFP GeoMean) that expresses the intensity of the EGFP fluorescence. In the presence of the WT form of Nac32HECT, both the % of GFP^+ and the GFP GeoMean are

reduced, compared to WT NEDD4. By contrast in the presence of Nac32HECT C/S these to parameters are not reduced.

To further support this conclusion, we tested the effect of Nac32HECT on endogenous aS intracellular amount over time. To this end we performed an experiment by overexpressing the selected ubiquibodies in cells where the *de novo* protein synthesis was inhibited by treatment with 20 µg/ml of cycloheximide (CHX), as previously reported (Tofaris et al., 2011). SHSY5Y cells were transduced with RLP expressing Nac32HECT either WT or C/S and after 24 hours, cells were incubated in fresh medium containing 20 µg/ml CHX. Cells were harvested at different time points (6, 8, and 10 hours) post-treatment and aS amount over time was checked by Western Blot (figure 27).

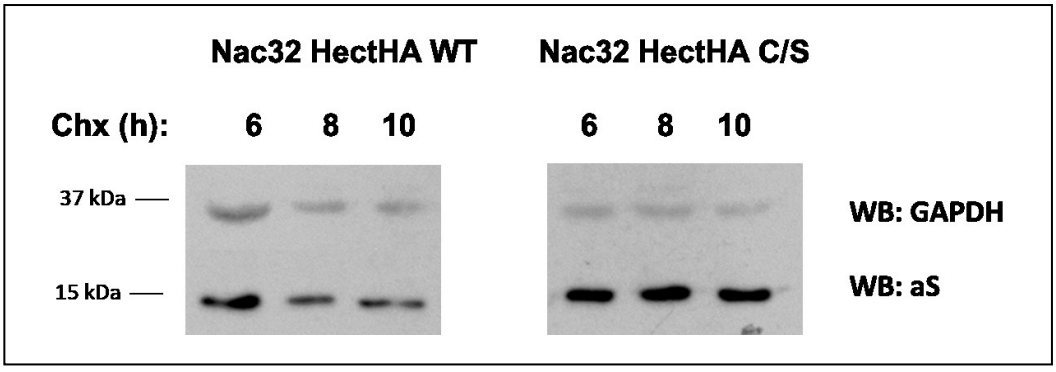


Figure 27. a**S intracellular amounts are affected by Nac32HECT expression**. SHSY5Y cells were transduced with RLP expressing ubiquibodies and 24 hours later, cells were incubated in fresh medium containing 20 µg/ml cycloheximide (CHX), harvested at different time points post-treatment (as indicated) and lysed. A Western Blot was performed to analyze the amount of intracellular aS over time. A loading control (GAPDH) was also added.

This result show that the Nac32HECT WT ubiquibody has an effect on endogenous aS levels, when compared to the catalytically inactive C/S control.

Overall our results show that Nac32HECT not only binds and ubiquitinates aS, but also leads to its degradation.

4.9 hiPSCs can be differentiated into dopaminergic neurons

Having selected an ubiquibody able to functionally interact with aS, we decided to move in further validating this chimeric protein and our overall strategy in a more relevant experimental model. To this end we decide to focus our attention on dopaminergic neurons since they are main cell type interested by aS aggregation in PD patients.

First of all, we developed and validate a protocol to differentiate human induced pluripotent stem cells (hiPSCs) to dopaminergic neurons (DNs). We adopted a differentiation protocol partially based on what has been published by Borg and collaborators (Borgs, 2016) and schematically reported in figure 28. As main differences with the published method, we adopted a different number of cells seeded in each plate (for 6-well plate $0.5x10^5$ cells/well); also, we introduced two steps during the growth of the neural stem cells (NSC): in the first step cells were named "progenitor NSC" and are grown in Neural Induction medium with 1% v/v of N-2 Supplement (17502-048 Gibco), 10 μM of ROCK inhibitor (Tocris Biochem) (described in paragraph 3.1 of the Materials), changing the medium every day for 5 days; in the second step cells were named "mature NSC" and are grown in polyornithine/laminin coated plates in Neural Expansion medium (Advanced DMEM along with Neurobasal Medium) with 1% v/v of N-2 Supplement. Also, we modified the differentiation step of DP in three steps all carried on in polyornithine/laminin coated plates: "1st week DP" in which the cells are grown in F12 DMEM and all the additional supplements for one week (see paragraph 3.1 of the Materials); "2nd week DP" in which cells are re-seed in polyornithine/laminin coated plates, changing the original medium with the F12 DMEM for one week; "3rd week DP" in which cells are grown in Neurobasal medium and the other additional supplements (see paragraph

3.1 of the Materials).

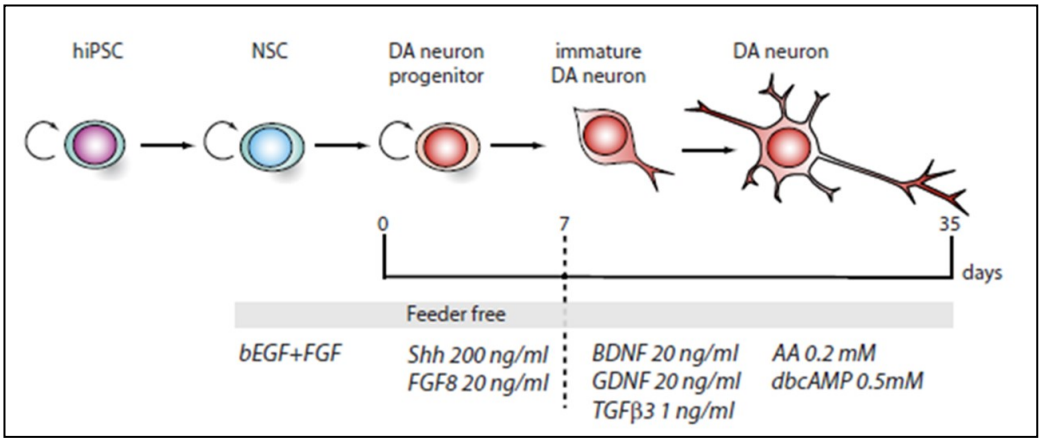


Figure 28**. Scheme showing the differentiation steps from hiPSCs to Dopaminergic neurons (Borgs, 2016).**

We verified by immunofluorescence each differentiation step, from NSC to DN, using appropriate differentiation markers (figure 29).

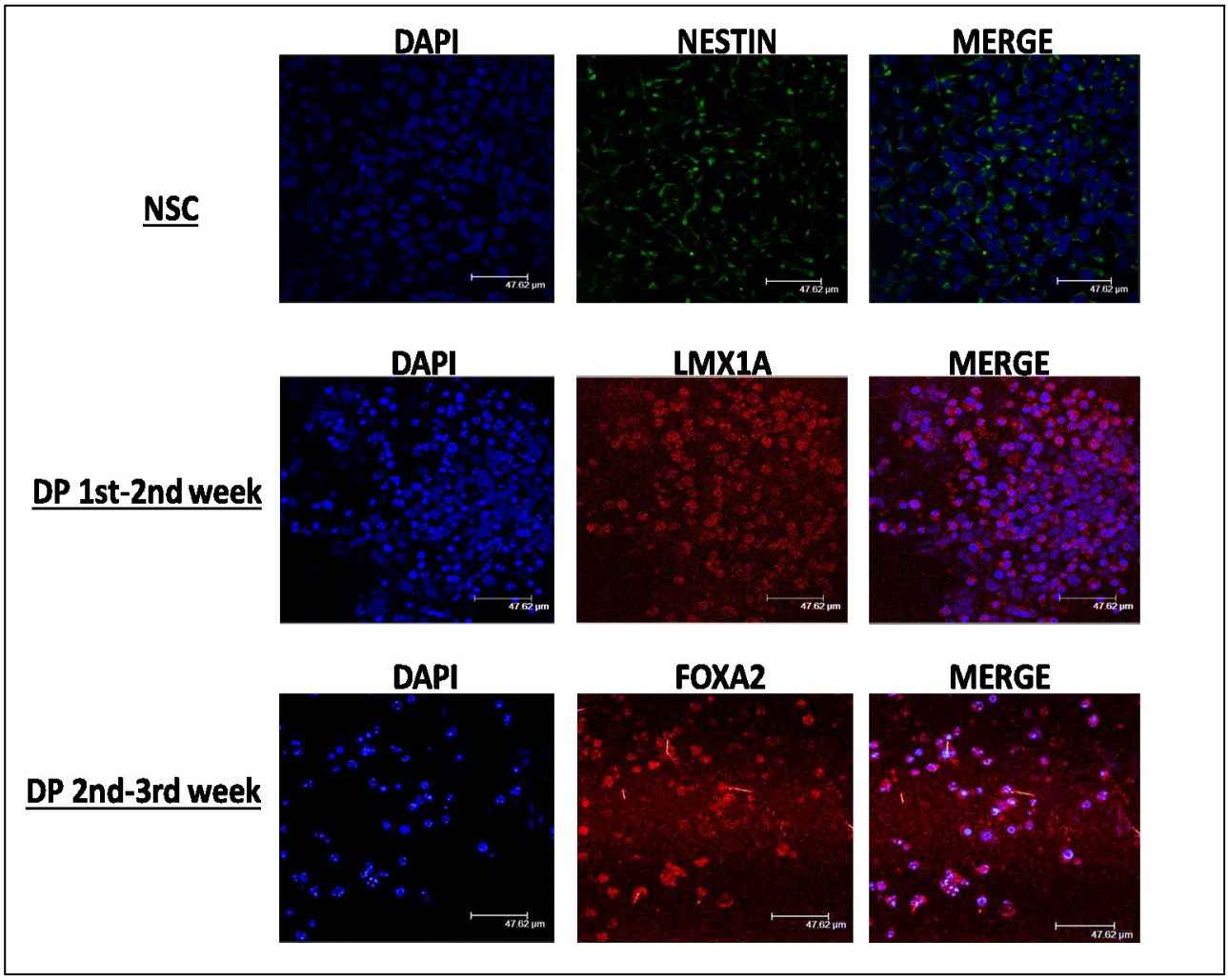


Figure 29**. Immunofluorescence for the differentiation from NSC to Dopaminergic progenitors (DP) at the 3rd week.** (NSC) immunolabeling of NSCs marker.In green Ab I: a-Nestin 1:200. Ab II: a-Rabbit 1: 500; nuclei Draq5 1:1500. (DP 1-2 week) immunolabeling of DP marker during 1st - 2nd week. In red. Ab I: a-Lmx1A 1:200 Ab II: a-Rabbit 1: 500; nuclei Draq5 1:1500 (DP 2-3 week) immunolabeling of DP marker during 2nd – 3rd week. In red. Ab I: a-FOXA2 1:200 Ab II: a-Rabbit 1: 500; nuclei Draq5 1:1500.

4.10 aS is expressed in neural stem cells and DN upon transduction with recombinant lentiviral particles

Next, the ectopic expression of aS fused in frame with EGFP was evaluated in NSC, obtained from hiPSCs, as previously described.

One-week post-transduction with RLP expressing the selected transgenes, NSCs were analyzed by FACS analysis, immunofluorescence, Western Blot and PCR end point for the presence of appropriate differentiation markers (Figure 30).

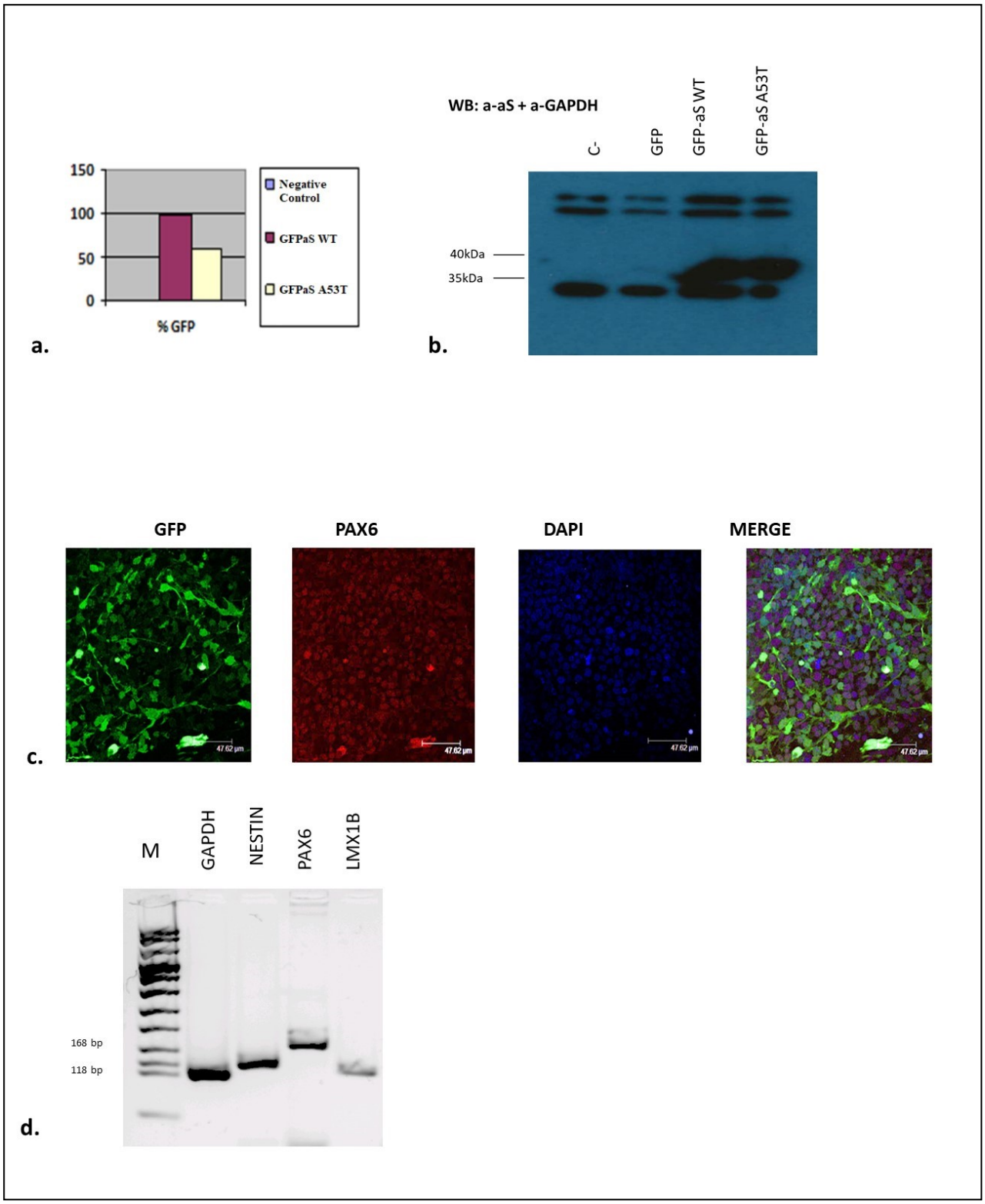


Figure 30. **RLP expressing A53T aS fused in frame with EGFP efficiently transduce NSC**. NSC were analyzed by FACS (a) and western blot (b) for aS expression 1-week post transduction with RLP encoding either WT or A53T aS fused in frame with EGFP. The In the western blot the primary antibody used is anti-aS and anti GAPDH, as a loading control. NSC transduced with RLP expressing A53T aS-EGFP were also analyzed by immunofluorescence (b), end point PCR (c) and western blot (d) for different differentiation

markers 1 week after transduction. In green: GFP; red: PAX6; blue:Draq5. Similar data were obtained with NSC transduced with WT aS expressing RLP (not shown)

The expression of A53T aS-EGFP was also followed by fluorescence microscopy over time during the differentiation of NSC to DN (Figure 31).

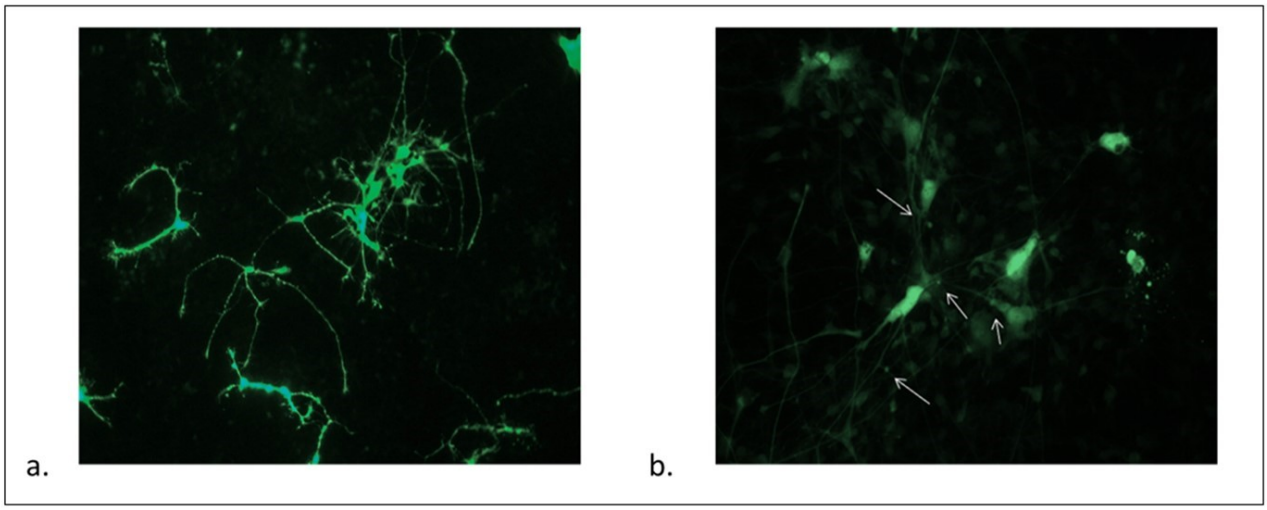


Figure 31. **A53T aS- EGFP is expressed in (a) Dopaminergic Progenitors 2 weeks post transduction with RLP, and (b) Dopaminergic Neurons. White arrows indicate aS aggregations.**

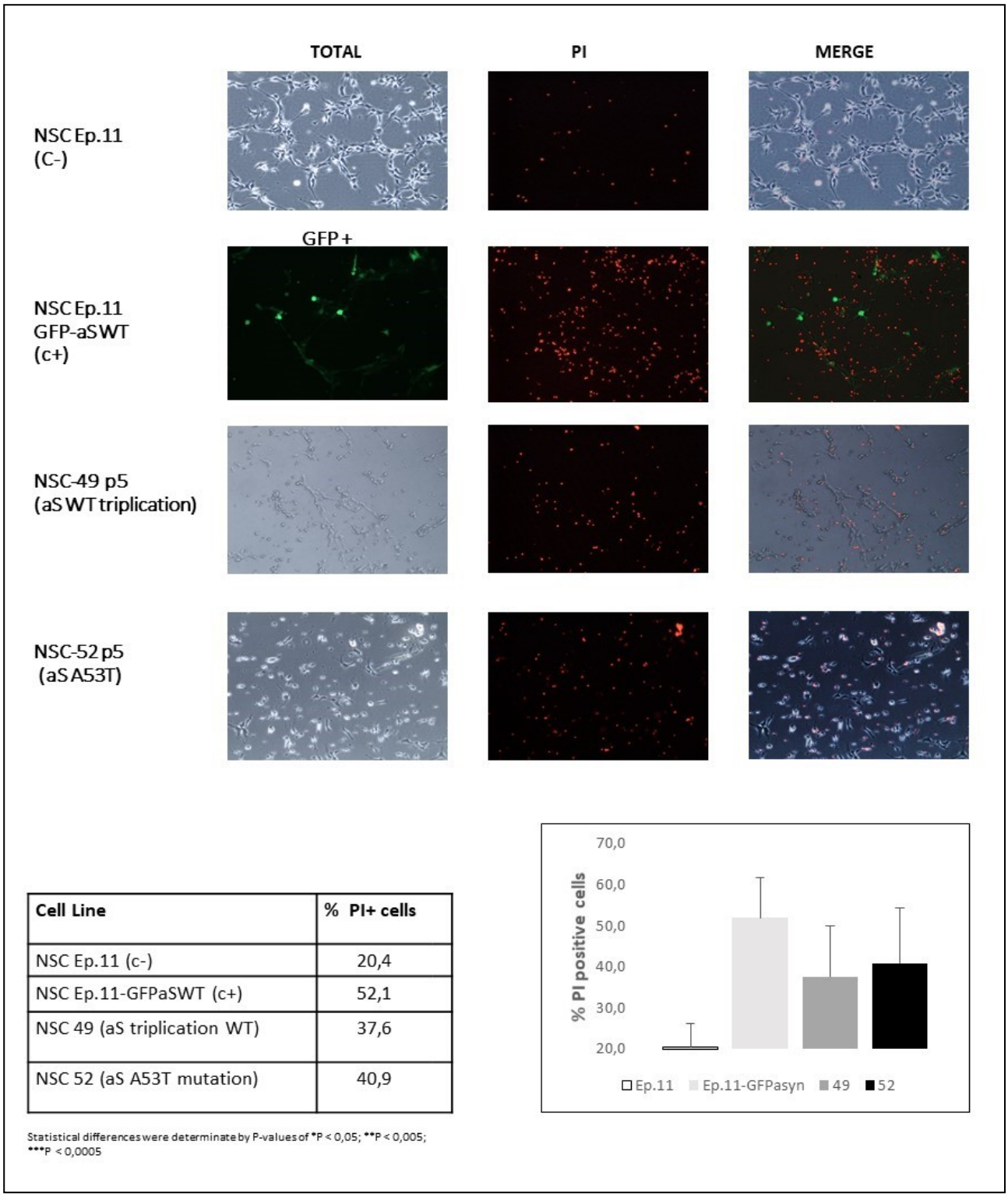
In figure 30.b is the presence of spots of possible aS accumulation are visible (indicated by white arrows), that might represent Lewy's bodies.

4.11 The expression of Nac32HECT ubiquibody rescue the Dopaminergic Progenitors

Since Oliveira and collaborators (Oliveira et al. 2015) have shown that high levels of aS, caused by a triplication of its encoding gene *SNCA* or by the A53T mutation, have a negative impact on hiPSC differentiation and subsequent maturation into NSCs (Oliveira et al., 2015), in order to functionally validate our strategy, we set up an experiment to investigate the effect of Nac32HECT on the ability of cells hiPSC overexpressing aS to differentiate into DN.

To this end, we obtained three lines of hiPSC, one from a healthy donor, and two from PD's patients, one characterized by *SNCA* triplication and the other one bearing A53T aS (described at the paragraph 3.1 of the Materials). Furthermore, we transduced NSC obtained from hiPSC, as previously described, with RLP expressing WT aS-EGFP.

First of all, we decided to confirm, in our experimental conditions, the observation of Oliveira and co-workers (Oliveira et al., 2015), whom have been shown that aS overexpression/mutation affect NSCs vitality. To this end, we analyzed the percentage of dead NSCs during the differentiation protocol. NSC were differentiate from "NSC progenitors" to "NSC mature" (see paragraph 4.10 of the Results), after one week of differentiation and the day before their differentiation in dopaminergic progenitors (DPs). To this end, propidium iodide staining was adopted (figure 32).



Cell Line	% PI+ cells
NSC Ep.11 (c-)	20,4
NSC Ep.11-GFPaSWT (c+)	52,1
NSC 49 (aS triplication WT)	37,6
NSC 52 (aS A53T mutation)	40,9

Statistical differences were determinate by P-values of *P < 0,05; **P < 0,005; ***P < 0,0005

Figure 32**. aS overexpression/mutation affects NSC vitality.** Cells were transduced with ubiquibodies and followed during the differentiation. Upper panels: representative imagines of the NSCs observed at the fluorescent microscopy upon propidium iodide (PI) staining. Bottom panels: table and graph report the percentage of PI positive NSC . Statistical differences were determinate by p-values of *p< 0.05; **p< 0.005.

The cell observation indicates that aS overexpression and mutation results in an increase in cell mortality as clearly highlighted in figure 31, confirming the results obtained by Oliveira and co-workers and thus validating our experimental system.

Based on these results, we tested the ability of Nac32HECT to rescue NSC physiological conditions and, as a consequence, their ability to differentiate into DP. To this end, we transduced all NSCs lines of the previous experiment with WT and C/S Nac32HECT and we followed cells during the differentiation steps from NSCs to DPs by microscopy (figure 33 and 34).

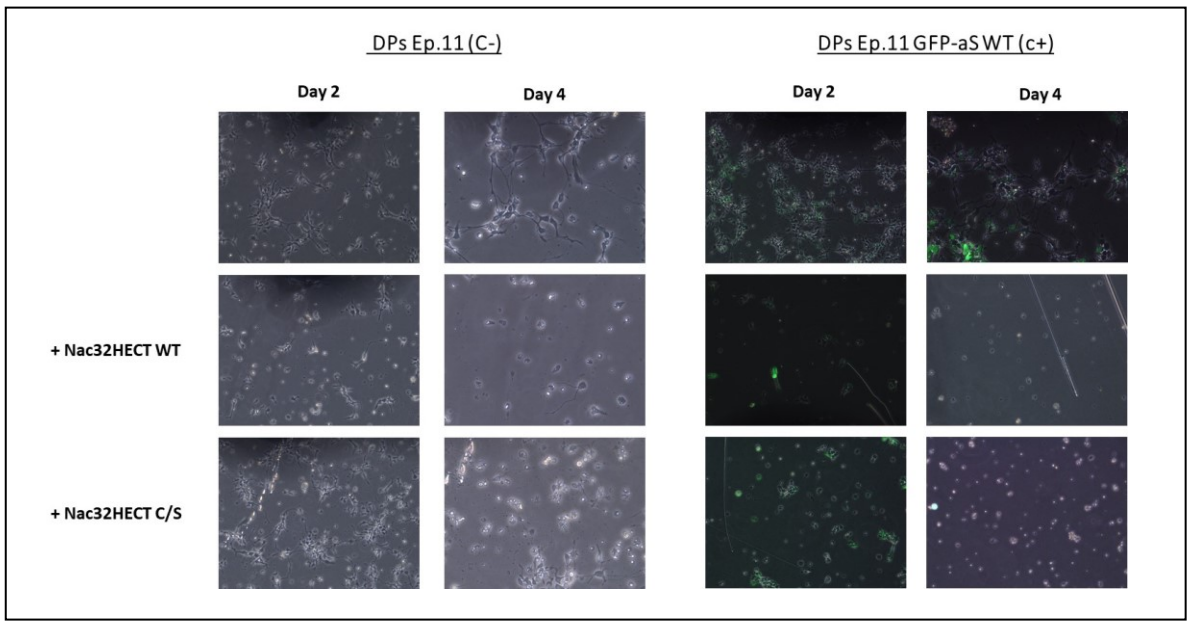


Figure 33. **Microscopy images of cells during the first week of differentiation.** (Left) images of the healthy control (c-) Ep.11 DP 1st week transduced with WT and C/S Nac32 ubiquibodies. (Right) images of the healthy control (c+) Ep.11 Dp1 1st week transduced with GFP-aS WT and WT or C/S Nac32 ubiquibodies.

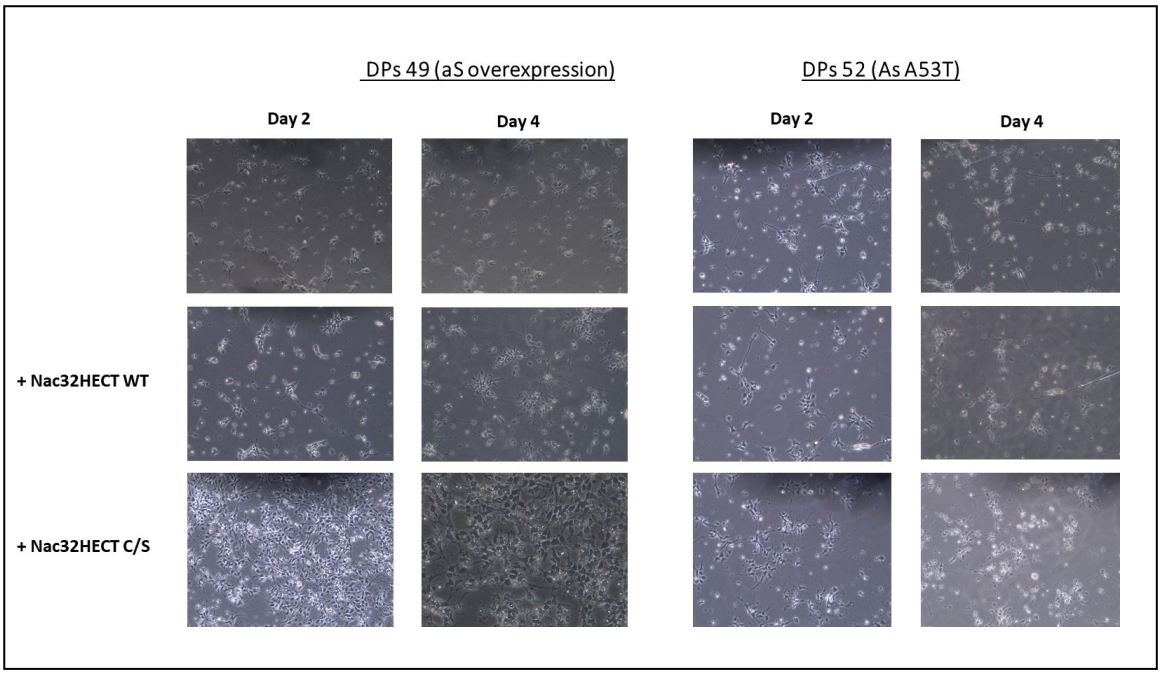


Figure 34. **Microscopy images of cells during the first week of differentiation**. (Left) images of the PD derived DP 1st week, which lead to the overexpression of WT aS (49) transduced with WT and C/S Nac32 ubiquibodies. (Right) images of PD derived DP 1st week which bearing the aS mutation A53Ttransduced with GFP-aS WT and WT or C/S Nac32 ubiquibodies.

In the case of aS-EGFP transduced cells we did not observe a rescue of cell vitality upon Nac32HECT transduction (figure 32). By contrast we noticed an increase in cell mortality likely due to the stress caused by the multiple transductions. It has to be mentioned that also Oliveira and coworkers had excluded ectopic over-expression of aS in their study, for similar reasons.

Instead, in the case of PD patient derived lines, cell observation showed an increase in the number of differentiated cells in the presence of WT Nac32HECT with respect to the controls, as confirmed by FACS analysis (table 4).

DP 1st week	*ubiquibodies*	*% FOXA2 positive cells*
49 (overexpression aS)	C- (not treated)	0.10
	+ Nac32HECT WT ubiquibody	23.6
	+ Nac32HECT C/S ubiquibody	18.70
52 (A53T aS)	C- (not treated)	0
	+ Nac32HECT WT ubiquibody	1.27
	+ Nac32HECT C/S ubiquibody	1.14

Table 4. **FACS analysis of positive cells to the differentiation marker FOXA2 expressing in DP 1st week of PD cells.**

These preliminary results but seem to indicate that indeed Nac32HECT is able to rescue the aS overexpressing/mutated NSC to differentiate into DP. If further confirmed with already ongoing experiments (figure 35) these data strongly support the validity of our approach to interfere with aS aggregation with biological and measurable consequences in relevant experimental models.

5. Discussion

PD is the second most frequent neurodegenerative disease after Alzheimer's, affecting more than 1% of people over 55 years old and more than 3% of over 75 years old (Dehay et al., 2015). PD is caused by the loss of dopaminergic neurons of the *pars compacta* of the *substantia nigra* and the subsequent striatal dopamine deficiency that it is primarily manifested as a motor disorder. In 80% of patients PD involves non-motor symptoms, including dementia (Perrett et al., 2015).

PD is also defined as a complex multifactorial disease, resulting from the combination of environmental and genetic factors, both of which play a decisive role in the development of the disease. The environment also seems to play a crucial role in PD pathogenesis. In particular, the toxic elements present in the environment, like pesticides, chemical agents, pollution or viruses may initiate PD pathogenesis at the olfactory bulb or at the gut enteric environment and, then, spread to the central nervous system (CNS) via olfactory or vagus nerves (Chen & Ritz, 2018).

The degeneration of the *substantia nigra pars compacta* and the presence of Lewy's bodies (LBs) which represent anomalous, cytoplasmic proteinaceous inclusions, mainly consisting of the alpha-synuclein protein (aS), are considered the hallmark of the disease (Kalia & Lang, 2015). In contrast to many proteins involved in neurodegeneration that are distributed throughout the neuron, however, aS localizes specifically to the nerve terminal, with relatively little in the cell body, dendrites or extrasynaptic sites along the axon. The presynaptic location of aS has been recognized since its original identification as a protein associated with synaptic vesicles (George et al., 1995) and it helps the excitatory transmission, mediating the docking of vesicles, transporting neurotransmitters, to the plasma membrane (Sulzer & Edwards, 2019). The role of LBs in the pathogenesis of PD is still controversial (Kalia & Lang, 2015), but the discovery that LBs contain deposits of misfolded aS has allowed the classification of the pathology as a synucleinopathy (Xilouri et al., 2013) and has focused the attention on this protein biology. Many evidence, including genetic ones, support the presence of misfolded aS in the cellular

environment that leads to the accumulation of aggregates and subsequently to cellular death (Xilouri et al., 2013).

Protein degradation systems are the main participants in the quality control of cellular proteins and are responsible for the removal of no more necessary, misfolded or damaged polypeptides (Villar-Piqué et al., 2016). aS, after being ubiquitinated, enters in multiple pathways of degradation, including the endo-lysosomal pathway (ELP). These proteolytic molecular pathways, can suffer alteration during physiological aging leading to an abnormal accumulation of aS and thus triggering a vicious circle (Martinez-Vicente & Vila, 2013).

Although the introduction of levodopa has revolutionized the treatment of PD, the therapies available today are aimed exclusively at alleviating symptoms and are not able to block or delay neurodegeneration (Dauer & Przedborski, 2003).

With this in mind, the present PhD project aimed at the generation of innovative potent tools to achieve degradation of aS aggregates in a specific manner. These tools will be adopted from one hand to further elucidate the role of aS and of its accumulation in PD. On the other hand, they could be adopted to develop an innovative therapeutic strategy to efficiently treat PD.

To this end, we took advantage of studies published by Tofaris and collaborators, which demonstrated that the ubiquitin ligase NEDD4 is the enzyme crucial for aS correct turn-over (Tofaris et al., 2011). Importantly, those Authors also convincingly showed that NEDD4 is over-expressed in the brain regions presenting Lewy pathology and that a single nucleotide polymorphism in its coding sequence is associated with a greater risk of PD onset (Tofaris et al., 2011). Overall this work correlates NEDD4 with aS turn-over and PD.

Furthermore, it has been shows that NEDD4 is protective against human aS toxicity in evolutionary distant models. In this context, a small molecule able to bind to and activate NEDD4 (NAB2) functions, was identified as neuroprotective agent in neuronal models of aS toxicity (Chung et al., 2013). Since Ub ligase activation cannot be easily obtained pharmacologically and despite the initial promising results with

compound screens, innovative strategies are needed to achieve a specific and targeted activation of NEDD4 towards aS.

Under this respect, Portnoff and collaborators were able to demonstrate that it is possible to generate chimeric ubiquitin ligases directed against a single specific target (Portnoff et al., 2014). In particular, those Authors have successfully developed a chimera that combines the enzymatic domain of Carboxyl terminus of Hsc70-interacting protein (CHIP) ubiquitin ligase with the target specificity of an scFv intrabody directed towards a protein of interest. At the same time, the engineered CHIP loses the ability to recognize its physiological targets (Portnoff et al., 2014).

Finally, Göttlinger and collaborators had already shown how it is possible to fuse the catalytic HECT domain of NEDD4 to heterologous proteins to achieve a specific ubiquitination of the structural Gag protein of the human immunodeficiency virus (HIV) (Weiss et al., 2010).

Based on all these literature findings, we decided to develop chimeric NEDD4 ubiquitin ligases specifically targeting aS, as a tool to interfere with its accumulation.

To this end, our experimental approach is based on the generation of chimeras constituted by the HECT catalytic domain of NEDD4 fused in frame to different intrabodies which are known to specifically bind aS. These novel chimeras, herein named ubiquibodies, should be capable of promoting ubiquitination and subsequent degradation of toxic forms and aggregates of aS through the endo-lysosomal pathway.

Specifically, we selected three previously characterized scFv intrabodies: Nac32, directed against the non-amyloid component (NAC domain) of human aS (Lynch et al., 2008); d5e, which exclusively binds the oligomeric form of aS and d10, which is pan-specific (Zhou & Przedborski, 2009b). Those scFvs were fused to a GSGSG spacer peptide, which provides suitable flexibility to the enzyme (Reddy Chichili et al., 2013), followed by the NEDD4, HECT catalytic domain and by an immunodominant sequence, the HA epitope.

For each ubiquibody we also developed a control in which the HECT catalytic domain is made not-functional due to an already well characterized mutation $C_{867}S$ (C/S) within the enzyme active site (Davies et al., 2014). Two additional controls were developed: the wild type (WT) and the mutated form (C/S) of the full length NEDD4 HA and the WT and C/S form of the isolated HECT domain.

All chimeras and controls were cloned into third-generation self-inactivating lentiviral vectors (LVs). The choice to use LVs is justified by the fact that they have shown a high efficiency in gene transfer *in vivo* and a stable and lasting expression of the transgene in multiple target tissues, including in non-dividing cells, like the neurons (Dull et al., 1998). Furthermore, last generation LVs are currently in several clinical trials also at advanced stages for the treatment of several genetic disorders (Cartier et al., 2009), thus supporting their safety and efficacy. Finally, we selected LVs over adeno-associated virus (AAV) based vectors, which are extremely efficient in transducing neurons, based on their superior ability to accommodate large and complex transgenes within their genome (Dull et al., 1998).

In order to characterize the generated LVs, first of all we tested their ability to express the recombinant proteins, by transfecting human embryonic kidney 293T cells, which are especially suitable for this application. The obtained results clearly showed that all the ubiquibodies are well expressed in a single chimeric protein within the intracellular environment. Starting from this encouraging result, we move to the production of pseudotyped recombinant lentiviral particles (RLPs). In particular, to pseudotype the RLPs we adopted the vesicular stomatitis virus (VSV) - G envelope glycoprotein. Indeed, VSV-G allows the transduction of a wide range of cell types, including neurons, and can be subjected to ultracentrifugation, a procedure required to increase the titer of the vectors especially before moving to *in vivo* experiments, without losing infectivity (Dull et al., 1998). We were able to show that the generated RLPs efficiently transduce 293T cells, leading to the expression of all the ubiquibodies and relative controls. This result indicate that the developed vectors are correctly incorporated into the RPLs.

Next, we moved to the characterization of the generated ubiquibodies in terms of ability to interfere with aS accumulation. To this end we started by setting up a suitable *in vitro* model. It has been previously reported that over-expression of WT aS under the transcriptional control of a strong promoter (e.g. the HCMV promoter) either alone or fused in frame with the reporter protein EGFP leads to aS accumulation in eukaryotic cells resembling what is found in dopaminergic neurons of PD patients (Falkenburger et al., 2016). Furthermore, is has been shown that aS bearing the mutation A53T, that is found in an autosomal dominant form of PD (Chaudhuri & Paul, 2006; Falkenburger et al., 2016; Conway et al., 1998), are particularly prone (Falkenburger et al., 2016) to forms fibrils when expressed *in vitro* in cell lines of different origin. Indeed, over-expression of WT or A53T aS alone or fused in frame with EGFP in cells as the 293T cells line is widely recognized and adopted as *in vitro* model to study drugs and strategies to interfere with aS accumulation (Falkenburger et al., 2016). We this in mind, we decided to generate constructs, and in particular LVs, expressing WT and A53T aS alone and fused to EGFP under the transcriptional control of the HCMV promoter. Once again the choice of adopting LVs was dictated by the need to develop a tool to efficiently over-express aS not only in cell lines, but also in primary cells as in dopaminergic neurons, that represent one of the most relevant cellular models to study aS biology in the context of PD. Also, in this case, we succeeded in generating LVs expressing WT and A53T aS fused either to a Flag tag or to EGFP and we were able to produce RLPs able to efficiently transduce target cells leading to the expression of all the recombinant proteins.

Having developed all the necessary tools, we set up to investigate whether ubiquibodies are able to i) interact with aS; ii) lead to aS ubiquitination; iii) affect aS intracellular amount. In particular, we were interested in compare the performance of the three different scFvs considering their ability to bind different forms/domains of aS (see above). We were able to show that all the developed ubiquibodies bind to and

ubiquitinate aS. However, Nac32HECT is overall the best performing recombinant ubiquitin ligase both in terms of binding and catalytic activity towards aS. In particular, we were able to show, by adopting two different assays [co-immunoprecipitation and proximity ligation assay (PLA)], that Nac32HECT ubiquibody efficiently interact with aS. Importantly, PLA was performed in immortalized murine dopaminergic-like cells, the MN9Dsyn cell line that are a well-recognized model to study the effects of aS accumulation (Feng et al., 2010). Furthermore, in this assay the ability of ubiquibodies to interact with an unrelated protein, i.e. the Glutamine Synthetase (GS), was checked, supporting the specificity of Nac32HECT, as well as of the additional two chimeric ubiquitin ligases towards aS.

This set of experiments allowed us to select Nac32HECT as the best performing ubiquibody to be employed in the following steps of the project aimed at further validating our strategy.

To this end, we started by investigating the ability of Nac32HECT to affect aS intracellular amount. By using two different approaches we were able to show that indeed the selected ubiquibody is able to reduce aS intracellular levels. Importantly, in one case we adopted the neuroblastoma cell line SHSY5Y and we looked at the effect of Nac32HECT expression on endogenous aS.

To move to an even more relevant *in vitro* system, we decided to employ dopaminergic neurons derived from human induced pluripotent stem cells (hiPSCs) obtained from PD patients bearing different mutations within aS encoding sequence. The reason of selecting this model stem from the fact that, as already mentioned, dopaminergic neurons are the cell type in the CNS most affected by aS accumulation in PD patients. Furthermore, the idea of deriving them from hiPSCs originate not only from the feasibility of this technique and the availability in the laboratory of all the tools and the expertise necessary to generate and/or manipulate this type of cells (Trevisan et al., 2018, 2015) but also from the fact that the process leading from hiPSCs to dopaminergic neurons (DNs) constitutes per se an excellent model to

biologically validate our strategy. Indeed, it has been previously demonstrated by Oliveira and collaborators that high levels of aS have a negative impact on the differentiation of hiPSCs towards neural stem cells (NSCs) till dopaminergic neurons (Oliveira et al., 2015). Importantly, when the authors silenced the expression of aS using small interfering RNAs in hiPSCs, they were able to recover the differentiation/maturation capacity of hiPSCs at levels correlated with the degree of silencing (Oliveira et al., 2015). Thus, we decided to adopt the same experimental setting to analyze whether Nac32HECT, by interfering with aS intracellular levels, was able to rescue the ability of aS overexpressing hiPSCs to differentiate into dopaminergic neurons.

First of all, we set up the protocol to differentiate hiPSCs to dopaminergic neurons by modifying what has been previously published by Borg and co-workers (Borgs, 2016). Next we selected two hiPSC, one deriving from a PD patient carrying a triplication of the aS encoding gene and the other one obtained from a PD patient characterized by A53T aS. Furthermore, we also over-express aS-EGFP by LV transducing NSC derived from hiPSC, kindly provided by Dr. Trevisan (Trevisan et al., 2015).

First of all, we confirmed that also in our hands, as reported by Oliveira and co-workers (Oliveira et al. 2015) aS over-expression affects the viability of NSCs, thus validating our experimental conditions. Next, we evaluated the ability of NSC to differentiate in DP in presence of the Nac32HECT ubiquibody. Our preliminary results show that, indeed, in presence of WT Nac32HECT there is an increase of differentiated cells compared to the negative controls, represented by untransduced cells and cells transduced with C/S Nac32HECT. Of note, we also confirm previous findings from Oliveira and co-workers showing that also in our hand LV transduction is not a suitable method to overexpress aS in this experimental setting. Indeed, cells are too stressed by multiple transductions and, most-likely, by the toxic effect of aS exacerbated by the efficiency of expression obtained thanks to the LV system. By contrast, patient derived hiPSCs represent the best choice.

We are currently setting up an experiment in which we will use NSC transduced with RLP expressing ubiquibodies and will differentiate in DN. During the differentiation step we will analyze cells with the appropriate differentiation markers by FACS analysis and Western Blot to verify weather in presence of ubiquibodies cells rescue the phenotype of DN. The future experiments are summarized in figure 35.

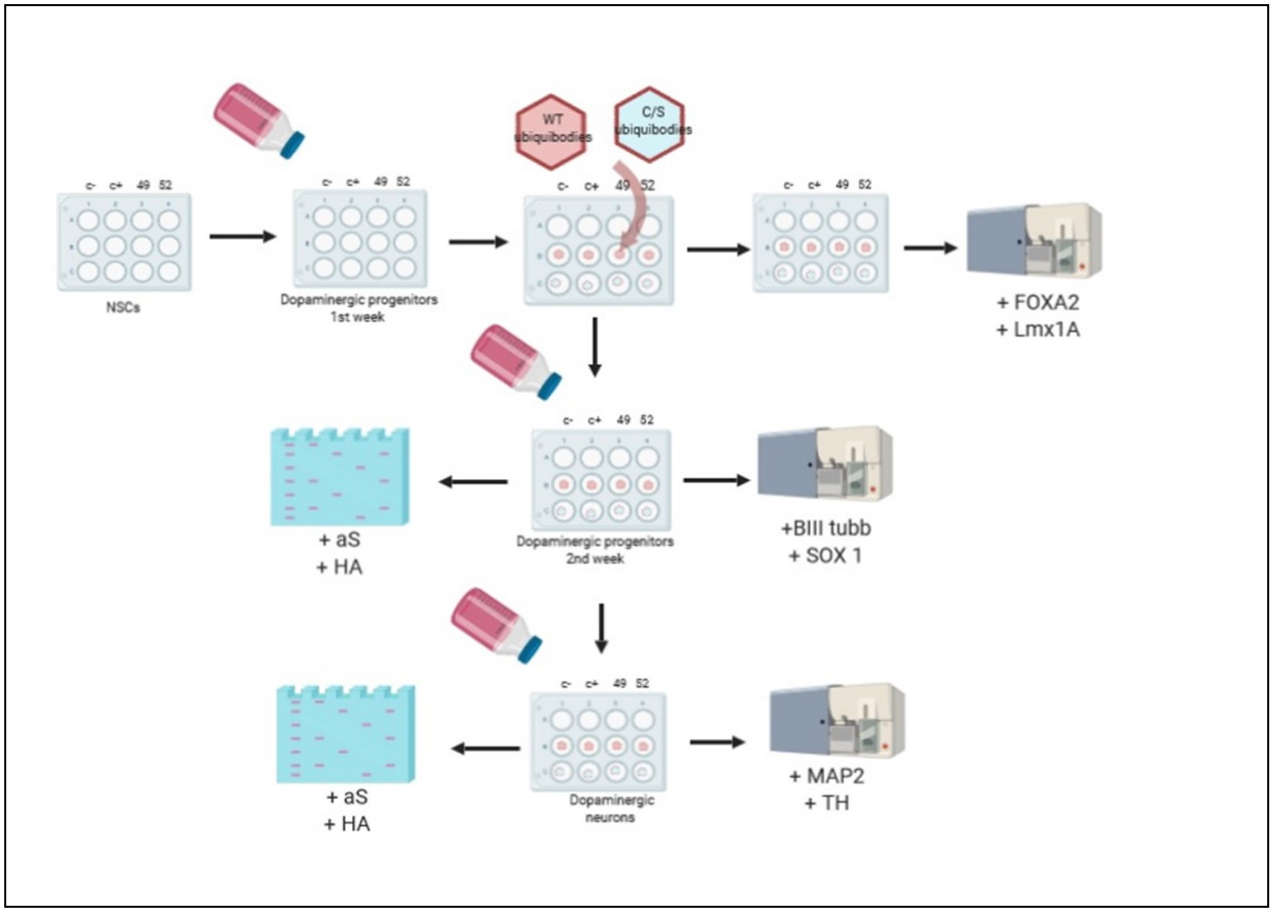


Figure 35**. Ongoing experiment to evaluate the rescue of DNs in presence of ubiquibodies.** We summarize the future experiments, in which we are going to use NSCs during the differentiation in DNs. After the transduction with RLP expressing WT and C/S ubiquibodies we will analyse cells with the appropriate differentiation markers by FACS analysis and Western Blot.

Furthermore, in collaboration with Professor Maguire-Zeiss form the Georgetown University (Washington D.C., USA), were I spent the last four months of my PhD program, we are currently setting up an experiment to test the ability of Nac32HCT to rescue aS pathology in a mouse model of aS overexpression (Decressac et al., 2012). I already prepared concentrated RLP stocks in PBS to be stereotaxicaly injected into

the mice substantia nigra. The effects of Nac32HECT expression on aS aggregates will be analyzed by immunohistochemistry (Béraud et al., 2013). Given the lack of boundaries between the SN and ventral tegmental area (VTA), in which 60% of neurons are dopaminergic as well, we will also investigate the possible effect of ubiquibody injections in these neurons. Indeed, VTA is mainly involved in motivational aspects of behavior and PD patients often show motivational deficits. Noteworthy, VTA is reciprocally connected with the olfactory bulb, the main dopaminergic brain area, and olfactory deficits are also among the early symptoms of PD. Thus, the olfactory system (olfactory bulb and amygdala) will be also analysed.

In conclusion, during my PhD work I was able to show that:

i) from the structural point of view, NEDD4 is a valid choice to generate functional chimeras with scFv;
ii) developed ubiquibodies interact with and ubiquitinate aS
iii) Nac32HECT, which contains the scFv binding to aggregate form of aS, is the best performing ubiquibody and it is able to affect aS intracellular levels
iv) Nac32HECT rescues the ability of hiPSCs to differentiate into dopaminergic neurons in the presence of aS overexpression/mutation.

Thus, Nac32HECT represents a suitable tool to interfere with aS accumulation and will be further characterized also in the mice model. The selected ubiquibody will be useful not only to clarify the role of aS aggregation in PD pathogenesis, but also as innovative therapeutic strategy. Previous attempts to develop gene therapy approaches against PD aimed at interfering with aS expression for instance by RNA interference failed since “normal” aS has physiological functions that need to be preserved (Gorbatyuk et al., 2010). This crucial issue should be overcome in our strategy, since we are performing a post-translational silencing specifically directed against aggregated forms of aS, thus maintaining unaltered those physiological functions.

6. References

Beach, T. G., Adler, C. H., Lue, L., Sue, L. I., Bachalakuri, J., Henry-Watson, J., … Arizona Parkinson's Disease Consortium, D. G. (2009). Unified staging system for Lewy body disorders: correlation with nigrostriatal degeneration, cognitive impairment and motor dysfunction. *Acta Neuropathologica*, *117*(6), 613–634. https://doi.org/10.1007/s00401-009-0538-8

Bendor, J. T., Logan, T. P., & Edwards, R. H. (2013). The Function of α-Synuclein. *Neuron*, *79*(6), 1044–1066. https://doi.org/10.1016/j.neuron.2013.09.004

Béraud, D., Hathaway, H. A., Trecki, J., Chasovskikh, S., Johnson, D. A., Johnson, J. A., … Maguire-Zeiss, K. A. (2013). Microglial Activation and Antioxidant Responses Induced by the Parkinson's Disease Protein α-Synuclein. *Journal of Neuroimmune Pharmacology*, *8*(1), 94–117. https://doi.org/10.1007/s11481-012-9401-0

Borsotti, C., Borroni, E., & Follenzi, A. (2016, December 1). Lentiviral vector interactions with the host cell. *Current Opinion in Virology*, Vol. 21, pp. 102–108. https://doi.org/10.1016/j.coviro.2016.08.016

Braak, H., Del Tredici, K., Rüb, U., de Vos, R. A. I., Jansen Steur, E. N. H., & Braak, E. (2003). Staging of brain pathology related to sporadic Parkinson's disease. *Neurobiology of Aging*, *24*(2), 197–211. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/12498954

Breil L. (2018). A gut-brain link for Parkinson's gets a closer look. *ScienceNews*, *194*, p.22.

Bruce, M. C., Kanelis, V., Fouladkou, F., Debonneville, A., Staub, O., & Rotin, D. (2008). Regulation of Nedd4-2 self-ubiquitination and stability by a PY motif located within its HECT-domain. *The Biochemical Journal*, *415*(1), 155–163. https://doi.org/10.1042/BJ20071708

Burré, J., Sharma, M., Tsetsenis, T., Buchman, V., Etherton, M. R., & Südhof, T. C. (2010). Alpha-synuclein promotes SNARE-complex assembly in vivo and in vitro. *Science (New York, N.Y.)*, *329*(5999), 1663–1667.

https://doi.org/10.1126/science.1195227

Cartier, N., Hacein-Bey-Abina, S., Bartholomae, C. C., Veres, G., Schmidt, M., Kutschera, I., … Aubourg, P. (2009). Hematopoietic Stem Cell Gene Therapy with a Lentiviral Vector in X-Linked Adrenoleukodystrophy. *Science*, *326*(5954), 818–823. https://doi.org/10.1126/SCIENCE.1171242

Cavazzana-Calvo, M., Payen, E., Negre, O., Wang, G., Hehir, K., Fusil, F., … Leboulch, P. (2010). Transfusion independence and HMGA2 activation after gene therapy of human β-thalassaemia. *Nature*, *467*(7313), 318–322. https://doi.org/10.1038/nature09328

Chaudhuri, T. K., & Paul, S. (2006). Protein-misfolding diseases and chaperone-based therapeutic approaches. *FEBS Journal*, *273*(7), 1331–1349. https://doi.org/10.1111/j.1742-4658.2006.05181.x

Chen, H., & Ritz, B. (2018). The search for environmental causes of Parkinson's disease: Moving forward. *Journal of Parkinson's Disease*, Vol. 8, pp. S9–S17. https://doi.org/10.3233/JPD-181493

Choi, H. K., Won, L. A., Kontur, P. J., Hammond, D. N., Fox, A. P., Wainer, B. H., … Heller, A. (1991). Immortalization of embryonic mesencephalic dopaminergic neurons by somatic cell fusion. *Brain Research*, *552*(1), 67–76. https://doi.org/10.1016/0006-8993(91)90661-e

Chung, C. Y., Khurana, V., Auluck, P. K., Tardiff, D. F., Mazzulli, J. R., Soldner, F., … Lindquist, S. (2013). Identification and Rescue of -Synuclein Toxicity in Parkinson Patient-Derived Neurons. *Science*, *342*(6161), 983–987. https://doi.org/10.1126/science.1245296

Conway, K. A., Harper, J. D., & Lansbury, P. T. (1998). Accelerated in vitro fibril formation by a mutant α-synuclein linked to early-onset Parkinson disease. *Nature Medicine*, *4*(11), 1318–1320. https://doi.org/10.1038/3311

Dauer, W., & Przedborski, S. (2003). Parkinson's disease: mechanisms and models. *Neuron*, *39*(6), 889–909. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/12971891

Davies, S. E., Hallett, P. J., Moens, T., Smith, G., Mangano, E., Kim, H. T., … Tofaris, G. K. (2014). Enhanced ubiquitin-dependent degradation by Nedd4 protects against α-synuclein accumulation and toxicity in animal models of Parkinson's disease. *Neurobiology of Disease*, *64*, 79–87. https://doi.org/10.1016/j.nbd.2013.12.011

Decressac, M., Mattsson, B., Lundblad, M., Weikop, P., & Björklund, A. (2012). Progressive neurodegenerative and behavioural changes induced by AAV-mediated overexpression of α-synuclein in midbrain dopamine neurons. *Neurobiology of Disease*, *45*(3), 939–953. https://doi.org/10.1016/j.nbd.2011.12.013

Dehay, B., Bourdenx, M., Gorry, P., Przedborski, S., Vila, M., Hunot, S., … Meissner, W. G. (2015). Targeting α-synuclein for treatment of Parkinson's disease: mechanistic and therapeutic considerations. *The Lancet. Neurology*, *14*(8), 855–866. https://doi.org/10.1016/S1474-4422(15)00006-X

Del Tredici, K., Rüb, U., de Vos, R. A. I., Bohl, J. R. E., & Braak, H. (2002). Where Does Parkinson Disease Pathology Begin in the Brain? *Journal of Neuropathology & Experimental Neurology*, *61*(5), 413–426. https://doi.org/10.1093/jnen/61.5.413

Dexter, D. T., & Jenner, P. (2013). Parkinson disease: from pathology to molecular disease mechanisms. *Free Radical Biology and Medicine*, *62*, 132–144. https://doi.org/10.1016/j.freeradbiomed.2013.01.018

Dull, T., Zufferey, R., Kelly, M., Mandel, R. J., Nguyen, M., Trono, D., & Naldini, L. (1998). A third-generation lentivirus vector with a conditional packaging system. *Journal of Virology*, *72*(11), 8463–8471. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/9765382

Ejlerskov, P., Rasmussen, I., Nielsen, T. T., Bergström, A.-L., Tohyama, Y., Jensen, P. H., & Vilhardt, F. (2013). Tubulin Polymerization-promoting Protein (TPPP/p25α) Promotes Unconventional Secretion of α-Synuclein through Exophagy by Impairing Autophagosome-Lysosome Fusion. *Journal of*

Biological Chemistry, *288*(24), 17313–17335. https://doi.org/10.1074/jbc.M112.401174

Emamzadeh, F. N. (2016). Alpha-synuclein structure, functions, and interactions. *Journal of Research in Medical Sciences : The Official Journal of Isfahan University of Medical Sciences*, *21*, 29. https://doi.org/10.4103/1735-1995.181989

Fahn, S. (2015). The medical treatment of Parkinson disease from James Parkinson to George Cotzias. *Movement Disorders*, *30*(1), 4–18. https://doi.org/10.1002/mds.26102

Falkenburger, B. H., Saridaki, T., & Dinter, E. (2016, October 1). Cellular models for Parkinson's disease. *Journal of Neurochemistry*, pp. 121–130. https://doi.org/10.1111/jnc.13618

Fauvet, B., Mbefo, M. K., Fares, M.-B., Desobry, C., Michael, S., Ardah, M. T., … Lashuel, H. A. (2012). α-Synuclein in Central Nervous System and from Erythrocytes, Mammalian Cells, and *Escherichia coli* Exists Predominantly as Disordered Monomer. *Journal of Biological Chemistry*, *287*(19), 15345–15364. https://doi.org/10.1074/jbc.M111.318949

Feng, L. R., Federoff, H. J., Vicini, S., & Maguire-Zeiss, K. A. (2010). α-Synuclein mediates alterations in membrane conductance: a potential role for α-synuclein oligomers in cell vulnerability. *European Journal of Neuroscience*, *32*(1), 10–17. https://doi.org/10.1111/j.1460-9568.2010.07266.x

Forsyth, C. B., Shannon, K. M., Kordower, J. H., Voigt, R. M., Shaikh, M., Jaglin, J. A., … Keshavarzian, A. (2011). Increased Intestinal Permeability Correlates with Sigmoid Mucosa alpha-Synuclein Staining and Endotoxin Exposure Markers in Early Parkinson's Disease. *PLoS ONE*, *6*(12), e28032. https://doi.org/10.1371/journal.pone.0028032

George, J. M., Jin, H., Woods, W. S., & Clayton, D. F. (1995). Characterization of a novel protein regulated during the critical period for song learning in the zebra finch. *Neuron*, *15*(2), 361–372. https://doi.org/10.1016/0896-6273(95)90040-3

Gorbatyuk, O. S., Li, S., Nash, K., Gorbatyuk, M., Lewin, A. S., Sullivan, L. F., … Muzyczka, N. (2010). In Vivo RNAi-Mediated α-Synuclein Silencing Induces Nigrostriatal Degeneration. *Molecular Therapy*, *18*(8), 1450–1457. https://doi.org/10.1038/mt.2010.115

Guhathakurta, S., Bok, E., Evangelista, B. A., & Kim, Y.-S. (2017). Deregulation of α-synuclein in Parkinson's disease: Insight from epigenetic structure and transcriptional regulation of SNCA. *Progress in Neurobiology*, *154*, 21–36. https://doi.org/10.1016/j.pneurobio.2017.04.004

Hasegawa, S., Goto, S., Tsuji, H., Okuno, T., Asahara, T., Nomoto, K., … Hirayama, M. (2015). Intestinal Dysbiosis and Lowered Serum Lipopolysaccharide-Binding Protein in Parkinson's Disease. *PLOS ONE*, *10*(11), e0142164. https://doi.org/10.1371/journal.pone.0142164

Hasegawa, T., Matsuzaki, M., Takeda, A., Kikuchi, A., Akita, H., Perry, G., … Itoyama, Y. (2004). Accelerated α-synuclein aggregation after differentiation of SH-SY5Y neuroblastoma cells. *Brain Research*, *1013*(1), 51–59. https://doi.org/10.1016/J.BRAINRES.2004.04.018

Hicke, L. (2001). A new ticket for entry into budding vesicles-ubiquitin. *Cell*, *106*(5), 527–530. https://doi.org/10.1016/S0092-8674(01)00485-8

Hill-Burns, E. M., Debelius, J. W., Morton, J. T., Wissemann, W. T., Lewis, M. R., Wallen, Z. D., … Payami, H. (2017). Parkinson's disease and Parkinson's disease medications have distinct signatures of the gut microbiome. *Movement Disorders*, *32*(5), 739–749. https://doi.org/10.1002/mds.26942

Houlden, H., & Singleton, A. B. (2012). The genetics and neuropathology of Parkinson's disease. *Acta Neuropathologica*, *124*(3), 325–338. https://doi.org/10.1007/s00401-012-1013-5

Ingham, R. J., Gish, G., & Pawson, T. (2004). The Nedd4 family of E3 ubiquitin ligases: functional diversity within a common modular architecture. *Oncogene*, *23*(11), 1972–1984. https://doi.org/10.1038/sj.onc.1207436

Jentsch, S. (1992). The Ubiquitin-Conjugation System. *Annual Review of Genetics*,

26(1), 179–207. https://doi.org/10.1146/annurev.ge.26.120192.001143

Kalia, L. V, & Lang, A. E. (2015). Parkinson's disease. *Lancet (London, England)*, *386*(9996), 896–912. https://doi.org/10.1016/S0140-6736(14)61393-3

Kim, C., Rockenstein, E., Spencer, B., Kim, H.-K., Adame, A., Trejo, M., … Masliah, E. (2015). Antagonizing Neuronal Toll-like Receptor 2 Prevents Synucleinopathy by Activating Autophagy. *Cell Reports*, *13*(4), 771. https://doi.org/10.1016/J.CELREP.2015.09.044

Krüger, R., Kuhn, W., Müller, T., Woitalla, D., Graeber, M., Kösel, S., … Riess, O. (1998). AlaSOPro mutation in the gene encoding α-synuclein in Parkinson's disease. *Nature Genetics*, *18*(2), 106–108. https://doi.org/10.1038/ng0298-106

Lehtonen, Š., Sonninen, T. M., Wojciechowski, S., Goldsteins, G., & Koistinaho, J. (2019). Dysfunction of cellular proteostasis in Parkinson's disease. *Frontiers in Neuroscience*, *13*(MAY), 1–19. https://doi.org/10.3389/fnins.2019.00457

Lévy, C., Verhoeyen, E., & Cosset, F.-L. (2015). Surface engineering of lentiviral vectors for gene transfer into gene therapy target cells. *Current Opinion in Pharmacology*, *24*, 79–85. https://doi.org/10.1016/J.COPH.2015.08.003

Lois, C., Hong, E. J., Pease, S., Brown, E. J., & Baltimore, D. (2002). Germline Transmission and Tissue-Specific Expression of Transgenes Delivered by Lentiviral Vectors. *Science*, *295*(5556), 868–872. https://doi.org/10.1126/science.1067081

Lynch, S. M., Zhou, C., & Messer, A. (2008). An scFv Intrabody against the Nonamyloid Component of α-Synuclein Reduces Intracellular Aggregation and Toxicity. *Journal of Molecular Biology*, *377*(1), 136–147. https://doi.org/10.1016/j.jmb.2007.11.096

Maguire-Zeiss, K. A., Wang, C. I., Yehling, E., Sullivan, M. A., Short, D. W., Su, X., … Federoff, H. J. (2006). Identification of human α-synuclein specific single chain antibodies. *Biochemical and Biophysical Research Communications*, *349*(4), 1198–1205. https://doi.org/10.1016/J.BBRC.2006.08.127

Maroteaux, L., Campanelli, J. T., & Scheller, R. H. (1988). Synuclein: a neuron-

specific protein localized to the nucleus and presynaptic nerve terminal. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience*, *8*(8), 2804–2815. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/3411354

Martinez-Vicente, M., & Vila, M. (2013, September). Alpha-synuclein and protein degradation pathways in Parkinson's disease: A pathological feed-back loop. *Experimental Neurology*, Vol. 247, pp. 308–313. https://doi.org/10.1016/j.expneurol.2013.03.005

Messer, A., & Joshi, S. N. (2013). Intrabodies as Neuroprotective Therapeutics. *Neurotherapeutics*, *10*(3), 447–458. https://doi.org/10.1007/s13311-013-0193-6

Miraglia, F., & Colla, E. (2019). Microbiome, Parkinson's Disease and Molecular Mimicry. *Cells*, *8*(3). https://doi.org/10.3390/cells8030222

Mor, D. E., Ugras, S. E., Daniels, M. J., & Ischiropoulos, H. (2016). Dynamic structural flexibility of α-synuclein. *Neurobiology of Disease*, *88*, 66–74. https://doi.org/10.1016/J.NBD.2015.12.018

Murphy, M. P., LeVine, H., & III. (2010). Alzheimer's disease and the amyloid-beta peptide. *Journal of Alzheimer's Disease : JAD*, *19*(1), 311–323. https://doi.org/10.3233/JAD-2010-1221

Naldini, L., Blomer, U., Gallay, P., Ory, D., Mulligan, R., Gage, F. H., … Trono, D. (1996). In Vivo Gene Delivery and Stable Transduction of Nondividing Cells by a Lentiviral Vector. *Science*, *272*(5259), 263–267. https://doi.org/10.1126/science.272.5259.263

Oliveira, L. M. A., Falomir-Lockhart, L. J., Botelho, M. G., Lin, K.-H., Wales, P., Koch, J. C., … Jovin, T. M. (2015). Elevated α-synuclein caused by SNCA gene triplication impairs neuronal differentiation and maturation in Parkinson's patient-derived induced pluripotent stem cells. *Cell Death & Disease*, *6*(11), e1994. https://doi.org/10.1038/cddis.2015.318

Pankratz, N., & Foroud, T. (2007). Genetics of Parkinson disease. *Genetics in Medicine : Official Journal of the American College of Medical Genetics*, *9*(12), 801–811. https://doi.org/10.1097GIM.0b013e31815bf97c

Papadimitriou, D., Antonelou, R., Miligkos, M., Maniati, M., Papagiannakis, N., Bostantjopoulou, S., … Stefanis, L. (2016). Motor and Nonmotor Features of Carriers of the p.A53T Alpha-Synuclein Mutation: A Longitudinal Study. *Movement Disorders*, *31*(8), 1226–1230. https://doi.org/10.1002/mds.26615

Parkinson, J. (2002). An Essay on the Shaking Palsy. *The Journal of Neuropsychiatry and Clinical Neurosciences*, *14*(2), 223–236. https://doi.org/10.1176/jnp.14.2.223

Perrett, R. M., Alexopoulou, Z., & Tofaris, G. K. (2015, May 1). The endosomal pathway in Parkinson's disease. *Molecular and Cellular Neuroscience*, Vol. 66, pp. 21–28. https://doi.org/10.1016/j.mcn.2015.02.009

Petersen, M. V. (2017). *(PDF) Tractography and Neurosurgical Targeting in Deep Brain Stimulation for Parkinson's Disease*. https://doi.org/10.13140/RG.2.2.16230.9376

Petrov, V. A., Saltykova, I. V., Zhukova, I. A., Alifirova, V. M., Zhukova, N. G., Dorofeeva, Y. B., … Sazonov, A. E. (2017). Analysis of Gut Microbiota in Patients with Parkinson's Disease. *Bulletin of Experimental Biology and Medicine*, *162*(6), 734–737. https://doi.org/10.1007/s10517-017-3700-7

Plotegher, N., Greggio, E., Bisaglia, M., & Bubacco, L. (2014). Biophysical groundwork as a hinge to unravel the biology of *α* -synuclein aggregation and toxicity. *Quarterly Reviews of Biophysics*, *47*(1), 1–48. https://doi.org/10.1017/S0033583513000097

Polymeropoulos, M. H., Higgins, J. J., Golbe, L. I., Johnson, W. G., Ide, S. E., Di Iorio, G., … Duvoisin, R. C. (1996). Mapping of a Gene for Parkinson's Disease to Chromosome 4q21-q23. *Science*, *274*(5290), 1197–1199. https://doi.org/10.1126/science.274.5290.1197

Ponsen, M. M., Stoffers, D., Booij, J., van Eck-Smit, B. L. F., Wolters, E. C., & Berendse, H. W. (2004). Idiopathic hyposmia as a preclinical sign of Parkinson's disease. *Annals of Neurology*, *56*(2), 173–181. https://doi.org/10.1002/ana.20160

Portnoff, A. D., Stephens, E. A., Varner, J. D., & DeLisa, M. P. (2014). Ubiquibodies, synthetic E3 ubiquitin ligases endowed with unnatural substrate

specificity for targeted protein silencing. *Journal of Biological Chemistry*, *289*(11), 7844–7855. https://doi.org/10.1074/jbc.M113.544825

Reddy Chichili, V. P., Kumar, V., & Sivaraman, J. (2013). Linkers in the structural biology of protein-protein interactions. *Protein Science : A Publication of the Protein Society*, *22*(2), 153–167. https://doi.org/10.1002/pro.2206

Rick, C. E., Ebert, A., Virag, T., Bohn, M. C., & Surmeier, D. J. (2006). Differentiated Dopaminergic MN9D Cells Only Partially Recapitulate the Electrophysiological Properties of Midbrain Dopaminergic Neurons. *Developmental Neuroscience*, *28*(6), 528–537. https://doi.org/10.1159/000095115

Sambrook, J., & Russell, D. W. (David W. (2001). *Molecular cloning : a laboratory manual*. Cold Spring Harbor Laboratory Press.

Sampson, T. R., Debelius, J. W., Thron, T., Janssen, S., Shastri, G. G., Ilhan, Z. E., … Mazmanian, S. K. (2016). Gut Microbiota Regulate Motor Deficits and Neuroinflammation in a Model of Parkinson's Disease. *Cell*, *167*(6), 1469-1480.e12. https://doi.org/10.1016/j.cell.2016.11.018

Savica, R., Carlin, J. M., Grossardt, B. R., Bower, J. H., Ahlskog, J. E., Maraganore, D. M., … Rocca, W. A. (2009). Medical records documentation of constipation preceding Parkinson disease: A case-control study. *Neurology*, *73*(21), 1752–1758. https://doi.org/10.1212/WNL.0b013e3181c34af5

Savica, Rodolfo, Grossardt, B. R., Bower, J. H., Ahlskog, J. E., Mielke, M. M., & Rocca, W. A. (2017). Incidence and time trends of drug-induced parkinsonism: A 30-year population-based study. *Movement Disorders*, *32*(2), 227–234. https://doi.org/10.1002/mds.26839

Schapira, A. H., & Jenner, P. (2011). Etiology and pathogenesis of Parkinson's disease. *Movement Disorders*, *26*(6), 1049–1055. https://doi.org/10.1002/mds.23732

Scheffner, M., & Kumar, S. (2014). Mammalian HECT ubiquitin-protein ligases: Biological and pathophysiological aspects. *Biochimica et Biophysica Acta (BBA)*

- *Molecular Cell Research*, *1843*(1), 61–74. https://doi.org/10.1016/J.BBAMCR.2013.03.024

Schulz-Schaeffer, W. (2015). Is Cell Death Primary or Secondary in the Pathophysiology of Idiopathic Parkinson's Disease? *Biomolecules*, *5*(3), 1467–1479. https://doi.org/10.3390/biom5031467

Srinivasan, B. S., Doostzadeh, J., Absalan, F., Mohandessi, S., Jalili, R., Bigdeli, S., … Ronaghi, M. (2009). Whole genome survey of coding SNPs reveals a reproducible pathway determinant of Parkinson disease. *Human Mutation*, *30*(2), 228–238. https://doi.org/10.1002/humu.20840

Strack, B., Calistri, A., Accola, M. A., Palu, G., & Gottlinger, H. G. (2000). A role for ubiquitin ligase recruitment in retrovirus release. *Proceedings of the National Academy of Sciences of the United States of America*, *97*(24), 13063–13068. https://doi.org/10.1073/pnas.97.24.13063

Sulzer, D., & Edwards, R. H. (2019). The physiological role of α-synuclein and its relationship to Parkinson's Disease. *Journal of Neurochemistry*, *150*(5), 475–486. https://doi.org/10.1111/jnc.14810

Thanvi, B. R., & Lo, T. C. N. (2004). Long term motor complications of levodopa: clinical features, mechanisms, and management strategies. *Postgraduate Medical Journal*, *80*(946), 452–458. https://doi.org/10.1136/pgmj.2003.013912

Tofaris, G. K., Kim, H. T., Hourez, R., Jung, J.-W., Kim, K. P., & Goldberg, A. L. (2011a). Ubiquitin ligase Nedd4 promotes -synuclein degradation by the endosomal-lysosomal pathway. *Proceedings of the National Academy of Sciences*, *108*(41), 17004–17009. https://doi.org/10.1073/pnas.1109356108

Tofaris, G. K., Kim, H. T., Hourez, R., Jung, J.-W., Kim, K. P., & Goldberg, A. L. (2011b). Ubiquitin ligase Nedd4 promotes -synuclein degradation by the endosomal-lysosomal pathway. *Proceedings of the National Academy of Sciences*, *108*(41), 17004–17009. https://doi.org/10.1073/pnas.1109356108

Trevisan, M., Alvisi, G., Barbaro, V., Barzon, L., Raffa, P., Migliorati, A., … Palù, G. (2018). Oral Mucosa-Derived Induced Pluripotent Stem Cells from Patients

with Ectrodactyly-Ectodermal Dysplasia-Clefting Syndrome. *Cellular Reprogramming*, *20*(4), 215–224. https://doi.org/10.1089/cell.2017.0064

Trevisan, M., Sinigaglia, A., Desole, G., Berto, A., Pacenti, M., Palù, G., … Barzon, L. (2015). Modeling Viral Infectious Diseases and Development of Antiviral Therapies Using Human Induced Pluripotent Stem Cell-Derived Systems. *Viruses*, *7*(7), 3835–3856. https://doi.org/10.3390/v7072800

Unger, M. M., Spiegel, J., Dillmann, K.-U., Grundmann, D., Philippeit, H., Bürmann, J., … Schäfer, K.-H. (2016). Short chain fatty acids and gut microbiota differ between patients with Parkinson's disease and age-matched controls. *Parkinsonism & Related Disorders*, *32*, 66–72. https://doi.org/10.1016/j.parkreldis.2016.08.019

Venda, L. L., Cragg, S. J., Buchman, V. L., & Wade-Martins, R. (2010). α-Synuclein and dopamine at the crossroads of Parkinson's disease. *Trends in Neurosciences*, *33*(12), 559–568. https://doi.org/10.1016/j.tins.2010.09.004

Villar-Piqué, A., Lopes da Fonseca, T., & Outeiro, T. F. (2016). Structure, function and toxicity of alpha-synuclein: the Bermuda triangle in synucleinopathies. *Journal of Neurochemistry*, *139*, 240–255. https://doi.org/10.1111/jnc.13249

Wakabayashi, K., Tanji, K., Mori, F., & Takahashi, H. (2007). The Lewy body in Parkinson's disease: Molecules implicated in the formation and degradation of α-synuclein aggregates. *Neuropathology*, *27*(5), 494–506. https://doi.org/10.1111/j.1440-1789.2007.00803.x

Weiss, E. R., Popova, E., Yamanaka, H., Kim, H. C., Huibregtse, J. M., & Göttlinger, H. (2010). Rescue of HIV-1 release by targeting widely divergent NEDD4-type ubiquitin ligases and isolated catalytic HECT domains to gag. *PLoS Pathogens*, *6*(9). https://doi.org/10.1371/journal.ppat.1001107

Xilouri, M., Brekk, O. R., & Stefanis, L. (2013). Alpha-synuclein and Protein Degradation Systems: a Reciprocal Relationship. *Molecular Neurobiology*, *47*(2), 537–551. https://doi.org/10.1007/s12035-012-8341-2

Xue, W.-F., Hellewell, A. L., Gosal, W. S., Homans, S. W., Hewitt, E. W., &

Radford, S. E. (2009). Fibril fragmentation enhances amyloid cytotoxicity. *The Journal of Biological Chemistry*, *284*(49), 34272–34282. https://doi.org/10.1074/jbc.M109.049809

Zarranz, J. J., Alegre, J., Gómez-Esteban, J. C., Lezcano, E., Ros, R., Ampuero, I., … de Yebenes, J. G. (2004). The new mutation, E46K, of α-synuclein causes parkinson and Lewy body dementia. *Annals of Neurology*, *55*(2), 164–173. https://doi.org/10.1002/ana.10795

Zennou, V., Serguera, C., Sarkis, C., Colin, P., Perret, E., Mallet, J., & Charneau, P. (2001). The HIV-1 DNA flap stimulates HIV vector-mediated cell transduction in the brain. *Nature Biotechnology*, *19*(5), 446–450. https://doi.org/10.1038/88115

Zhou, C., Emadi, S., Sierks, M. R., & Messer, A. (2004). A human single-chain Fv intrabody blocks aberrant cellular effects of overexpressed α-synuclein. *Molecular Therapy*, *10*(6), 1023–1031. https://doi.org/10.1016/j.ymthe.2004.08.019

Zhou, C., & Przedborski, S. (2009a). Intrabody and Parkinson's disease. *Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease*, *1792*(7), 634–642. https://doi.org/10.1016/J.BBADIS.2008.09.001

Zhou, C., & Przedborski, S. (2009b, July). Intrabody and Parkinson's disease. *Biochimica et Biophysica Acta - Molecular Basis of Disease*, Vol. 1792, pp. 634–642. https://doi.org/10.1016/j.bbadis.2008.09.001

Zufferey, R., Donello, J. E., Trono, D., & Hope, T. J. (1999). Woodchuck hepatitis virus posttranscriptional regulatory element enhances expression of transgenes delivered by retroviral vectors. *Journal of Virology*, *73*(4), 2886–2892. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/10074136

7. Abbreviation

aa: amminoacid

Ab: antibody

aS: alpha-synuclein

Aβ: β-amyloid

BCA: bicinchoninic acid

bp: base pair

BSA: bovine serum albumin

LB: Lewy body

cPPT: central poly-Purine Tract

DBPs: designer binding proteins

DEPC: diethylpyrocarbonate

DMEM: Dulbecco's Modified Eagle's medium

dNTPs: deoxynucleoside triphosphate

DP: dopaminergic progenitors

DN: dopmaniergic neurons

EGFP: enhanced green fluorescence protein

ELP: Endo-Lysosomal Pathway

ER: endoplasmatic reticulum

FBS: Fetal Bovine Serum

HCMV: human cytomegalovirus

hiPSC: human induced pluripotent stem cell

HRP: horseradish peroxidase

LB: Luria-Bertani

LBA: LB-Agar

Nac: non Amyloid-β Component

NSC: Neural Stem Cell

nt: nucleotide

PBS: Primer Binding Site

PBS: Phosphate Buffered Saline

PCR: Polymerase Chain Reaction

PFA: paraformaldehyde

scFv: single-chain Fragment variable

SDS-PAGE: Sodium Dodecyl Sulphate – Polyacrylamide Gel Electrophoresis

SIN: Self-Inactivating

SNARE: Soluble N-ethylmaleimide sensitive factor attachment protein receptor 81

SV40: Simian virus 40, Virus Vacuolante della Scimmia

TBE: Tris-borate EDTA

TH: Tyrosine Hydroxylase

Ub : Ubiquitin

VSV: Vesicular Stomatitis Virus

WPRE: Woodchuck Hepatitis C Virus Post-transcriptional Regulatory Element

wt: wild type

8. Aknoledgments

Ringrazio Arianna Calistri per essere stata un'ottima mentore e insegnante. La tua conoscenza ed esperienza mi hanno guidato durante tutto il ciclo di dottorato. La tua pazienza e il tuo continuo supporto mi hanno spinto a migliorarmi e a maturare come scienziata. Nessuno di questi esperimenti sarebbe stato possibile senza di te. Per questo, ti sarò per sempre grata.

Ringrazio Claudia Del Vecchio per essere stata sempre il mio consulente professionale e non, per la sua competenza e per la sua presenza costante nel mio percorso. Gli scambi di sguardi complici e divertiti oltre il “muro” di fogli hanno accompagnato i nostri pomeriggi insieme!

Ringrazio Marta Trevisan per avermi preso per mano e accompagnato nel mondo delle staminali, per avermi insegnato con pazienza e dedizione a lavorare con esse. Ho sempre potuto contare su di te per un parere e per questo mille grazie!

A special thank is for Kathy Maguire-Zeiss for your scientific input, for gave me the possibility to work in your laboratory. Your analysis has helped make my results and myself more critical and for this thank you.

I also want to thank Deran, Sean (Big Brain!), Katryn and Mondona that have always helped me and made me laugh and feel welcomed even at a distance of home, you will always remain in my heart.

Uno speciale ringraziamento va alla mia compagnia di avventure, Daniela con la quale ho condiviso questi tre anni. Grazie per essere stata la mia spalla, la mia amica e per avermi sempre supportato, questi tre anni senza di te non sarebbero MAI stati gli stessi.

Infine ringrazio Valeria, Mattia, Mavi, Silvia, Martina, Silvia e Hanieh perché oltre ad essere stati compagni sono stati Amici, per questi tre anni insieme e per essere stati in grado di farmi sentire a casa. Per questo, grazie di cuore!