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INSIGHT IN THE MOLECULAR CONSEQUENCES OF LRRK2 KINASE INHIBITION

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Samenvatting

De ziekte van Parkinson is de tweede meest voorkomende neurodegeneratieve aandoening, na de ziekte van Alzheimer, en de meest voorkomende bewegingsstoornis. Wereldwijd lijden 7 tot 10 miljoen mensen aan deze ziekte en dit aantal zal waarschijnlijk alleen maar stijgen door de optredende vergrijzing. Het zijn namelijk vooral mensen ouder dan 60 jaar, die door deze ziekte worden getroffen. Het exacte ontstaansmechanisme van de ziekte van Parkinson is nog niet volledig gekend. Momenteel zijn er nog geen behandelingen beschikbaar die patiënten kunnen genezen, enkel behandelingen die de symptomen (tijdelijk) kunnen onderdrukken.

Sinds 1997 hebben genetische studies al verschillende mutaties geïdentificeerd, die aan de oorzaak liggen van de ziekte. Zo zijn mutaties in het leucine-rich repeat kinase 2 (LRRK2) gen tot op heden de meest voorkomende gekende oorzaak van genetische vormen van de ziekte van Parkinson. Het klinische fenotype dat patiënten met LRRK2 mutaties vertonen, is sterk gelijkend aan het fenotype van patiënten die lijden aan een sporadische vorm, wat zou kunnen wijzen op eenzelfde ontstaansmechanisme voor de beide vormen. Bijgevolg zou onderzoek naar de moleculaire processen, die aan de basis liggen van de genetische vormen van de ziekte van Parkinson, ook kunnen leiden naar de ontwikkeling van nieuwe therapieën voor sporadische vormen.

Men heeft ontdekt dat pathogene mutaties in het LRRK2 eiwit een verhoogde kinase activiteit veroorzaken, waardoor het blokkeren van deze verhoogde activiteit met inhibitoren werd voorgesteld als mogelijke therapie. Uit verschillende studies blijkt dat LRRK2 kinase inhibitoren kunnen beschermen tegen neurodegeneratie. Dit werd reeds in celcultuur en in proefdieren aangetoond en dit zowel in modellen op basis van LRRK2 mutaties als in modellen voor de ziekte die geen veranderingen in het LRRK2-gen dragen. Hoe dan ook is enige voorzichtigheid geboden, aangezien we mogelijk nog niet alle gevolgen van LRRK2 kinase inhibitie begrijpen.

Een eerste gevolg van LRRK2 kinase inhibitie is defosforylatie van het LRRK2 eiwit. Opmerkelijk is dat defosforylatie ook een kenmerk is van pathogeen gemuteerd LRRK2 eiwit en bijgevolg betrokken zou kunnen zijn bij de pathogenese van de ziekte. Het al dan niet gefosforyleerd zijn van een eiwit, speelt vaak een cruciale rol in de signaaltransductie in een cel of tussen cellen onderling en komt tot stand door interactie met andere eiwitten. Het is van belang te weten te komen of dezelfde fosfatasen verantwoordelijk zijn voor defosforylatie van LRRK2 na kinase inhibitie en in een pathogene vorm. In het verleden werd in ons labo de katalytische subeenheid van proteïne fosfatase 1 (PP1) geïdentificeerd als fosfatase voor pathogene vormen van LRRK2 en na LRRK2 kinase inhibitie. De regulatorische eiwitten of andere fosfatasen die bij deze processen betrokken zijn, zijn nog ongekend. We beoogden in een eerste deel van dit werk de identificatie van fosfatasen of regulatorische eiwitten die betrokken zijn bij LRRK2 defosforylatie na LRRK2 kinase inhibitie. Vooreerst werd in ons laboratorium al een ‘small interfering’ (si) RNA screening uitgevoerd, waarbij gekeken werd naar de effecten op LRRK2 fosforylatie na het uitschakelen van specifieke fosfatasen of regulatorische eiwitten. In dit project willen we de meest belovende kandidaten van de siRNA screening verder bestuderen en valideren in cellen door gebruik te maken van RNA interferentie met behulp van lentivirale vector transductie. We hebben gevonden dat naast PP1, ook het proteïne fosfatase 2A (PP2A) complex betrokken is bij de defosforylatie van LRRK2, en dan vooral na LRRK2 kinase inhibitie. Verdere identificatie van het exacte fosfatasecomplex dat

inwerkt op LRRK2 in pathogene vormen en na LRRK2 kinase inhibitie, kan leiden tot nieuwe therapeutische inzichten.

Daarnaast zorgt een langdurige behandeling met LRRK2 kinase inhibitoren voor een verlaging van de totale hoeveelheid LRRK2 eiwit. Dit konden we waarnemen in celcultuur, maar ook in weefsel van dieren die werden behandeld met LRRK2 kinase inhibitoren. Andere onderzoeksgroepen beschreven ook reeds dat in dieren die behandeld werden met LRRK2 kinase inhibitoren een kenmerkende longpathologie kan worden vastgesteld, die eveneens wordt waargenomen in LRRK2 knockout dieren. Bijgevolg zou deze longpathologie gelinkt kunnen zijn aan een verlaagde hoeveelheid LRRK2 eiwit. In een volgend deel van dit werk, trachtten we het onderliggende werkingsmechanisme van LRRK2 kinase inhibitor-geïnduceerde eiwit destabilisatie te identificeren. Door verschillende synthesewegen en afbraakmechanismen van eiwitten in de cel te bestuderen en/of te blokkeren, vonden we dat LRRK2 na inhibitie wordt afgebroken door het proteasomaal systeem. Daarnaast wilden we ook LRRK2 kinase inhibitor-geïnduceerde destabilisatie verder karakteriseren door na te gaan hoe verschillende pathogene mutanten van LRRK2 reageren op langdurige LRRK2 kinase inhibitie. We zagen dat niet alle pathogene mutanten van LRRK2 destabilisatie vertonen na behandeling. Merkwaardig genoeg vertonen sterk gedefosforyleerde mutanten geen verlaagde LRRK2 eiwitniveaus na inhibitie, in tegenstelling tot een slechts deels gedefosforyleerde mutant. Dit deed ons vermoeden dat de basale fosforylatieniveaus van belang kunnen zijn voor de inductie van destabilisatie. We onderzochten het belang van reeds gekende fosforylatieplaatsen door mutaties aan te brengen in LRRK2 die het (de)fosforylatieproces verhinderen. Zo vonden we dat de reeds gekende fosforylatieplaatsen niet van cruciaal belang zijn voor de inductie van destabilisatie. Caseïne kinase 1α (CK1α) werd geïdentificeerd als het kinase verantwoordelijk voor de fosforylatie van deze gekende fosforylatieplaatsen en mogelijks ook van enkele nieuwe fosforylatieplaatsen in LRRK2. Na CK1 inhibitie zagen we dat destabilisatie van LRRK2 optreedt, zowel in het wild-type LRRK2 als in zijn pathogene varianten, in de variant waarbij we de gekende fosforylatieplaatsen gemuteerd hebben en in de long van muizen. Hieruit concludeerden we dat (de)fosforylatie van enkele vooralsnog ongekende fosforylatieplaatsen, die onder invloed staan van het kinase CK1, mogelijks aan de basis liggen van de verlaging in LRRK2 eiwit niveau na LRRK2 kinase inhibitie. De volgende stap is de identificatie van deze ongekende fosforylatieplaats(en). De studie in het tweede deel van dit werk biedt nieuwe inzichten in de eiwithomeostase van LRRK2, wat eveneens bijdraagt tot een bredere kennis over de werking van LRRK2 kinase inhibitoren, één van de meest belovende potentiële therapeutische strategieën voor de ziekte van Parkinson vandaag de dag.

Summary

Parkinson's disease is the second most common neurodegenerative disorder, after Alzheimer's disease, and the most common movement disorder. Worldwide, 7 to 10 million people suffer from Parkinson's disease. It is estimated that this number will only increase due to the general ageing of the population, as the disease primarily affects people older than the age of 60. Current therapies fail to cure, halt or slow down disease progression, partly because the exact etiology of Parkinson's disease is still poorly understood. However, treatment options that are able to (temporarily) suppress a patient's symptoms do exist.

Since 1997, different genetic studies revealed that mutations in several genes underlie the development of Parkinson's disease. To date, mutations in leucine-rich repeat kinase 2 (LRRK2) are the most common known cause of genetic forms of Parkinson's disease. Moreover, the clinical phenotype of patients carrying LRRK2 mutations strongly resembles the phenotype of patients with a sporadic form of the disease, which might indicate a common etiology. Consequently, more insight in the molecular processes that underlie the development of genetic Parkinson's disease could provide important insights for the development of potential new therapies, both for genetic and sporadic forms of the disease.

For example, studies have shown that pathogenic mutations in LRRK2 display increased kinase activity. As a result, kinase inhibition was proposed as a potential therapeutic strategy. Studies in cell culture and in animal models, both LRRK2-based and non-LRRK2-associated PD models, have shown the protective effect of LRRK2 kinase inhibition. Regardless, some caution is warranted, as we might not yet fully comprehend all the consequences of LRRK2 kinase inhibition.

First of all, LRRK2 kinase inhibition induces dephosphorylation of the LRRK2 protein, which is also seen in pathogenic LRRK2 variants. This finding indicates a potential contribution of dephosphorylation to LRRK2 pathogenesis in Parkinson's disease. Signal transduction in a cell or between cells often relies on whether or not certain proteins are phosphorylated. It is of great importance to identify the different phosphatase complexes involved in LRRK2 dephosphorylation after LRRK2 kinase inhibition or in pathogenic conditions. Our lab identified the catalytic subunit of protein phosphatase 1 (PP1) as the phosphatase acting on pathogenic variants of LRRK2 and during LRRK2 kinase inhibition. The regulatory proteins or other phosphatases involved in the process, remain elusive. In the first part of this work, we aimed to identify phosphatases or regulatory proteins involved in LRRK2 dephosphorylation after LRRK2 kinase inhibition. Therefore, a small interfering (si) RNA screen was executed, in which the effects on LRRK2 phosphorylation were investigated after knockdown of specific phosphatases or regulatory proteins. In this project, we aimed to further validate the top hits from the siRNA screen in cells, using lentiviral vector-mediated knockdown. Besides PP1, we found that also the protein phosphatase 2A (PP2A) complex is involved in the LRRK2 dephosphorylation cycle, especially after LRRK2 kinase inhibition. Further identification of the LRRK2-phosphatase complex in pathogenic variants or after kinase inhibition, will provide more insight in potential therapies for Parkinson's disease.

Besides, we and others found that prolonged treatment with LRRK2 kinase inhibitors causes a decrease in total LRRK2 protein levels. This could be observed both in cell culture and in animals treated with LRRK2 inhibitors. A characteristic pathology has been described in the lung of inhibitor-treated animals. Moreover, this phenotype was also observed in LRRK2 knockout animals. In the next part of this work, we aimed to identify the underlying mechanism of LRRK2 kinase inhibitor-induced destabilization. By studying and/or blocking the different ways for synthesis or degradation of proteins in the cell, we found that LRRK2 is mainly degraded by the proteasomal system after LRRK2 kinase inhibition. Next, we aimed to further characterize LRRK2 kinase inhibitor-induced destabilization in different pathogenic LRRK2 mutants. We observed that not all pathogenic variants of LRRK2 show a decrease in total LRRK2 protein levels after treatment. Remarkably, mutants that are strongly dephosphorylated, do not destabilize after LRRK2 kinase inhibition, in contrast to an only partly dephosphorylated mutant. Consequently, we assumed that the basal phosphorylation levels of the LRRK2 protein could be important in the regulation of protein homeostasis and induction of destabilization. We investigated the importance of already known phosphorylation sites, by mutating these residues. We found that none of the already known phosphorylation sites were crucial residues in the induction of protein degradation. Casein kinase 1α (CK1α) was identified as the kinase phosphorylating these known, and potentially other unknown, phosphorylation sites in LRRK2. After inhibition of CK1, destabilization of the LRRK2 protein still occurs, both in wild type LRRK2, in pathogenic LRRK2 variants, in the LRRK2 variant in which we mutated known phosphorylation sites and in the lung of mice. Consequently, we hypothesize that currently unknown phosphorylation sites, regulated by CK1, could be the primary inductors of LRRK2 kinase inhibitor-induced destabilization of LRRK2. The next crucial step is the further identification of this/these phosphorylation site(s). The second part of the study offers important new insights in the protein homeostasis of LRRK2, which will contribute to a broader knowledge on the working mechanism of LRRK2 kinase inhibitors, one of the prevailing therapeutic strategies for the treatment of Parkinson’s disease nowadays.

List of abbreviations

AA	amino acid
AADC	aromatic L-amino decarboxylase
AMP	adenosine monophosphate
AMPK	adenosine monophosphate-activated protein kinase
ANK	ankyrin
ANOVA	analysis of variance
APLRCKW	truncated form of LRRK2 which lacks the armadillo domain
ARM	armadillo
ATP	adenosine triphosphate
ATP13A2	ATPase 13A2
AAV	adeno-associated viral vector
BAC	bacterial artificial chromosome
BBB	blood-brain barrier
BCA	bicinchoninic acid assay
CalA	calyculin A
CAMKK-β	calcium-dependent protein kinase β
CHIP	C-terminus of Hsc70-interacting protein
CK1	casein kinase 1
COMT	catechol-o-methyltransferase
COR	C-terminal of ROC
CSF	cerebrospinal fluid
DAPK1	death-associated protein kinase 1
DBS	deep brain stimulation
DLP1	dynamin-like protein 1
DMEM	Dulbecco modified eagle medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DVL	dishevelled
4E-BP1	eukaryotic translation initiation factor 4E-binding protein 1
EDTA	ethylenediaminetetraacetic acid
eIF4E	eukaryotic initiation factor 4E
ER	endoplasmic reticulum
ERM	ezrin/radixin/moesin
FADD	fas-associated protein with death domain
fLuc	firefly luciferase
GABA	γ-aminobutyric acid
GAD*	glutamic acid decarboxylase
GAD**	G-protein activated by nucleotide-dependent dimerization
GAK	cyclin G-associated kinase
GAP	GTPase activating protein
GBA	glucocerebrosidase
GCH	GTP cyclohydrolase
GDNF	glial cell-derived neurotrophic factor
GDP	guanosine diphosphate
GEF	guanine nucleotide exchange factor
GMP	guanosine monophosphate
GPi	globus pallidus interna
GTP	guanosine triphosphate
GWAS	genome-wide association study
HEK	human embryonic kidney
IBD	inflammatory bowel disease
IKK	inhibitor of nuclear factor Κ-B kinase
i.p.	intraperitoneal
JIP	JNK-interacting proteins
L-DOPA	L-3,4-dihydroxyphenylalanine
LAMP2a	lysosome-associated membrane protein 2a
LB	Lewy body
LN	Lewy neurite
LPS	lipopolysaccharide

LRP6	low-density lipoprotein receptor-related protein 6
LRR	leucine-rich repeat
LRRK1	leucine-rich repeat kinase 1
LRRK2	leucine-rich repeat kinase 2
LV	lentiviral
MAO-B	monoamine oxidase-B
MAPK	mitogen-activated protein kinase
MAPT	microtubule-associated protein tau
MAPKK	mitogen-activated protein kinase kinase
MAPKKK	mitogen-activated protein kinase kinase kinase
MASL1	MFH-amplified sequences with leucine-rich tandem repeats 1
MFHAS1	MFH-amplified sequences with leucine-rich tandem repeats 1
miRNA	micro RNA
MLK	mixed lineage kinase
MPP+	1-methyl-4-phenylpyridinium
MPTP	1-methyl-4-fenyl-1,2,3,6-tetrahydropyridine
mRNA	messenger RNA
MSA	multiple system atrophy
NAADP	nicotinic acid adenine dinucleotide phosphate
NFAT	nuclear factor of activated T-cells
OA	okadaic acid
PAK6	p21-activated kinase 6
PBS	phosphate buffered saline
PBS-T	phosphate buffered saline supplemented with 0.1% Triton X-100
PD	Parkinson's disease
PKA	protein kinase A
PLRCKW	truncated form of LRRK2 which lacks both the armadillo and the ankyrin domain
PP1	protein phosphatase 1
PP2A	protein phosphatase 2A
PPP1C	catalytic subunit of protein phosphatase 1
PPP2C	catalytic subunit of protein phosphatase 2
PPP2R	regulatory subunit of protein phosphatase 2
qPCR	quantitative polymerase chain reaction
RIPK	receptor-interacting serine/threonine-protein kinases
RNA	ribonucleic acid
ROC	ras of complex
RT-qPCR	real-time quantitative polymerase chain reaction
shRNA	short hairpin RNA
siRNA	small interfering RNA
SNpc	substantia nigra pars compacta
STN	subthalamic nucleus
SYT11	synaptotagmin 11
TGN	trans-Golgi network
TH	tyrosine hydroxylase
TKL	tyrosine kinase-like
TLR	toll-like receptor
TNF	tumor necrosis factor
TRADD	tumor necrosis factor receptor type 1-associated DEATH domain protein
TR-FRET	time resolved-fluorescence resonance energy transfer
UPDRS	unified Parkinson's disease rating scale
WD	dipeptide tryptophan-aspartic acid
WT	wild type

Chapter 1 – General introduction

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1. Parkinson's disease

1.1 Epidemiology and clinical features

Parkinson's disease (PD) is the second most common neurodegenerative disorder after Alzheimer's disease and the most common movement disorder. The disease was first described in 1817 by James Parkinson in his work 'An assay on the shaking palsy' and later named after him[1]. Age is the greatest risk factor for developing PD and the incidence of PD increases with age from 1-2% in people over the age of 60 up to 4% in people over the age of 80[2,3] with a median age of onset being 60 years old[4]. However, 5-10% of the patients show young-onset PD, in which symptoms develop before the age of 50[3]. In line with the increasing ageing of the general population, the number of individuals suffering from PD was predicted to double between 2005 and 2030[5]. Moreover, PD affects individuals of all ethnic origins[3], but men have a higher chance to develop the disease than women, potentially due to a protective effect of estrogen in the brain[6].

PD patients typically suffer from four cardinal motor symptoms: an initially asymmetric resting tremor that eventually becomes bilateral, bradykinesia (slowness in movement), muscle rigidity and postural instability[2]. Rating scales, like the Unified Parkinson's Disease Rating Scale (UPDRS), are used for diagnosis and to evaluate motor impairment of patients[7]. Moreover, responsiveness to dopamine replacement therapy is considered conclusive[4] (see further). However, a final diagnosis can only be made after post-mortem brain examination[3]. Besides, non-motor related brain regions become affected as well, which results in symptoms including cognitive and sensory decline, depression, constipation, apathy, behavioral and psychiatric issues and dysautonomia[8]. Non-motor symptoms are believed to occur already in prodromal stages of PD[9], yet, they are rarely diagnosed correctly as PD symptoms. Consequently, there is an urgent need for early biomarker assays to detect the disease in earlier stages (reviewed in [10,11]).

1.2 Neuropathology

PD is recognized by two neuropathological hallmarks, of which the primary and most predominant feature is the progressive degeneration of dopaminergic neurons in the substantia nigra pars compacta (SNpc)[2,12], which results in dopamine depletion in the striatum. The nigrostriatal pathway regulates fine motor function by directly stimulating the striatum. Consequently, the striatum inhibits the globus pallidus interna (GPi), which inhibits the thalamus when activated. In turn, the thalamus excites the motor cortex to initiate voluntary movement. Decreased dopaminergic input results in increased inhibition of the thalamus and decreased activation of the motor cortex, a mechanism which underlies motor disability[13]. Motor symptoms generally manifest at the point of 70% dopaminergic neuronal cell death in the SNpc[2]. The second key feature is the presence of Lewy bodies (LBs) and Lewy neurites (LNs) in the cytoplasm and neurites of remaining neurons. These intraneuronal, eosinophilic inclusion bodies mainly consist of phosphorylated and ubiquitinated α-synuclein[14], a key protein in the pathogenesis of PD[15]. LBs and LNs are not exclusively present in the SNpc, but throughout the brain and this could underlie the development of the non-motor symptoms. Remarkably, the majority but not all PD patients show LB pathology[16]. Several other neurodegenerative disorders are characterized by the abnormal accumulation of α-synuclein, like dementia with LBs and multiple system atrophy (MSA), grouping these disorders as 'synucleinopathies'[17,18].

1.3 Treatment

Current treatment options for PD are purely symptomatic and thus unable to reverse or slow down disease progression. First of all, most pharmacotherapeutical options focus on replacing the void of dopamine by administering dopamine agonists, dopamine precursors (L-3,4-dihydroxyphenylalanine or L-DOPA) or inhibitors of dopamine degradation (monoamine oxidase-B (MAO-B) or catechol-o-methyltransferase (COMT) inhibitors). However, none of these drugs affects non-motor symptoms.

For almost 60 years, the use of the dopamine precursor L-DOPA is considered the golden standard. L-DOPA is always administered in combination with a peripheral dopa-decarboxylase inhibitor (carbidopa or benserazide)[3], to reduce the conversion of L-DOPA to dopamine in the periphery. Unlike dopamine, L-DOPA is able to cross the blood-brain barrier (BBB), where it is further metabolized to dopamine. However, long-term use of L-DOPA results in the development of severe side effects like motor fluctuations and dyskinesia. Initially, patients experience a wearing-off effect of L-DOPA, which is characterized by predictable periods of reoccurrence of motor symptoms. With time, these periods become unpredictable resulting in an on-off phenomenon and periods of complete immobility. Consequently, combinatory treatment with COMT or MAO-B inhibitors is preferred as it alleviates the motor fluctuations by preventing the degradation of dopamine[3,19]. The use of dopamine agonists, that bind the dopaminergic receptors post-synaptically, is less effective to treat motor symptoms, but rarely initiates dyskinesia. In the first years, dopamine agonists seem sufficient to treat motor symptoms, yet, the majority of patients needs additional L-DOPA treatment within 5 years[3,19].

Secondly, deep brain stimulation (DBS) is used to modulate the output of the basal ganglia by sending electrical pulses to the subthalamic nucleus (STN) or, which is less frequently applied, to the GPi. Two hypotheses exist on how DBS can alleviate motor symptoms. On the one hand, DBS is thought to directly influence the output of the thalamus. On the other hand, it is possible that DBS interrupts more randomly the pathological network firing that was established during disease. DBS is considered superior to medical treatment options in patients that suffer from motor fluctuations. However, due to the risks of invasive surgery

and the risk to develop both motor and non-motor complications afterwards, DBS is preferably applied in elder PD patients only[3,19].

In addition, several options exist for the treatment of non-motor symptoms (reviewed in [3] and [19]). Eventually, most patients require polypharmacy treatment to control their symptoms.

As mentioned before, there is an unmet need for disease-modifying therapies, as none of the existing therapeutic options has proven to be neuroprotective. To date, several strategies have already been put forward. First, several clinical trials investigated the potential of dopaminergic cell replacement therapy as a disease-modifying therapy for PD. Although some studies described significant graft cell survival after fetal mesencephalic tissue transplantation[3], this type of therapeutic strategy did not lead to a major breakthrough due to inconsistent results, (partly) because of the lack of rigorous scientific data, debate on the efficacy and potential as well as the high costs for this therapy (reviewed in [20]).

Second, several gene therapy trials have already been executed and aimed to either increase striatal dopamine levels or the delivery of neurotrophic factors to protect the remaining neurons using adeno-associated viral (AAV) or lentiviral (LV) vectors. Two distinct phase I gene therapy trials delivered aromatic L-amino decarboxylase (AADC), the enzyme that converts L-dopa to dopamine, in the putamen of PD patients. This approach, using an AAV vector, was proven safe and well tolerated. Moreover, significant, but modest, motor improvement could be observed in the first year[21,22]. Unfortunately, a 3-year follow-up study showed deterioration of motor improvement, despite constant AADC expression[23]. A second approach, which delivered ProSavin, a combination of 3 enzymes; AADC, tyrosine hydroxylase (TH) and GTP cyclohydrolase (GCH), by injection of LV vectors in the putamen of PD patients, was proven successful at 6 months. Patients are currently enrolled in a 9-year follow-up study[24]. Gene therapy, based on the injection of the glutamic acid decarboxylase (GAD*) gene, which encodes the enzyme responsible for γ-aminobutyric acid (GABA) production, was proposed as an alternative approach, since PD patients show decreased GABA input in the STN. In phase 1/2 clinical trials, patients were injected with AAV2-GAD* in the STN and showed significant motor improvement 6 months after surgery[25,26]. Another approach, where an AAV2 vector with the gene encoding the neurotrophic factor neurturin was injected in the putamen of PD patients, showed disappointing results. Although this strategy was proven safe and well-tolerated, it failed to improve motor function in the first year[27]. Moreover, a phase 1 clinical trial, which delivered glial cell derived neurotrophic factor (GDNF) in the putamen of PD patients, showed a widespread symptomatic improvement[28]. However, this study was followed by another trial in which the clinical benefit could not be reproduced[29]. Taken together, gene therapy is believed to offer great promises as potential PD therapy, yet some burdens like efficacy and a life-long effect still need to be addressed.

Furthermore, the main component of LB's, α-synuclein, is believed to contribute to the PD pathogenesis. It is hypothesized that different α-synuclein monomers can aggregate into toxic species. Subsequently, these structures spread to and affect different brain regions. Consequently, active or passive immunization against α-synuclein is considered as a potential disease-modifying therapy, which is currently tested in clinical trials. Moreover, small molecules that have the capacity to interfere with the aggregation of α-synuclein into toxic species or partial antisense gene silencing to lower the total amount of protein, could be promising as a PD therapy as well[19,30].

Additionally, kinase inhibitors against leucine-rich repeat kinase 2 (LRRK2) could be a promising option as potential PD therapy[30] and are discussed in detail in the next part of the introduction.
In conclusion, in order to develop new disease-modifying therapies or personalized medicine for PD, knowledge on the etiology of the disease is critical.

1.4 Etiology and pathogenesis

PD is envisaged as a complex and multifactorial disease, in which the combination of both genetics and environmental factors underlies disease onset[31]. As mentioned before, ageing is the greatest risk factor for PD[2,3]. Besides, exposure to pesticides (e.g. rotenone or paraquat[32]) or analogous, living in rural areas and consuming well water have been linked to an increased risk to develop PD[3]. For example, 1-methyl-4-fenyl-1,2,3,6-tetrahydropyridine (MPTP), found as a side product in synthetic heroine, is able to cross the BBB where it is converted to the neurotoxin, 1-methyl-4-phenylpyridinium or MPP+, that selectively damages dopaminergic neurons and results in parkinsonian symptoms[3,33]. On the contrary, the consumption of caffeine, intake of inosine and exposure to nicotine have been associated with a reduced risk to develop PD[19].
The majority of PD cases is considered idiopathic, however in 10% of the patients a clear genetic cause underlies the disease[34]. To date, mutations in more than 40 loci (PARK loci) have been described to clearly segregate with monogenetic familial forms of PD (the first 23 are listed in Table 1.1.). Most of these PARK loci were identified via linkage analysis[35,36]. Moreover, genome-wide association studies (GWAS) revealed risk factors associated with genes that are also linked to familial PD, like *SNCA* and *LRRK2*[36]. Genes linked to other disorders were also reported as a risk factor for PD, like *MAPT* (microtubule-associated protein tau, a protein linked to Alzheimer's disease, a neurodegenerative disorder that affects short-term memory) or *GBA* (glucocerebrosidase, a protein linked to Gaucher's disease, a lysosomal storage disorder)[37,38].

The PARK8 locus encodes the protein leucine-rich repeat kinase 2 or LRRK2, which will be discussed extensively in the next section.

PARK locus	Gene	Position	Gene product	Inheritance	Disease onset	Function
PARK1/4	*SNCA*	4q21	α-synuclein	AD	EO	Protein aggregation / prion-like transmission / synaptic function
PARK2	*PARKIN*	6q25-q27	E3 ubiquitin protein ligase	AR	EO	Mitochondrial maintenance / mitophagy / ubiquitin--proteasome
PARK3	*SPR ?*	2p13	Sepiapterin reductase	AD	LO	Dopamine biosynthesis
PARK5	*UCH-L1*	4p14	Ubiquitin C-terminal hydrolase L1	AD	LO	Ubiquitin hydrolase
PARK6	*PINK1*	1p36	PTEN induced putative kinase 1	AR	EO	Mitochondrial function / mitophagy
PARK7	*DJ-1*	1p36	DJ-1	AR	EO	Mitochondrial function / cell stress response
PARK8	*LRRK2*	12q12	Leucine-rich repeat kinase 2	AD	LO	Protein and membrane trafficking / neurite structure / lysosomal autophagy / synaptic function
PARK9	*ATP13A2*	1p36	ATPase 13A2	AR	EO	Lysosomal autophagy / mitochondrial function
PARK10	*RNF11?*	1p32	RING-finger protein 11	unclear	unclear	unknown
PARK11	*GIGYF2*	2q36-37	GRB10 interacting GYF protein 2	AD	LO	Tyrosine kinase receptor signaling / insulin like growth factor pathway
PARK12	unknown	Xq21-q25		unclear	unclear	unknown
PARK13	*HTRA2*	2p12	Serine peptidase 2	AD	LO	Mitochondrial function
PARK14	*PLA2G6*	22q12-q13	Phospholipase A2 group VI	AR	EO	Mitochondrial function
PARK15	*FBX07*	22q12-q13	F-box protein 7	AR	EO	Mitochondrial maintenance / mitophagy / ubiquitin-proteasome
PARK16	RAB7L1?	1q32	RAS oncogene family-like 1	unclear	unclear	Protein and membrane trafficking / lysosomal autophagy
PARK17	*VPS35*	16q11.1	Vacuolar protein sorting 35 retromer complex component	AD	LO	Lysosomal autophagy / endocytosis
PARK18	*EIF4G1*	3q27.1	Eukaryotic translation initiation factor 4 gamma 1	AD	LO	Protein translation
PARK19	*DNAJC6*	1p31.3	DnaJ heat shock protein family (Hsp40) member C6	AR	EO	Synaptic function / endocytosis
PARK20	*SYNJ1*	21q22.11	Synaptojanin 1	AR	EO	Synaptic function / endocytosis
PARK21	*DNAJC13*	3q22.1	DnaJ heat shock protein family (Hsp40) member C13	AD	EO	Endocytosis
PARK22	*CHCHD2*	7p11.2	Coiled-coil-helix-coiled-coil-helix-domain-containing protein 2	AD	LO	Mitochondrial function / cell stress response
PARK23	*VPS13C*	15q22.2	Vacuolar protein sorting 13C	AR	EO	Mitochondrial function

Table 1.1. - Overview of the PARK loci. AD, autosomal dominant; AR, autosomal recessive; EO, early onset; LO, late onset. Based on [36,39,40,41].

2. Leucine-rich repeat kinase 2 (LRRK2)

2.1. Identification of LRRK2

In 2002, linkage analysis of a Japanese family with autosomal dominant parkinsonism revealed a new PARK locus, PARK8, mapped to chromosome 12[42]. Missense mutations in the gene, that encodes the protein LRRK2, were identified as the cause of inherited autosomal dominant PD two years later[43,44]. To date, LRRK2 is known as one of the most common genes linked to PD[43–45]. Besides, LRRK2 has been implicated in other diseases, like inflammatory bowel disease (IBD), leprosy and cancer[46–50]. More than 40 mutations in LRRK2 have already been described, of which eight (N1437H, R1441C/G/H/S, Y1699C, G2019S and I2020T) cause PD[51,52] and four (A419V, R1628P, M1646T and G2385R) are considered risk factors as they significantly increase the risk to develop PD[53–58]. Interestingly, the combination of LRRK2 N551K, R1398H and K1423K is identified as a protective haplotype[55,56,58] (Figure 1.1.). Worldwide, the most common pathogenic mutation, G2019S, shows an incomplete and age-dependent penetrance of 28% at the age of 59, 51% at the age of 69 and 74% at the age of 79[59]. Moreover, reports describe G2019S-carriers at the age of 89 without parkinsonian symptoms[60]. LRRK2 G2019S accounts for about 4% of familial PD cases and 1% of apparent sporadic PD cases[45,51,61,62]. Interestingly, large ethnic differences exist; while the G2019S mutation is rather rare in Asian populations, it accounts for up to 40% of all PD cases in North African Arabs and Ashkenazi Jews[63,64]. Residue R1441 is the second most common pathogenic hotspot in LRRK2, in which the substitutions R1441C, R1441G, R1441H and R1441S have already been reported in patients[41]. Again, large population specific differences can be found. For the R1441G mutation, different reports describe a prevalence between 8-46% in Basque familial PD cases and between 2,5-4% in Basque sporadic PD cases[43,65,66], while the mutation is almost absent in other large populations studied[67]. Consequently, the formulation of a general prevalence of specific LRRK2 mutations requires some caution.

In addition, GWAS have revealed that variations in the LRRK2 locus can increase the risk to develop sporadic PD[55,68–71]. Interestingly, the majority of LRRK2-PD patients is clinicopathologically indistinguishable from idiopathic PD patients. However, recent studies reported that LRRK2-PD patients may show a slightly slower disease progression and fewer non-motor symptoms like cognitive decline, REM sleep behavior disorder or hyposmia. Yet, LRRK2-PD patients show an analogous response to treatment compared to non-LRRK2-PD patients[72]. This suggests that a common pathological mechanism could underlie both forms of the disease and that studying the molecular features that contribute to familial PD might give insight into the pathogenesis of sporadic PD as well[73–78]. Taken together, LRRK2 is a key player in PD and one of the most attractive candidates when it comes to disease-modifying targets. Therefore, understanding LRRK2 (dys)function will be fundamental to develop therapies that cure, stop or at least delay PD progression.

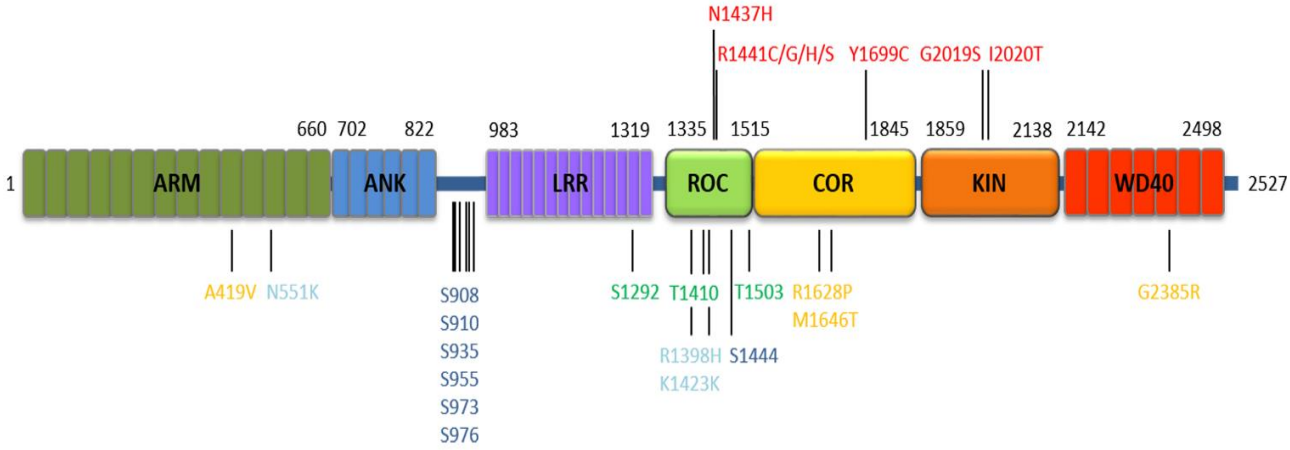

Figure 1.1. – Schematic representation of LRRK2 with domain boundaries indicated. Pathogenic mutations are depicted in red and risk factors in orange. The protective haplotype N551K-R1398H-K1423K is indicated in light blue. Autophosphorylation sites that are validates in cells (T1410 and T1503) or *in vivo* (S1292) are depicted in green. The most studied heterologous phosphorylation sites are depicted in dark blue. An extensive overview of other LRRK2 auto- and heterologous phosphorylation sites can be found in [66]. The different domains in LRRK2 are abbreviated as: ARM, armadillo domain; ANK, ankyrin domain; LRR, leucine-rich repeat domain; ROC, Ras of complex domain; COR, C-terminal of ROC domain; KIN, kinase domain; WD40, WD40 domain.

2.2. Tissue distribution

LRRK2 is widely expressed throughout the brain and the peripheral organs.

In the brain, high LRRK2 mRNA and protein expression can be detected in the cerebral cortex, hippocampus, cerebellum and striatum, whereas LRRK2 levels are rather low in the dopaminergic neurons of the SN. LRRK2 mRNA is expressed in different brain cell types like neurons, astrocytes and oligodendrocytes. In the rodent striatum, LRRK2 gene expression can only be detected starting from one week after birth. The concentration of LRRK2 mRNA then increases to reach a maximum after 1 month. From then on, LRRK2 levels stay constant over time[79].

In the periphery, LRRK2 expression levels are high in lung, kidney, spleen, heart, liver and in cells of the immune system[44,79–82]. LRRK2 protein levels are highest in a subpopulation of peripheral blood mononuclear cells, the monocytes and B cells[83–86], while low LRRK2 levels are reported in T-cells[83,84,87].

2.3. Structure of the LRRK2 protein

The *LRRK2* gene encompasses 51 exons, encoding a large protein, which consists of 2527 amino acids with an estimated molecular weight of 286 kDa. LRRK2 consists of 7 protein domains (Figure 1.1.) of which 2 enable the enzymatic functions of LRRK2 (a kinase function and a GTPase function). Based on homology studies, 4 domains (the armadillo (ARM), ankyrin (ANK), leucine-rich repeat (LRR) and the WD40 domain) were designated as protein-protein interaction domains. The presence of different enzymatic domains and protein-interaction domains might indicate that LRRK2 acts as an upstream central regulator or as a scaffold protein of different cellular processes, which is supported by the fact that LRRK2 has already been associated with a multitude of cellular functions (see previously). Moreover, based on recent bioinformatics studies on LRRK2 interacting proteins, it was suggested that LRRK2 can be termed as a 'date-hub', a dynamic protein which is capable of forming different protein complexes at different locations and times and consequently regulating diverse functions based on the complexes formed in different tissues, cell types, stages of development or under specific stimuli[88–90].

2.3.1. Protein-protein interaction domains

Each ARM-repeat is about 40 amino acids in length and consists each of 3 α-helices that together form an α-solenoid structure. ARM-repeats between different proteins share a related tertiary structure, rich in hydrophobic residues, rather than sequence identity and are related to several cellular functions like adhesion and signaling, regulation of gene expression and Wnt signalling[91–93].

The ANK-domain exists of seven ANK-repeats that each consists of two antiparallel α-helices followed by a β-hairpin. These repeats form a solenoid-like structure and are found in proteins important for transcriptional regulation, cytoskeleton dynamics, signaling and cell cycling.

The LRR-domain of LRRK2 consists of 13 LRRs that each form a β-sheeted structure, followed by an α-helix. The LRR-repeats form a U-shaped structure in which the β-sheets can be found on the inside and the α-helices on the outside. This type of domain is known to play crucial roles in cell adhesion processes and cellular transport.

Besides, the WD40-domain of LRRK2 comprises seven WD40-repeats in which each repeat consists of approximately 40 amino acids that form a four-stranded, anti-parallel β-plated sheet and ends in the dipeptide tryptophan-aspartic acid (WD). Altogether, the WD40-repeats form a propeller-like structure and this type of domain is likely involved in signal transduction processes, vesicle formation and trafficking or cytoskeleton dynamics[44,93,94].

2.3.2. Catalytic functions

The catalytic core of LRRK2 consists of two enzymatic functions; a GTPase domain called ROC (Ras of Complex), linked to a COR (C-terminal of ROC) domain and a kinase domain. Remarkably, all pathogenic mutations cluster in the catalytic core of the protein, suggesting that aberrant LRRK2 enzymatic activity contributes to PD pathogenesis (see previously).

2.3.2.1. GTPase function

The family of the ROCO-proteins is characterized by the presence of both a ROC and a COR domain. Often, a kinase domain and several regulatory protein-protein interaction domains are present in this family, as in LRRK2. In mammals, only four ROCO-protein family members are identified; LRRK1, LRRK2, MFH-amplified sequences with leucine-rich tandem repeats 1 (MFHAS1 or MASL1) and death-associated protein kinase 1 (DAPK1). The GTPase function of LRRK2 is catalytically active[74,95–98] and belongs to the family of small GTPases, which are known to cycle between a GTP-bound active state, which initiates further cell signaling, and a GDP-bound inactive state. Residues K1347 and T1348 are key for guanine nucleotide phosphate binding[99]. Most small GTPases are regulated by GTPase activating proteins (GAPs) and guanine nucleotide exchange factors (GEFs), which respectively promote the hydrolysis of GTP to GDP (rendering the GTPase inactive) or the exchange of GDP with GTP (rendering the GTPase active). For LRRK2, some GAPs and GEFs have already been proposed, like ArhGEF7[100], ArfGAP1[101,102] and RGS2[103]. On the other hand, a second mechanism of GTPase cycling has been proposed, where LRRK2 is suggested to function as a G-protein activated by nucleotide-dependent dimerization (GAD**) and does not rely on GEFs or GAPs. As only limited evidence supports the latter hypothesis, additional studies will have to provide more insight in the regulation of the GTPase cycle[99].

Pathogenic mutations in the ROC-COR bidomain (N1437H, R1441C/G/H/S and Y1699C) show decreased GTPase activity, while their effects on GTP binding are rather discrepant[95,97,98,104–106]. On the other hand, pathogenic mutations in the kinase domain of LRRK2 do not show significant effects on GTPase activity, nor on GTP binding[96,105,107]. Whether or not LRRK2 GTPase activity is directly related to cell toxicity remains uncertain.

2.3.2.2. Kinase function

The LRRK2 kinase domain is member of the tyrosine kinase-like family[108], which consists of several serine/threonine kinases that share sequence similarity with tyrosine kinases, but lack the tyrosine kinase specific motifs. Moreover, the LRRK2 kinase domain share homology with the RIPKs and MLKs, which are kinases that function in the cellular stress response by activating the MAPK pathway[94,109]. The kinase activity depends on an intact ROC domain and the presence of the WD40-domain[110–112], whereas the N-terminus plays an inhibitory role on LRRK2 kinase activity[113].

A more detailed description on what is known on LRRK2 kinase activity, its contribution to cellular functions and the effect of pathogenic mutations and LRRK2 kinase inhibition, is discussed further.

2.3.2.3. Interplay between GTPase and kinase function

GTPases are known to activate several tyrosine kinase-like (TKL) family members. Therefore, it was suggested that the main enzymatic output of LRRK2 is its kinase function and that the LRRK2 GTPase activity can be found upstream of the kinase activity. Several studies showed that the binding of non-hydrolyzable GTP analogues increase kinase activity[74,95,96,98] and GDP/GTP binding-dead mutants, like T1348N or K1347A, showed alterations in their kinase activity. However, mutations in the kinase domain did not show any effect on the GTPase activity or GTP binding capacity[105,114]. Moreover, Jaleel et al. demonstrated that the presence of an intact ROC domain in essential for the LRRK2 kinase activity[110]. Although the function of the COR-domain is rather speculative, it is believed to be essential for stimulation of GTPase activity and it might act as a molecular link between the GTPase/ROC and a kinase function[115–120]. Lastly, a recent study proposed that GTP binding to LRRK2 affects LRRK2 kinase activity and consequently induces phosphorylation of Rab7L1, which eventually results in LRRK2 recruitment at the TGN and increased activation of LRRK2 kinase activity[121].

However, some discrepancies exist as not all GDP/GTP binding-dead mutants consistently affect kinase activity. Consequently, a second hypothesis was postulated, based on the evolutionary conservation of the ROC-COR bidomain. Herein, the LRRK2 GTPase activity is most likely the functional output of LRRK2 activity, whereas the kinase domain is able to regulate the GTPase domain[99,114]. Several lines of evidence support this hypothesis, starting with the identification of autophosphorylation sites in the ROC domain that, based on their phosphorylation status, modulate GTP binding. Mutations at two autophosphorylation residues T1348 and T1503 result in decreased GTP binding and decreased GTPase activity[122]. Moreover, no alterations in kinase activity could be observed after the co-incubation of cell lysates with GTP, GDP or GMP[123,124].

Several lines of evidence support either of the above-mentioned hypothesis. Yet, an intact ROC domain seems essential for LRRK2 kinase activity and autophosphorylation sites tend to cluster in the ROC-COR bidomain. These observations favor the idea of a complex mechanism of intrinsic regulation between LRRK2 kinase and GTPase activity, in which both functions work in a reciprocal fashion, rather than independently of one another[114,125].

2.3.2.4. Dimerization

Both *in vitro* and cellular assays showed the existence of monomeric and dimeric forms of LRRK2. However, LRRK2 is believed to mainly exist as a dimer, which is the conformation that represents the active kinase state of the protein[116,126,127]. Dimerization is well known to regulate the transition between inactivity and activity in several protein kinases, including MAPKKK's, a protein family with which LRRK2 shares structural similarities[116]. Moreover, the COR domain has been suggested to be involved in dimerization processes[104,120]. Also, the WD40 domain of LRRK2 seems to play a crucial role in LRRK2 dimerization[111,128], while the kinase function itself seems of secondary importance[129].

In general, it is now believed that monomeric LRRK2 is inactive and resides in the cytosol. Whenever LRRK2 gets activated, dimers are formed that shuttle to membranous structures[126,130]. Unfortunately, the exact stimulus, which induces this transition, remains elusive. One report describes the induction of dimeric LRRK2 at the membranes in microglia after lipopolysaccharide (LPS) stimulation[131]. More

recently, it was suggested that increased GTP-binding results in increased Rab7L1-mediated activation of LRRK2 via the ankyrin-domain and shuttling to the membrane[121]. Both observations suggest a physiological function for LRRK2 at the membrane (see previously). Moreover, dimeric LRRK2 shows increased autophosphorylation due to its increased kinase activity, which is established after intramolecular autophosphorylation, where one LRRK2 monomer autophosphorylates itself rather than to affect the second LRRK2 monomer[119,132]. The effect of dimerization on heterologous phosphorylation sites, which are under control of other kinases than LRRK2, is not as clear as for autophosphorylation sites[121,126].

2.4. LRRK2 kinase activity and inhibition

All pathogenic mutations of LRRK2 are located in the catalytic core of the protein[94] (Figure 1.1., in red). Clustering of disease-causing mutations at these domains suggests a role for altered enzymatic function in PD pathogenesis. Initial *in vitro* kinase assays were based on LRRK2 autophosphorylation or the phosphorylation of a generic (peptide) substrate and only showed a consistent increase in kinase activity for the G2019S mutation[110,133–135]. However, more recent studies assessed kinase activity in a cellular context by using a phospho-specific LRRK2 antibody that recognizes the autophosphorylation site S1292. In contrast to the *in vitro* studies, increased autophosphorylation at S1292 could be detected in cultured cells for most of the pathogenic mutations (N1437H, R1441C/G/H, Y1699C, G2019S and I2020T), in brain lysates of bacterial artificial chromosome (BAC) transgenic mice overexpressing the G2019S mutation[132,136,137] and very recently also in the striatum of G2019S knockin mice with endogenous levels of LRRK2 protein[138]. Interestingly, also in idiopathic PD patients, increased autophosphorylation on S1292 has been reported in urinary exosomes and substantia nigra dopamine neurons[139–141]. Several studies have now shown that increased LRRK2 kinase activity is linked to cellular toxicity in cell culture models[74,142] and *in vivo*[143–145], although other reports failed to link LRRK2 overexpression with toxic effects[146–149]. Increased levels of cell death induced by overexpression of pathogenic LRRK2 variants could be avoided when introducing a mutation that abolishes kinase activity; for example after transfecting cultured cells or primary cortical neurons[74,142,144,150]. The observation that treatment with LRRK2 kinase inhibitors can rescue cell toxicity induced by pathogenic LRRK2 in cell culture[132,144,145,151] and in rodent brain[143,144] has made LRRK2 kinase activity one of the most prevailing disease-modifying targets. The finding that LRRK2 kinase inhibition can also protect against alpha-synuclein-induced toxicity in rodent brain[143] even suggests that LRRK2 kinase activity could be a valid target for PD patients without LRRK2 mutations. Consequently, many companies have sought to develop potent and selective LRRK2 kinase inhibitors (overview of patents in [152,153]). Although beneficial effects of LRRK2 kinase inhibition have been reported in independent studies, some caution is warranted, as we might not yet fully comprehend all consequences of LRRK2 kinase inhibitor treatment.

2.5. The complex regulation of LRRK2 (de)phosphorylation

2.5.1. LRRK2 phosphorylation: one term, different concepts

LRRK2 contains many serine and threonine residues, of which several are found to be phosphorylated [154–156]. In general, these sites can be subdivided into two groups: autophosphorylation sites (Figure 1.1., in green), which are identified as being phosphorylated by the LRRK2 kinase itself, and sites that are considered to be phosphorylated by kinases other than LRRK2, which we will refer to as heterologous phosphorylation sites (Figure 1.1., in dark blue).

Autophosphorylation sites were identified in *in vitro* assays and although they are found within the different domains of LRRK2, many of them cluster in or around the central ROC domain. LRRK2 is suggested to prefer threonine residues as autophosphorylation sites [157,158]. However, up till now only two threonine residues, T1410[154,157] and T1503[125,159,160], and one serine residue, S1292[132,154], have been confirmed in a cellular context[132,136,157,159,160] or *in vivo*[132] (Figure 1.1., in green).

The serines of the heterologous phosphorylation sites are highly conserved[134] and most of them cluster between the ANK- and the LRR-domain. Different findings support the idea that these sites are phosphorylated by kinases other than LRRK2[161]. First, when LRRK2 is dephosphorylated at residues S910 and S935 by LRRK2 kinase inhibition, immunoprecipitated and washed to remove the inhibitor, no increase in phosphorylation at these sites can be observed after addition of LRRK2 protein and ATP [134]. Second, the lack of correlation between kinase activity and the phosphorylation status of the heterologous phosphorylation sites[136,162] supports the role of cellular upstream kinases other than LRRK2. LRRK2 mutations, G2019S and K1906M, display either increased or strongly reduced kinase activity compared to wild-type (WT) LRRK2, respectively, but have phosphorylation levels at S935 comparable to WT LRRK2[134,163]. Third, different kinase-dead mutants have different effects on heterologous phosphorylation levels as both reduced or similar levels of S935 phosphorylation compared to WT LRRK2 were observed[162]. Finally, several pathogenic mutants (R1441C/G/H, Y1699C and I2020T) display decreased heterologous phosphorylation at S935[134], despite their increased autophosphorylation at S1292, which indicates increased LRRK2 kinase activity.

Heterologous phosphorylation sites are rapidly dephosphorylated after exposure to LRRK2 kinase inhibitors (>80% dephosphorylation after 90 minutes[164]). We previously showed that there is a strong correlation between the efficacy of compounds to inhibit *in vitro* LRRK2 kinase activity and their ability to dephosphorylate LRRK2 in cells[165]. This observation, together with the fact that detection of phosphorylated autophosphorylation sites in cells is significantly weaker than detection of phosphorylated S935, resulted in the use of dephosphorylated S935 as an indirect readout for LRRK2 kinase inhibitor activity[161,165,166]. The inhibition of LRRK2 kinase activity thus induces both decreased phosphorylation levels at S1292 (directly) and at the heterologous phosphorylation sites (indirectly). Intriguingly, dephosphorylated heterologous phosphorylation sites are also found in the most of pathogenic mutations, as well as in different brain regions, including the SNpc of idiopathic PD patients[167]. The finding that a potential protective agent induces the same PP1-mediated LRRK2 dephosphorylation as seen in pathogenic mutants[163] (see next paragraph) is a fascinating observation in light of the therapeutic use of LRRK2 kinase inhibitors and might indicate that LRRK2 dephosphorylation at the heterologous phosphorylation sites is not a pathogenic mechanism.

2.5.2. Upstream regulators of LRRK2 phosphorylation

Identification of upstream kinases, phosphatases or other regulatory proteins of LRRK2 phosphorylation sites will be crucial to understand how LRRK2 can influence its phosphorylation status, directly or indirectly (Figure 1.2.). CK1α was identified as an upstream kinase of LRRK2 at S908, S910, S935, S955, S973 and S976 in cell culture as well as *ex vivo* in striatum[168]. Interestingly, the interaction between LRRK2 and CK1α is diminished upon LRRK2 kinase inhibitor treatment of WT LRRK2, but not of the kinase-dead variant K1906M[168]. This finding suggests a role for LRRK2 kinase activity in CK1α recruitment or for the heterologous phosphorylation status given that K1906M LRRK2 does not get dephosphorylated (at least at S910 and S935) upon kinase inhibition[162,169]. Other upstream regulators of LRRK2 phosphorylation include inhibitor of nuclear factor Κ-B kinase subunits α and β (IKKα and β) and the IKK-related kinases[170]. These regulators were shown to phosphorylate LRRK2 S910 and S935 in bone marrow-derived macrophages upon stimulation of the toll-like receptor (TLR) pathway[170]. A role for IKK-kinases in the regulation of LRRK2 S935 phosphorylation was also observed in the neuronal SH-SY5Y cell line and in HEK293 cells for LRRK2 S910, S935, S955 and S973[171]. A third upstream kinase that has been suggested in different independent studies is protein kinase A (PKA) (reviewed in [172]). Initial evidence was provided a decade ago, when Ito and colleagues showed decreased metabolic labeling of overexpressed LRRK2 after treating HEK293 cells with different PKA inhibitors[95]. However, PKA-mediated LRRK2 phosphorylation was only confirmed many years later by two research groups after S910 and S935 phosphospecific antibodies became available[173,174]. The specific link with LRRK2 is however still unresolved since other studies were not able to confirm PKA as an upstream kinase of LRRK2. Indeed, a significant decrease of phosphorylation at S910, S935, S955 and S973 sites was described in conditions of PKA activation[136] in HEK293 cells and no effect of PKA inhibition or activation was observed on the phosphorylation status of S935[171]. In addition to these well-characterized heterologous phosphorylation sites of LRRK2, S1444 was identified as a PKA phosphorylation site. This PKA recognition site encloses the pathogenic hotspot R1441 and is not recognized when the arginine is mutated, leading to reduced phosphorylation at S1444, reduced 14-3-3 binding (which will be further discussed in a following section) and increased kinase activity[174]. It should be noted that this observation awaits confirmation and might be cell type specific as another study reported that forskolin-induced activation of PKA was not able to affect S1292 autophosphorylation, a measure for LRRK2 kinase activity[136]. Altogether, PKA appears to be part of the regulatory complex of LRRK2 phosphorylation, albeit unresolved whether this is a direct effect and dependent on specific experimental conditions. The conflicting reports, together with the accumulating evidence for a functional interaction between the two kinases in different cell types[172], suggests that a PKA-dependent regulation of LRRK2 phosphorylation results from a much more complex functional interaction in different cellular pathways. Indications that individual phosphorylation sites are regulated by different proteins comes from experiments using the LRRK2 A2016T variant, which shows a strongly reduced ability to bind inhibitors. LRRK2 kinase inhibitor treatment of this LRRK2 variant induces dephosphorylation at S955, but not at S910 and S935. This could imply that the kinase responsible for S955 phosphorylation is non-specifically inhibited, leading to the observed dephosphorylation at S955, but not at S910/S935[162]. Finally, besides the ones discussed

above, several other potential kinases have been proposed to regulate LRRK2, but still await confirmation (summarized in [155]).

Rab7L1 (also known as Rab29) is one of the Rab GTPase proteins that have been proposed as LRRK2 substrates[137,175] and is known as a functional interaction partner of LRRK2[137,176–181]. This interaction is of particular interest given that Rab7L1 is a candidate gene located in the PD risk-associated locus PARK16[68]. Only very recently, Rab7L1 was found to be a major regulator of LRRK2 kinase activity and its phosphorylation[121,182]. Rab7L1 induces recruitment of LRRK2 to the trans-Golgi network, where its kinase activity is stimulated, resulting in increased S1292 phosphorylation. This process appears to depend on LRRK2 GTP-binding capacity as the presence of pathogenic mutations R1441C/G and Y1699C further enhance this recruitment and the subsequent S1292 phosphorylation, in contrast to the GTP binding dead mutant T1348N, which completely inhibits S1292 autophosphorylation. In addition, phosphorylation at the heterologous phosphorylation sites S910, S935, S955 and S973 also depends on Rab7L1 activity as reduced phosphorylation at these sites is observed in conditions of decreased Rab7L1 interaction or in Rab7L1 knockout cell lines[121].

When it comes to phosphatases, only the catalytic subunit α of PP1 (PPP1Cα) is identified to affect the heterologous phosphorylation sites so far. We and others showed previously that increased recruitment of PP1α underlies the dephosphorylation at S910, S935, S955 and S973 induced by LRRK2 kinase inhibition, the pathogenic mutation R1441G and most likely also the other dephosphorylated pathogenic mutants[163,183,184]. In contrast, phosphatases involved in the regulation of the LRRK2 autophosphorylation sites remain ambiguous and interpretation of the regulation at S1292 is complicated by the fact that kinase inhibitor treatment also reduces the direct phosphorylation at this site, in contrast to S910, S935, S955 and S973, which are not directly phosphorylated by LRRK2. LRRK2 kinase inhibitor-induced reduction of phosphorylated S1292 cannot be prevented by exposure to calyculin A (CalA, an inhibitor of PP1 and protein phosphatase 2A (PP2A)), or to okadaic acid (OA, an inhibitor of PP2A)[136]. Still, PP1 and/or PP2A are most likely involved in the basal regulation of S1292 phosphorylation, at least in the G2019S and R1441G variants, given the upregulated S1292 phosphorylation upon OA or CalA treatment[136]. In addition, PP2A was suggested to play a role in LRRK2 dysfunction as LRRK2 R1441C or G2019S-induced toxicity could be aggravated or reduced by PP2A knockdown or activation, respectively[185]. Whether this is related to a direct regulation of the LRRK2 phosphorylation status is unclear.

The LRRK2 phosphorylation balance appears to be a regulating element of several cellular functions including its interaction with 14-3-3 proteins, its cellular distribution and its role during an immune response (see next paragraphs). Identification of the regulators of this balance will therefore be crucial to understand how LRRK2 is (de)regulated to execute its (dys)function and thus to interfere with pathogenic processes.

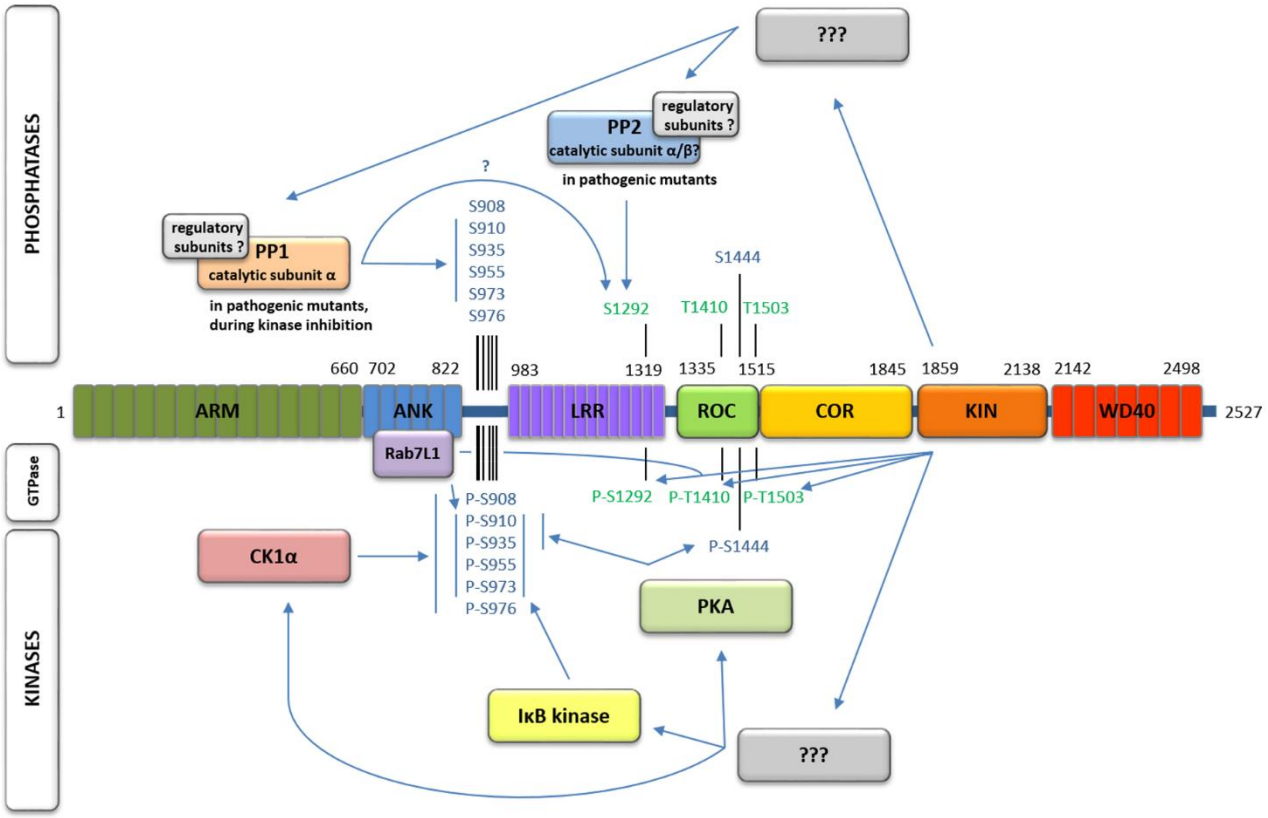

Figure 1.2. - Schematic representation of reported regulators of LRRK2 phosphorylation sites.

2.6. Biological effects related to LRRK2 (de)phosphorylation

2.6.1. Interaction with 14-3-3 proteins

14-3-3 proteins are among the best described protein interactors of LRRK2. The LRRK2-14-3-3 relationship is particularly exciting since 14-3-3 proteins are highly expressed in the brain, play a key role in cell survival[186] and have already been implicated in several neurological disorders, including PD [187–193]. 14-3-3 protein dimers are known to interact with LRRK2 in a phospho-dependent manner [134,156,161,174]. However, how this interaction exactly occurs is not completely resolved. A 14-3-3 dimer was suggested to bind to LRRK2 at both P-S910 and P-S935[134]. Yet, another study only found binding of 14-3-3 to phosphorylated S910, in addition to a much higher affinity interaction at P-S1444[174] (Figure 1.3.). The fact that also LRRK2 exists as a dimer further complicates the interpretation since 14-3-3 dimer interactions might occur at phosphorylated residues of two different LRRK2 molecules. 14-3-3 proteins can protect interaction partners from dephosphorylation[194], which might also be the case for LRRK2. Alanine substitutions at positions S910 or S935 strongly reduce phosphorylation at S935 and S910 respectively[134], while alanine mutations at S955 or S973 do not affect S910 or S935 phosphorylation[195]. We can therefore hypothesize that a 14-3-3 dimer binds P-S910/P-S935, and that loss of one of the phosphorylated binding sites abolishes this interaction. With 14-3-3 unable to bind, the remaining phosphorylated serine would be left exposed to phosphatases for subsequent dephosphorylation. Indeed, every condition that leads to dephosphorylation of at least one of the heterologous phosphorylation sites S910, S935 or S1444 results in a loss or strongly reduced interaction with 14-3-3 proteins. A similar reduced 14-3-3 binding is observed for most of the pathogenic mutants (N1437H, R1441C/G/H, Y1699C and I2020T) and the risk factor G2385R[134,163,168]. Also inhibition of LRRK2 kinase activity[161,163,164,168,173,195] or of the upstream kinase CK1α[168] leads to weakened 14-3-3 interaction. The cellular consequences of reduced LRRK2-14-3-3 binding are not completely understood. 14-3-3 binding is proposed to play a role in the release of LRRK2 in urinary exosomes[196] and is related to LRRK2 distribution and protein ubiquitination in the cell[184]. Interestingly, binding of 14-3-3 proteins to LRRK2 S910 and S1444 was shown to decrease kinase activity *in vitro*[174]. This is in line with the observation that overexpression of 14-3-3θ proteins can decrease LRRK2 G2019S kinase activity and rescue the reduced neurite length of primary neurons overexpressing LRRK2 G2019S or R1441G[160]. These findings suggest a protective role for 14-3-3θ proteins, one of the seven isoforms of 14-3-3 proteins, in LRRK2-related toxicity. However, a more complex relation with 14-3-3 proteins might be in place since LRRK2 kinase inhibition exerts its protective effects while disrupting the 14-3-3 binding [160].

2.6.2. Subcellular localization

Within the cell, LRRK2 displays a diffuse and homogenous cytoplasmic distribution under basal conditions[134,197]. LRRK2 co-localizes mainly with membrane-associated cell organelles like lysosomes, synaptic vesicles, the outer mitochondrial membrane, the endoplasmic reticulum (ER) and the Golgi apparatus, in spite of the fact that LRRK2 lacks a transmembrane domain. Moreover, some reports describe the association of LRRK2 with the cytoskeleton, specifically with microtubules.

At the molecular level, both monomeric and dimeric LRRK2 exists. Dimeric LRRK2 is believed to represent the active state of the protein and to be recruited and associate with membrane structures as a functional dimer, most likely through intermolecular interaction of the ROC-COR domains[106,111,119,126,131]. In the presence of several disease-associated LRRK2 mutations[127,134,161,163,197–199] or after pharmacological LRRK2 kinase inhibition[128,156,161,164], LRRK2 can be found in intracellular inclusions, often described as filamentous, skein-like structures. These structures are associated with microtubules[161,199] and were described by Alegre-Abarrategui and colleagues as 'membrane-surrounded elongated structures composed of a fine granular material enclosing a densely packed core positive for LRRK2[197]. LRRK2 phosphorylation is most likely one of the determining factors in the formation of these structures, since they are linked to conditions of decreased phosphorylation at S910/S935 and thus abrogated 14-3-3 interaction. Pathogenic mutants show a strongly decreased phosphorylation at heterologous phosphorylation sites, which would correspond to the observation that 14-3-3-associated LRRK2, which depends on phosphorylated heterologous residues, resides in the cytosol[134,200]. Moreover, we showed that inhibition of phosphatases PP1 and PP2A can prevent LRRK2 to relocalize in pathogenic conditions[163]. Still, additional factors are probably involved since the LRRK2 G2385R risk factor displays impaired 14-3-3 binding and S910/S935 phosphorylation[128,134], but does not accumulate in skein-like structures[128]. Strikingly, abolishing kinase activity of dephosphorylated pathogenic mutants by introducing the kinase-dead K1906R mutation induced a homogenous distribution of these mutants as seen in WT LRRK2[199]. This is in line with the observation that the G2385R mutant displays reduced kinase activity compared to WT LRRK2[128]. The observation that both pharmacological LRRK2 kinase inhibition and pathogenic mutants with increased kinase activity result in LRRK2 relocalization [128,142,156,161,163,164,197,199,201] (Figure 1.3.) raises questions whether LRRK2 kinase activity is involved in the regulation of LRRK2 distribution and to what extent these filamentous structures are comparable in condition of inhibition and pathogenic mutations. Recently, it has been suggested that the GTP-binding capacity of LRRK2 is the main determinant of LRRK2 relocalization. Indeed, non-hydrolyzable GTP strongly induced the formation of filamentous structures, while treatment with LRRK2-GTP-binding inhibitors or the presence of mutations that disrupt GTP-binding prevented relocalization[202]. Although the skein-like structures are a remarkable phenomenon, it should be pointed out that their physiological relevance is still under debate given that skein-like structures have only been reported in conditions of LRRK2 overexpression so far. However, if physiological and/or pathological relevance can be demonstrated, the promotion of monomers over dimers, could be considered as a therapeutic option. To this end, the crystallographic structure of a LRRK2 monomer and dimer would provide tremendous amounts of information. However, technical issues prevented the purification of sufficient amounts of recombinant LRRK2 protein until now. It was once suggested that two monomers

interact in an antiparallel manner to form dimers[106,116]. However, this hypothesis was based on observations from isolated ROC-domains, instead of full length LRRK2, indicating the need for a thoughtful interpretation.

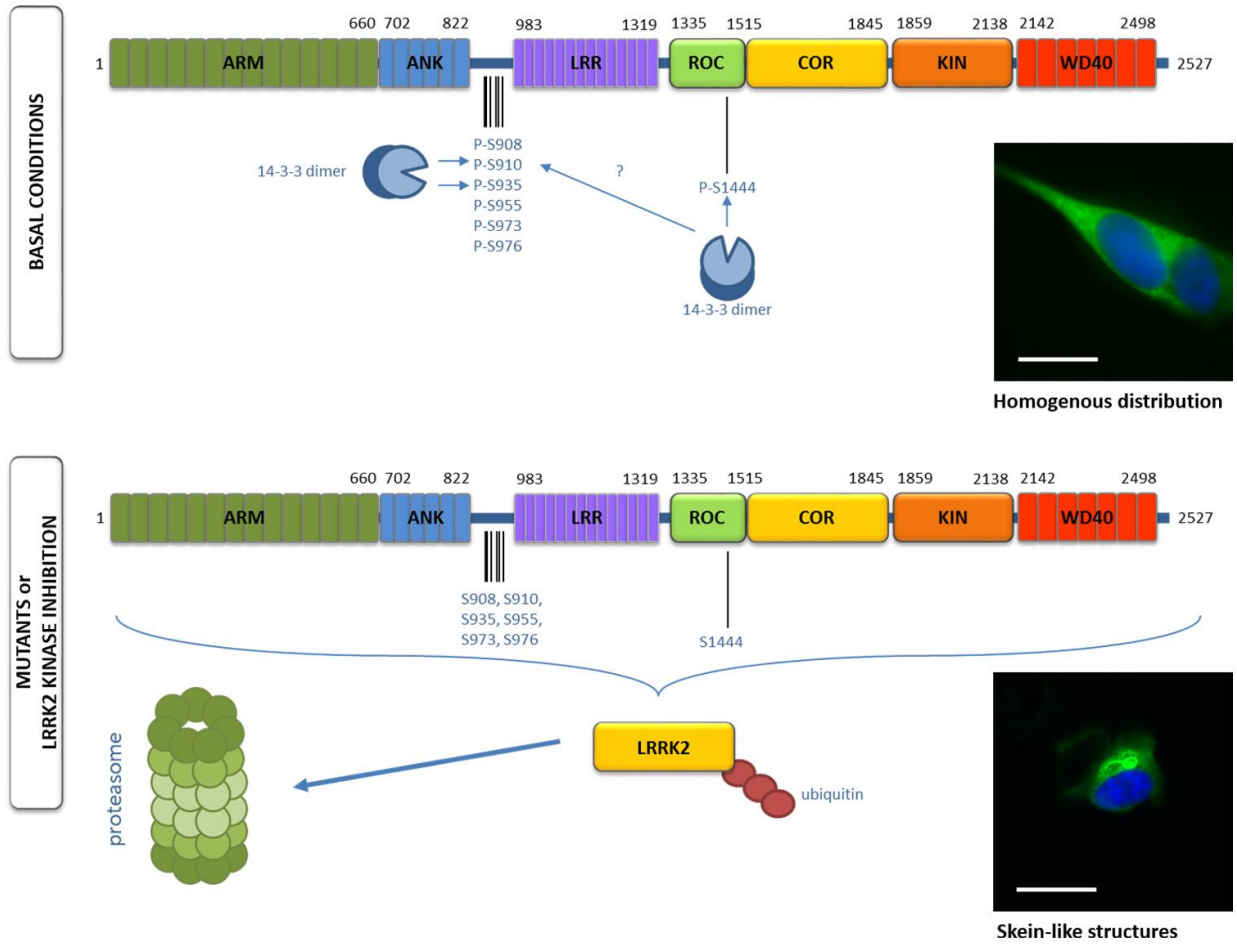

Figure 1.3. : Molecular changes observed upon 14-3-3 binding to LRRK2. Pictures show HEK293T cells with stable eGFP-LRRK2 expression that were stained with DAPI (blue). LRRK2 protein was detected by immunofluorescence using eGFP (green). Scale bars represent 15 µm.

2.6.3. LRRK2 kinase inhibition and destabilization

We and others have shown that sustained LRRK2 kinase inhibition can result in reduced LRRK2 protein levels[169,184,198,203–205]. This phenomenon has long been undetected given the fast kinetics of LRRK2 kinase inhibition on dephosphorylation of both heterologous and autophosphorylation sites [143,161,164,165,206]. Consequently, most studies applied short-term kinase inhibitor treatment and thus did not report inhibitor-induced LRRK2 protein loss. An exception is the reduction of a ~170 kDa truncated, but not full length, form of LRRK2, in neutrophils treated with MLi-2 for 30 minutes. The observation that treatment with high doses of PF-06447475 did not affect truncated LRRK2 protein levels[207], might point to effects of MLi-2 on other proteins (e.g. cell type-specific proteases), rather than LRRK2-specific effects. Nevertheless, the differential regulation of this truncated form of LRRK2 is intriguing as we have shown a reduction of full length, but not ~170 kDa truncated, LRRK2 in the kidney of mice treated with MLi-2 for two days[169]. More insight in the exact sequence, distribution, regulation and specific function of this truncated protein will be of major interest to understand the (peripheral) function of LRRK2.

Several studies have described the interaction of LRRK2 with proteins involved in the chaperone machinery, which are crucial in the regulation of proteostasis both under physiological and stress conditions. The LRRK2 protein is stabilized after interaction with Hsp90 and its co-chaperone cdc37[129,208–210], which prevent the ubiquitination and degradation of LRRK2. LRRK2 can be degraded after binding to the E3 ubiquitin ligase C-terminus of Hsc70-interacting protein (CHIP)[208,210]. LRRK2 stability seems to be related to cell toxicity. For example, inhibition of Hsp90 or overexpression of CHIP results in increased LRRK2 degradation and decreased LRRK2 toxicity, while knockdown of CHIP increased toxicity[208,209].

Reduced LRRK2 protein levels have also been reported *in vivo* in transgenic knockin mice expressing a kinase-dead variant, pointing to LRRK2 kinase activity as a potential regulator of LRRK2 protein homeostasis[203]. In line with this, functional mutants with reduced Rab7L1 interaction, and consequently reduced kinase activity and phosphorylation at heterologous phosphorylation sites, display reduced protein stability[121]. Still, LRRK2 levels in brain or kidney of Rab7L1 knockout mice are not reduced[178]. Therefore, it would be interesting to assess LRRK2 kinase activity and phosphorylation in these mice.

A more detailed study, using S910/S935 phosphodead mutants and difopein, which induces LRRK2 dephosphorylation by inhibiting 14-3-3 protein interaction, identified S935 dephosphorylation as a mediator of LRRK2 ubiquitination and subsequent proteasomal degradation[184]. However, our recent observations that S910A or S935A LRRK2 variants are degraded upon LRRK2 kinase inhibition, to the same extent as their WT counterpart, add another layer of complexity. These data suggest that dephosphorylation at one of these sites is not a critical factor in inhibitor-induced destabilization[169] (Figure 1.3.). In addition, cell type specific mechanisms might be important for LRRK2 stability since destabilization is not observed in all cell types after LRRK2 kinase inhibitor treatment (e.g. mouse primary astrocytes)[169]). The observation that LRRK2 is degraded after LRRK2 kinase inhibition has important implications for the development of LRRK2 kinase inhibitors as a PD drug. On the one hand, preclinical toxicity studies using distinct LRRK2 kinase inhibitors have revealed morphological changes in type II pneumocytes of the lung, in rodents[204] and non-human primates[205]. These observations are reminiscent of changes seen in the lung of LRRK2 knockout mice[211]. This suggests a direct correlation

between decreased LRRK2 protein levels and LRRK2 kinase inhibition. However, not all treated non-human primates with a lung phenotype showed reduced LRRK2 levels in the lung[205]. Besides possible cell type specific effects, it is currently unclear why LRRK2 kinase inhibitors induce dephosphorylation in different organs and different species, but do not always induce destabilization[143,169,184,204,205]. Although initial studies suggest that the lung phenotype is reversible and without serious functional implications, it is an important feature of LRRK2 kinase inhibition to take into account during clinical development. It is worth mentioning that preclinical safety studies that showed a lung phenotype, have assessed the effect of LRRK2 kinase inhibitor treatment on lungs with normal WT LRRK2 kinase activity [204,205]. These observations leave open the possibility that reducing the elevated kinase activity to basal WT levels in a pathogenic condition, rather than eliminating all kinase activity, can prevent potential peripheral side effects. Interestingly, heterozygous LRRK2 knockout mice do not display morphological lung changes[203], suggesting that a more modest reduction of kinase activity can by-pass the observed lung phenotype. However, it is still unclear, how such a restricted kinase inhibition could be obtained and how it would impact the potential neuroprotective effects. In addition, identification of the determinants of LRRK2 destabilization could provide crucial information to prevent potential side-effects of LRRK2 inhibition. On the other hand, inhibitor-induced destabilization might entail a new therapeutic strategy, as it has been suggested that the beneficial effects of LRRK2 kinase inhibition can be attributed to reduced LRRK2 protein levels, rather than a reduction of its kinase activity *per se*[198]. However, protective effects exerted by LRRK2 kinase inhibitors cannot merely be attributed to a reduction in proteins levels since no LRRK2 destabilization was observed in a viral vector-based α-synuclein rat model that was protected from neurodegeneration by LRRK2 kinase inhibitor treatment[143]. Still, with regard to drug development, the idea of reducing total LRRK2 protein levels is interesting and recently supported by the finding that LRRK2 antisense oligonucleotides can reduce the formation of α-synuclein inclusions in a PD mouse model[212].

In addition to inhibitor-induced destabilization, also the regulators of LRRK2 protein homeostasis in basal conditions have to be identified. To date, only few studies have reported on the putative effect of pathogenic mutations on LRRK2 protein stability. Interestingly, the dephosphorylated LRRK2 variants I2020T[213], R1441C[214] and G2385R[215] are suggested to display increased degradation rates compared to WT LRRK2, which is in line with the observation that pathogenic mutations with reduced S935 phosphorylation display increased ubiquitination[184]. In contrast, the G2019S mutant was reported to have an increased protein half-life[216], although this was not observed by others[208].

Lastly, when assessing LRRK2 protein stability, the choice of protease inhibitor[207] or lysis buffer composition (own unpublished observations) should be well-considered given possible effects on LRRK2 protein preservation.

2.7. Regulation of LRRK2 phosphorylation and expression levels in immune cells

Multiple evidence suggests that disturbed and elevated peripheral and central immune responses contribute to the pathogenesis of PD[217]. For example, elevated tumor necrosis factor α (TNFα) and interferon γ (IFN-γ) levels were found in the cerebrospinal fluid (CSF) and in postmortem brain tissue of PD patients[218,219]. Recent studies showed that both monocytes and T-cells from PD patients produce an increased amount of pro-inflammatory cytokines after stimulation with IFN-γ[220] or α-synuclein peptides and fibrils[221]. High LRRK2 protein levels are found in different types of immune cells including monocytes, neutrophils and B-cells[83–86,207] in the peripheral immune system and microglia in the central nervous system[159,222]. However, emerging databases now suggest that LRRK2 mRNA is very low in microglia (eg. RNA sequencing database from the B. Barres lab). Interestingly, PD patients were shown to have increased LRRK2 protein levels in different types of monocytes[220,223], B-cells and T-cells[220], compared to healthy controls.

In the peripheral immune system, increased LRRK2 phosphorylation and total protein levels have been reported in different cell types upon stimulation[83–85,159,220,224,225]. Stimulation of the TLR pathway in bone marrow-derived macrophages or a macrophage cell line induced increased LRRK2 phosphorylation levels at S910 and S935[131,170]. IκB kinase members were identified as upstream kinases of LRRK2 phosphorylation, but only after stimulation[170]. Under these conditions, increased LRRK2 phosphorylation could not be reversed by kinase inhibition, while treatment with LRRK2 kinase inhibitors induced LRRK2 dephosphorylation in basal conditions[131,170]. The increase in LRRK2 phosphorylation in LPS-stimulated macrophages was shown to precede LRRK2 dimerization and membrane association, representing the active state of LRRK2[131]. The exact role of LRRK2 (phosphorylation) in LPS-induced activation in macrophages is currently unclear, but various independent studies were unable to detect changes in cytokine release under various experimental conditions including LRRK2 deficiency [50,83,170,226] and overexpression of WT or pathogenic LRRK2 variants[83,227], suggesting that LRRK2 is not a critical mediator in this process.

In the central nervous system, LRRK2 phosphorylation and protein levels appear to be strictly controlled in immune cells and differentially regulated during cell activation. Activation of the TLR-4 pathway by LPS results in increased LRRK2 protein levels in microglia *in vivo* and in primary microglia *in vitro*[159,222,228], although one study could not confirm this finding in BV2 cells and primary microglia[229]. Moreover, pathogenic LRRK2 mutant mice show exacerbated immune response after LPS exposure, accompanied with LRRK2 upregulation and increased neurodegeneration, compared to control mice[230]. The increase in LRRK2 protein levels *in vivo* were accompanied by increased LRRK2 kinase activity as shown by increased autophosphorylation after LRRK2 immunoprecipitation from LPS-injected SNpc[159]. This is in line with the finding that LRRK2 in LPS-activated microglia displays increased S935 phosphorylation and subsequent dimerization and membrane association[131], which might represent the active state of the protein, as described earlier in macrophages[119,126,127]. LRRK2 is proposed to act as a positive regulator of neuroinflammation in primary microglia, since reduced LRRK2 levels or LRRK2 kinase inhibition were found to result in an attenuated inflammatory response upon LPS or α-synuclein fibril treatment[159,229,231,232]. In contrast, LPS-mediated stimulation of microglia overexpressing the

R1441G pathogenic mutant, exhibit an increased cytokine expression and secretion. The finding that conditioned medium from these activated R1441G microglia, but not from WT microglia, induced a significant increase in cell death when neuronal cultures are exposed to the medium[222] further points to a role for LRRK2 in neuroinflammation and related neurotoxicity. This was further corroborated by the finding that double transgenic A53T α-synuclein/LRRK2$^{-/-}$ mice do not display elevated astrocytosis or microgliosis in contrast to A53T α-synuclein transgenic mice with a LRRK2 WT background[233]. In addition, LRRK2 deficient rats were protected from LPS- or α-synuclein-induced toxicity and neuroinflammation[228], while rats with a G2019S background displayed exacerbated LPS[227] and α-synuclein-induced toxicity and neuroinflammation, which could be blocked by LRRK2 kinase inhibition[143]. In conclusion, gaining insight in this microglia-neuron interaction and in the role of LRRK2 (phosphorylation) in the (central) immune response will be indispensable to understand the processes that contribute to the pathogenesis of PD. Moreover, some caution is warranted with regard to the interpretation of the above-mentioned observations, as recent findings from RNA sequencing experiments now suggest very low LRRK2 mRNA levels in microglia.

2.8. Physiological and pathological role of LRRK2

The general physiological function of LRRK2 and its corresponding role in PD pathology remain poorly understood, yet its cellular and subcellular distribution favors its importance in cellular processes like mitochondrial function[234], microtubule function[235,236], translation[237], vesicular trafficking[238], autophagy[88,234,236] and neuroinflammation[239]. The number of cellular processes in which LRRK2 is implicated, is long. However, if and how these different functions might correlate to one another and how they might contribute to PD pathogenesis remains elusive.

2.8.1. Neurite outgrowth & cytoskeleton dynamics

Several studies reported decreased neurite length and decreased neurite branching[105,144,208,240–242] after overexpression of pathogenic LRRK2 variants G2019S[240,243,244], R1441C[243], Y1699C[245] or I2020T[240,246]. These experiments were performed in different cell lines overexpressing mutant LRRK2[240,243] or in primary cultures from transgenic animals[240,245,246]. It was suggested that LRRK2 kinase activity is important for regulating neurite morphology as overexpression of the LRRK2 kinase domain alone, which was thought to have aberrant kinase activity, was sufficient to induce neurite shortening. Moreover, expression of the kinase dead variant K1906M was able to increase neurite length in primary cultures[240]. The effect of overexpression of WT LRRK2 on neurite length is indistinct as both reduced[243,247], increased[248] and equal[128,240,241,245] neurite length compared to control conditions has been reported. Also reports on the effects of knockout of LRRK2 in cell lines, primary cultures or animals show discrepancies as both increased[240,242,243,245,247] and decreased[101,248] neurite length has been described in these conditions. We should keep in mind that the discrepancies observed might be influenced by specific experimental factors used in the different studies like differences in cell type, expression system, experimental set-ups etc. Still, most reports describe disturbed neurite outgrowth in the presence of pathogenic LRRK2 mutations, for which several potential mechanisms have already been suggested.

One possible underlying mechanism might be related to the link between LRRK2 and the cytoskeleton, as suggested by several studies. The cytoskeleton is a complex and dynamic network of filamentous proteins throughout the cell that provides shape to the cell and connects to the cell membrane and the cell organelles. The ERM (ezrin/radixin/moesin) proteins are important proteins for controlling neurite outgrowth by stabilizing F-actin and linking filaments to the plasma membrane. Jaleel et al. showed that LRRK2 is able to phosphorylate ERM proteins[110] and F-actin and actin-related proteins were shown to interact with LRRK2[249]. Primary neurons from transgenic G2019S mice showed increased ERM-phosphorylation, increased F-actin enriched filopodia and reduced neurite outgrowth. Conversely, knockout of LRRK2 led to a decrease in ERM-phosphorylation and an increase in neurite length. Moreover, inhibition of ERM phosphorylation or actin polymerization could rescue the G2019S-mediated reduction in neurite length[242]. LRRK2 was also shown to interact with Rac1 and dishevelled (DVL) proteins, other proteins important for the remodeling of the actin cytoskeleton (further discussed in the next paragraph)[248,250]. Besides, several studies showed the interaction of LRRK2 with β-tubulin[251–254]. LRRK2 is able to phosphorylate β-tubulin, resulting in stabilization of the microtubule complex and enhanced microtubule polymerization[233,253]. Consequently, knockout of LRRK2 results in increased amounts of free β-tubulin[253], while β-tubulin from transgenic mutant LRRK2 brain shows strong

polymerization[246]. Thirdly, LRRK2 has been shown to interact with and to phosphorylate tau[247] and overexpression of mutant LRRK2 induces increased tau phosphorylation and pathology[240,255–259]. Tau is a microtubule-associated protein that is able to stabilize proteins, when it is dephosphorylated. In GWAS studies, tau was identified as a PD risk locus[68,69] and tauopathies can be observed post-mortem in PD brains[260,261].

Strong evidence points to a role for LRRK2 in the regulation of cytoskeleton dynamics. However, the exact mechanism by which LRRK2 contributes to this process remains elusive. Other cell processes that have been linked to LRRK2, like vesicle dynamics and autophagy (reviewed in [262]), have already been suggested to contribute to the regulation of cytoskeleton dynamics as well[144,241,243].

2.8.2. Wnt signaling

Wnt signaling can be considered as the main regulator of cell development as it closely regulates gene transcription and cytoskeleton dynamics. Wnt ligands bind to the Frizzled receptors, which in turn activate DVL proteins. DVL proteins recruit β-catenin from the cytoplasm, preventing its degradation, and promoting its translocation to the nucleus, where β-catenin affects gene regulation[263]. LRRK2 co-localizes and interacts with DVL proteins[250]. Knockdown of LRRK2 results in increased Wnt signaling, while the presence of pathogenic mutations R1441C, Y1699C and G2019S decreases Wnt signaling[264]. Moreover, the interaction between LRRK2 and DVL proteins is abolished in the Y1699C mutant and enhanced in the R1441C/G/H mutant. Other components of Wnt signaling are linked to LRRK2 as well, for example low-density lipoprotein receptor-related protein 6 (LRP6) and β-catenin itself. Also the LRP6-LRRK2 interaction is abolished in LRRK2 pathogenic mutants[264].

2.8.3. Transcription and translation

LRRK2 might also be involved in translational processes, as it interacts with eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1), which regulates translation in stressed conditions. Moreover, when LRRK2 phosphorylates 4E-BP1, the latter cannot bind to eukaryotic initiation factor 4E (eIF4E), resulting in increased translation[265]. Whether LRRK2 directly affects phosphorylation of 4E-BP1 is currently under debate. First of all, *in vitro* phosphorylation of 4E-BP1 by LRRK2 was found to be rather weak. In addition, changes in 4E-BP1 phosphorylation after overexpression of LRRK2 in HEK293T cells could not be confirmed[266]. Remarkably, Drolet et al. found increased phosphorylation of 4E-BP1 after LRRK2 kinase inhibition in primary neurons[267]. The role of LRRK2 in translation is further supported by a more recent study, where 19 ribosomal proteins were identified as potential candidate LRRK2 substrates[268].

For transcription, LRRK2 has been described as a negative regulator of nuclear factor of activated T-cells (NFAT), an important transcription factor as it was hypothesized that LRRK2 is part of an NFAT repressing complex[50]. In conclusion, several lines of evidence support a role for LRRK2 in transcription and translation. However, more research is needed for thorough validation of the direct influence of LRRK2 in these processes.

2.8.4. MAPK signaling

LRRK2 shows homology with mixed lineage kinases (MLK). These proteins regulate the stress response and proliferation in the cell via activation of the mitogen-activated protein kinase (MAPK) cascade. MAPK kinase kinase (MAPKKK), MAPK kinase (MAPKK) and MAPK are sequentially activated in this cascade, eventually affecting transcription. Proteins like p38, JNK and ERK1/2[269] belong to the MAPK family and LRRK2 was shown to be involved in signal pathways for all three proteins via phosphorylation of MKK3/6, MKK4/7 and JNK-interacting proteins (JIPs)[264,270–272].

2.8.5. Apoptotic signaling

Moreover, LRRK2 shows homology to receptor-interacting serine/threonine-protein kinases (RIPKs) as well. RIPKs are involved in extrinsic apoptotic signaling, in which apoptosis is induced after activation of dead domain receptors. LRRK2 can interact with Fas-associated protein with death domain (FADD), tumor necrosis factor receptor type 1-associated DEATH domain protein (TRADD) and RIP1. Pathogenic LRRK2 mutations R1441C, Y1699C, G2019S and I2020T increase the binding between LRRK2 and FADD, resulting in increased activation of caspase 8. Moreover, inhibition of FADD in primary neurons is able to prevent LRRK2-mediated toxicity, which suggests a role for LRRK2 in the extrinsic apoptotic pathway[273]. Next to extrinsic apoptotic signaling, LRRK2 is also implicated in the intrinsic apoptotic signaling cascade, where pro-apoptotic signals arise from the mitochondria. Eventually, these signals activate intrinsic caspases and overexpression of mutant LRRK2 results in increased cytochrome c release and activation of caspase 3[274]. On the other hand, LRRK2 was suggested to interact with Akt, a pro-survival protein that prevents apoptosis. However, mutant LRRK2 shows decreased binding to Akt, which might indicate that pathogenic activity of LRRK2 is established due to defects in cell survival and apoptotic regulation[275].

2.8.6. Mitochondrial dynamics

The main function of mitochondria is the provision of enough energy for the cell, which is produced by respiration[276]. Mutations in mitochondria-regulating proteins like PINK1 and Parkin, two PD-associated genes that have been linked to autosomal recessive PD and sporadic PD (in the case of Parkin[277–280]), result in severe mitochondrial deficits and cell death[281]. Yet, growing evidence links the pathobiology of LRRK2 to mitochondrial dysfunction, as well. First of all, LRRK2 co-localizes with the outer mitochondrial membranes[80,133]. Next, LRRK2 toxicity was also linked to increased mitochondrial cytochrome c release, which results in increased apoptosis[274]. Moreover, LRRK2 interacts with the mitochondrial fission factor, dynamin-like protein 1 (DLP1). In case of pathogenic mutations, the DLP1 levels in mitochondria increase significantly and increased mitochondrial fission, a key factor in mitochondrial dysfunction[282], is observed. However, this phenotype can be rescued by silencing of DLP1[281,283,284]. Lee et al. observed that the number of mitochondria in axons is significantly higher after LRRK2 knockout, while overexpression of LRRK2 shows a decreased number of mitochondria. Co-expression of the mitochondria-related protein Futsch is able to re-normalize the number of mitochondria in axons. LRRK2 seems to affect the motility of mitochondria as overexpression of LRRK2 is able to increase mitochondrial motility, both in an anterograde and retrograde fashion. However, motility of mitochondria

in case of mutant LRRK2 is mostly retrograde, resulting consequently in a decreased number of mitochondria in the synapse[150,285].

Peroxiredoxin 3 was further suggested as a LRRK2 substrate, in which phosphorylated peroxiredoxin 3 negatively affects mitochondrial function, resulting in increased oxidative damage[286].

Moreover, patients carrying the G2019S mutation display impaired mitochondrial function and morphology[287] and patients with idiopathic PD present mitochondrial complex-I deficiency in the SN[281].

In conclusion, mitochondrial dysfunction is most likely to contribute to LRRK2-PD pathobiology, although the exact mechanism remains elusive.

2.8.7. Autophagy-endolysosomal system

The purpose of autophagy is the specific removal and degradation of unnecessary, damaged or toxic components in the cell. On the other hand, autophagy can induce programmed cell death in response to stress. The cellular components are engulfed by the autophagosome, which later on fuses with the lysosome to form an autolysosome, in which the degradation and recycling occurs.

GWAS have revealed variations in autophagy-linked genes with an increased risk to develop PD, like RAB7L1, cyclin-G-associated kinase (GAK), GBA, lysosome-associated membrane protein 2a (LAMP2a), ATPase 13A2 (ATP13A2) and synaptotagmin 11 (SYT11) (reviewed in [88])[71,176]. Moreover, the list of PARK genes includes several autophagy-linked genes, like PINK1, Parkin, ATP13A2, VPS35, SYNJ1 and FBX07 (Table 1.1.).

Several studies have reported autophagic dysfunction in a mutant LRRK2 background. For example, an increased number of autophagosomes can be observed in several cell lines that overexpress G2019S or R1441C LRRK2[197,241]. Moreover, transgenic mice that express either G2019S or R1441C LRRK2 display an increased number of autophagic vacuoles throughout their brain[244]. It was hypothesized that the observed phenotype is due to a decreased autophagic activity as small-interfering (si) RNAs against LRRK2 result in an increased autophagic flux. This is in line with a report describing decreased autophagic response after induction of starvation or activation of the mTOR pathway in primary fibroblasts from individuals carrying R1441G, Y1699C or G2019S LRRK2[288]. On the other hand, increased basal autophagic flux was observed in fibroblasts expressing G2019S LRRK2[289]. More conflicting data were published in two studies where decreased degradative capacity was observed both in immune cells with knockdown of LRRK2[131] and after overexpression of G2019S LRRK2 in SH-SY5Y cells[290].

Moreover, LRRK2-deficient animals consistently show peripheral pathology that is linked to autophagic defects as an increased number and size of secondary lysosomes can be observed in lung and kidney[203,291,292]. The autophagic impairment was shown to be biphasic as young mice display increased autophagic activity, while older mice show decreased autophagic activity[292] (see further)[293]. It should be noted that in these studies, no autophagic-related defects were observed in the brain. However, in a G2019S LRRK2 mouse model, autophagic abnormalities could only be observed in the brain of aged mice[244].

Several studies postulated on the mechanism by which LRRK2 is able to influence autophagy. First of all, overexpression of WT LRRK2 led to the activation of nicotinic acid adenine dinucleotide phosphate (NAADP) receptors and of the calcium-dependent protein kinase β (CAMKK-β)/adenosine monophosphate (AMP)-activated protein kinase (AMPK) pathway, leading to reduced autophagic degradation by elevating lysosomal pH.

Taken together, studies in cell culture and in *in vivo* models have provided convincing results on the role of LRRK2 in autophagy. Unfortunately, mechanistic insight on how LRRK2 acts on the autophagic pathway in diseased conditions is still lacking. Altogether, it is possible that different degrees of autophagic defects depend on the cell type investigated, the developmental stage of animals, or specific experimental conditions.

2.8.8. Vesicle dynamics

Moreover, LRRK2 has been implicated in vesicular trafficking related to endocytic uptake, endosome-lysosome and autophagosome-lysosome trafficking as well as retromer-mediated trafficking to and from the Golgi complex[105,294]. For example, LRRK2 co localizes with vesicular structures and overexpression of (pathogenic) LRRK2 (variants) leads to vesicular deficits[80,105,179,240]. Moreover, in primary neurons, both overexpression and silencing of LRRK2 leads to decreased synaptic vesicle endocytosis[295,296].

Several LRRK2 substrate proteins important for vesicle dynamics have already been proposed. For example, Matta and colleagues showed that LRRK2 phosphorylates endophilinA and thereby affects synaptic vesicle endocytosis[297]. LRRK2 also phosphorylates Rab proteins, important proteins in vesicle dynamics. Rab proteins belong to the GTPase family and localize to different intracellular membranes and modulate vesicle budding, transport and fusion. Phosphorylation of Rab proteins by LRRK2 is thought to inactivate Rab proteins and their further functioning[294]. Different Rab small GTPase proteins have already been suggested as LRRK2 substrates[137,176,177,179,295,298,299], further pointing for a role for LRRK2 in vesicle dynamics. For example, LRRK2 was shown to interact with Rab5b[295], Rab7[179,298], Rab7L1 (also known as Rab29)[121,177,300] and Rab32[299]. Rab7B has been shown to colocalize with LRRK2 in human brain[301]. Moreover, decreased synaptic vesicle endocytosis, which is observed in primary neurons after overexpression or silencing of LRRK2, can be rescued after co-overexpression with Rab5b[295,296].

Several studies report on the relation between PD, LRRK2 and Rab7L1. Rab7L1 resides in the PARK16 locus and has been implicated in sporadic PD in GWAS studies[69,177,180,300,302]. Beilina and colleagues proposed that LRRK2 and Rab7L1 form a protein complex that promotes clearance of Golgi-derived vesicles through the autophagy-lysosomal pathway, which is increased in pathogenic LRRK2 variants. After co-overexpression with Rab7L1, Golgi clearance is even further increased[176], suggesting an additive effect of LRRK2 and Rab7L1. Another study reported that Rab7L1 or Rab7L1 ortholog silencing induces degeneration of primary rodent neurons and *Drosophila* dopaminergic neurons, comparable to the degeneration observed in pathogenic LRRK2. Also deficits in endolysosomal and Golgi apparatus sorting could be observed. Moreover, Rab7L1 overexpression is able to rescue the toxicity of pathogenic LRRK2 variants[177]. More mechanistic insight in the LRRK2-Rab7L1 interaction comes from a recent study from Purlyte and colleagues. They suggest that LRRK2 phosphorylates Rab7L1, and thereby

activates itself as activated Rab7L1 recruits LRRK2 to the trans-Golgi network (TGN) and further activates LRRK2 kinase activity. Remarkably, pathogenic LRRK2 mutants R1441C/G and Y1699C get activated to a greater extent by Rab7L1 than WT LRRK2[121]. Altogether, it is very likely that the pathogenic mechanism of LRRK2 includes defective vesicle dynamics.

Chapter 2 – Aims of the thesis

In this PhD project, we aimed to gain more insight in two molecular consequences of pharmacological LRRK2 kinase inhibition. First of all, we and others have shown that heterologous phosphorylation sites of LRRK2 are rapidly dephosphorylated after treatment with LRRK2 kinase inhibitors. It is remarkable that dephosphorylation at these sites is also observed in pathogenic LRRK2 mutants. Second, total LRRK2 protein levels decrease after longer-term LRRK2 kinase inhibitor treatment, which might be closely related to the peripheral pathology observed in animals treated with a LRRK2 kinase inhibitor, as it is reminiscent of the pathology reported in LRRK2$^{-/-}$ mice.
Therefore, the work in this PhD project can be subdivided into following specific aims:

1. **Identification and validation of phosphatases important in the LRRK2 dephosphorylation cycle**

Our research group has identified the catalytic subunit α of protein phosphatase 1 as the phosphatase responsible for dephosphorylation of heterologous phosphorylation sites, both after LRRK2 kinase inhibition and in pathogenic mutants. To identify the regulatory subunits and other potential phosphatases involved in the protein complexes that regulate LRRK2 phosphorylation, a small interfering (si) RNA screen was performed in the lab. In this part of the project, we wanted to validate the top hits from this screen. We aimed to investigate the identified regulators in neuronal and kidney cells using lentiviral vector-mediated knockdown. The effect of knockdown of candidate proteins on LRRK2 phosphorylation was studied in basal conditions and after LRRK2 kinase inhibition.
This part of the project is discussed in Chapter 3.

2. **Understanding LRRK2 kinase inhibitor-induced LRRK2 destabilization**

In this part of the project, we aimed to characterize LRRK2 kinase inhibitor-induced LRRK2 destabilization and to identify the underlying mechanism. To this end, we investigated the destabilization in different cell types and *in vivo*. Moreover, we wanted to identify the determinant of LRRK2 destabilization and the relationship between LRRK2 kinase inhibitor-induced dephosphorylation and destabilization. Therefore, we tested the effect of LRRK2 kinase inhibition and casein kinase 1 inhibition on several pathogenic and functional LRRK2 mutants.
This part of the project is discussed in Chapter 4 & 5.

Chapter 3 – The holoenzyme PP2A regulates the LRRK2 dephosphorylation cycle

This chapter is part of an article in preparation as:

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PP2A holoenzyme regulates LRRK2 phosphorylation and expression.

* shared first authorship, § shared last authorship

Scientific acknowledgement & personal contribution

Evy Lobbestael, Jean-Marc Taymans, Jeremy Nichols and Veerle Baekelandt designed the research. Only data from Marc F. Bolliger, Evy Lobbestael, Jeremy Nichols, Jean-Marc Taymans and myself are included in this chapter. Evy Lobbestael designed the shRNA's for cloning of knockdown constructs, which I cloned in the pGAE-SFFV-eGFP-P2A-ZeoR-miRNa30 stem loop Esp3I plasmid. Evy Lobbestael and I performed experiments of the follow-up screening using lentiviral vector mediated knockdown. Evy Lobbestael determined the selection criteria, which led to the identification of 12 top hits that were included in further validation experiments. Marc F. Bolliger and Jeremy Nichols optimized, executed and analyzed the overexpression experiments (Figure 3.3), while I optimized, executed and analyzed the knockdown experiments (Figure 3.4). I prepared Figure 3.4 and wrote this chapter. Matthieu Drouyer and Jean-Marc Taymans will write the paper in its final form. All authors will read and approve the paper before submission**.** We thank the Leuven Viral Vector Core for the production of lentiviral vectors (https://gbiomed.kuleuven.be/english/ research/50000715/laboratory-of-molecular-virology-and-gene-therapy/lvvc). Financial support from the MJFox Foundation, KU Leuven, Flanders Innovation & Entrepreneurship (VLAIO) (doctoral fellowship TDW IWT.141396) and Research Foundation Flanders (FWO, projects G0E1917N and SBOS006617N) is gratefully acknowledged.

Conflict of interest statements

The authors declare no competing financial interests.

1. Abstract

The phosphorylation cycle of heterologous phosphorylation sites in LRRK2 is important in the pathobiology of PD as several heterologous phosphorylated serines residues are found to be dephosphorylated in pathogenic mutants. Moreover, LRRK2 kinase inhibition, currently one of the prevailing disease-modifying strategies for PD, induces rapid dephosphorylation of these heterologous phosphorylation sites. The finding that a potential protective agent induces the same LRRK2 dephosphorylation as seen in pathogenic mutants is a fascinating observation in light of the therapeutic use of LRRK2 kinase inhibitors and might indicate that LRRK2 dephosphorylation at the heterologous phosphorylation sites is not a pathogenic mechanism. The contrary might also be valid, e.g. that dephosphorylation at the heterologous phosphorylation sites can be directly linked to LRRK2

pathobiology, rendering LRRK2 kinase inhibition invalid as a potential therapeutic strategy. However, this hypothesis is not supported by much experimental evidence to date. We have shown previously that dephosphorylation of these sites, after LRRK2 kinase inhibition or in the presence of pathogenic mutations, is mediated by the catalytic subunit α of protein phosphatase 1 (PPP1Cα), yet regulatory proteins or other phosphatases involved in this process remain unknown. To identify the specific complex of phosphatases involved in LRRK2 dephosphorylation after LRRK2 kinase inhibition, we performed a small interfering (si) RNA screen and further validated the top hits, both in basal conditions and after LRRK2 kinase inhibition. Here, we provide evidence that the PP2A phosphatase complex is involved in the LRRK2 dephosphorylation cycle, especially after LRRK2 kinase inhibition.

2. Introduction

Missense mutations in LRRK2 cause autosomal dominant PD and are the most common cause of familial PD[43,44]. Eight point mutations in LRRK2 (N1437H, R1441C/G/H/S, Y1699C, G2019S and I2020T) are known to cause PD[51,52], of which the most common mutation, G2019S, accounts for 4% of familial PD cases and 1% of apparent sporadic PD cases[51]. In addition, GWAS have revealed that variations in the LRRK2 locus can increase the risk to develop sporadic PD[55,68–71]. Moreover, LRRK2-PD patients are clinicopathologically indistinguishable from idiopathic PD patients, suggesting a potential common pathological mechanism for both forms of the disease[72–78]. These observations have made LRRK2 one of the most interesting proteins for studying PD pathobiology.

All pathogenic mutations of LRRK2 are located in the catalytic core of the protein which consists of a GTPase domain (ROC/Ras of complex proteins), linked to a COR-domain (C-terminal of ROC), and a kinase domain. This suggests a role for altered enzymatic function in PD pathogenesis which is supported by the fact that most pathogenic mutations show increased kinase activity[136] and consequently, autophosphorylation[132]. Several studies have shown that increased LRRK2 activity is linked to toxicity in cell culture models and *in vivo*[143–145], which can be counteracted when introducing a kinase-dead mutation[144] or after treatment with LRRK2 kinase inhibitors[143–145]. These observations have made LRRK2 kinase activity one of the prevailing disease-modifying targets and many companies have sought to develop potent and selective LRRK2 kinase inhibitors, which are currently tested in a phase I clinical trial. Several independent studies have reported beneficial effects of LRRK2 kinase inhibition; yet, some caution is needed, as we do not yet fully comprehend all consequences of LRRK2 kinase inhibition.

On the molecular level, LRRK2 kinase inhibition induces rapid dephosphorylation of LRRK2 autophosphorylation sites. LRRK2 also contains residues that are phosphorylated by kinases other than LRRK2 (reviewed in [293]), termed heterologous phosphorylation sites, of which S908, S910, S935, S955, S973 and S976 are best known. These residues are also dephosphorylated after LRRK2 kinase inhibition. Remarkably, the heterologous phosphorylation sites are also dephosphorylated in pathogenic mutations with increased kinase activity[303]. Moreover, dephosphorylation of heterologous phosphorylation sites is also found in different brain regions, including the SNpc, of idiopathic PD patients[167]. These findings suggest the importance for LRRK2-dephosphorylation of heterologous

phosphorylation sites in PD pathogenesis and indicates that caution is advised with regard to LRRK2 kinase inhibition as a potential PD therapy.

Findings from our group show that dephosphorylation of heterologous sites after LRRK2 kinase inhibition or in the presence of pathogenic mutations is mediated by the PPP1Cα[163], a protein which is highly and ubiquitously expressed in all eukaryotic cells. PP1 is one of the best structurally and functionally conserved proteins[304]. For example, the human and yeast gene that encode PP1 show more than 80% sequence identity. Moreover, human PP1 is able to rescue lethality caused by PP1 deletion in yeast[305], indicating the evolutionary conservation of the PP1 protein. The PP1 holoenzyme consists of a catalytic subunit (PPP1Cα, PPP1Cβ or PPP1Cγ) and one of many regulatory subunits, which directs the complex to its substrate and determines specificity and activity of the PP1 complex[304,305]. PP1 catalyzes, together with protein phosphatase 2A (PP2A), more than 90% of all dephosphorylation reactions in eukaryotes and consequently is involved in the majority of cellular processes (reviewed in [304])[306]. As for PP1, PP2A is ubiquitously expressed and the sequence conservation between yeast, drosophila and mammals is high (between 78-93%)[307]. Both PP1 and PP2A belong to the same phosphatase superfamily of Ser/Thr phosphatases, yet the structure of the PP2A holoenzyme is slightly different. Besides a catalytic and a regulatory subunit, the PP2A complex consists of a structural subunit as well, which facilitates the interaction between the different subunits and substrates[307,308]. Three families of regulatory subunits exist for the PP2A complex: the B (PR55), the B' (PR56) and the B'' (P72) family[309].

As the regulatory subunit involved in dephosphorylation of heterologous LRRK2 phosphorylation sites remains unknown, we aimed to identify regulatory subunits of PP1 or other phosphatases important in the LRRK2 phosphorylation cycle.

3. Results

3.1. siRNA screen

To identify phosphatases and/or their regulators involved in LRRK2 dephosphorylation, an initial siRNA screen was performed in collaboration with Invitrogen. Cells from the human bone osteosarcoma epithelial cell line U2OS were transduced with BacMam LRRK2(WT)-GFP vector and further transfected with target specific siRNA oligos against 298 phosphatase proteins (Table 3.S1). Three unique siRNA's per target protein were tested. The siRNA screen was set up to select hits based on their potential to affect LRRK2 phosphorylation at S935. Therefore, cells were assayed via LanthaScreen®, a technology based on Time Resolved-Fluorescence Resonance Energy Transfer (TR-FRET), with LRRK2 phosphorylation at S935 as readout (Figure 3.1.). 39 proteins induced ≥75% change in phosphorylation in at least one of three replicate experiments or ≥60% change in phosphorylation in at least two out of the three replicate experiments and were selected for further validation (Table 3.1.). Interestingly, both PP1 and PP2A related subunits showed great effects on LRRK2 S935 phosphorylation as well as some tyrosine phosphatases.

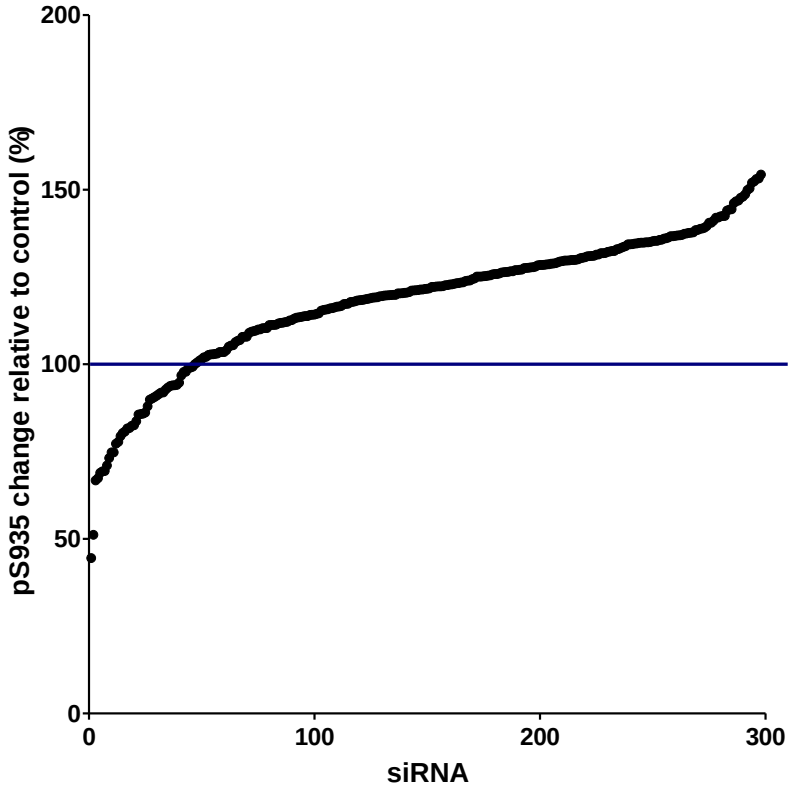


Figure 3.1. – Graphical overview of the results from the phosphatome-wide siRNA screen from which 39 top hits for the present study have been selected. Changes in LRRK2 S935 phosphorylation induced by siRNA-mediated knockdown of 298 phosphatases and phosphatase regulators are depicted in the diagram.

Selected protein	Full name	siRNA screen	2nd selection	Final selection
ASNA1	arsA arsenite transporter, ATP-binding, homolog 1	X		
CBP-P22	Calcium binding protein P22	X		
CDC14B	Cell division cycle 14B	X	X	
CDC25A	Cell division cycle 25A	X		
CDC25B	Cell division cycle 25B	X		
CDC25C	Cell division cycle 25C	X		
DUPD1	Dual specificity phosphatase and pro-isomerase domain	X		
DUSP3	Dual specificity phosphatase 3	X		
ENTPD1	Ectonucleoside triphosphate diphosphohydrolase 1	X		
ENTPD4	Ectonucleoside triphosphate diphosphohydrolase 4	X		
IMPA1	Inositol(Myo)-1(or 4)-monophosphatase 1	X	X	
INPLL1	Inositol polyphosphate phosphatase-like 1	X	X	
MINPP1	Multiple inositol-polyphosphate phosphatase 1	X		
PPM1A	Protein phosphatase, Mg^{2+}/Mn^{2+} dependent, 1A	X		
PTPN13	Protein tyrosine phosphatase, non-receptor type 13	X		
PTPN23	Protein tyrosine phosphatase, non-receptor type 23	X		
PTPRM	Protein tyrosine phosphatase, receptor type M	X		
PTPRN	Protein tyrosine phosphatase, receptor type N	X	X	
PTPRR	Protein tyrosine phosphatase, receptor type R	X		
PTPRS	Protein tyrosine phosphatase, receptor type S	X		
PPP1R1A	Protein phosphatase 1, regulatory (inhibitor) subunit 1A	X	X	
PPP1R1B	Protein phosphatase 1, regulatory (inhibitor) subunit 1B	X		
PPP1R3B	Protein phosphatase 1, regulatory subunit 3B	X		
PPP1R3D	Protein phosphatase 1, regulatory subunit 3D	X		
PPP1R3F	Protein phosphatase 1, regulatory subunit 3F	X		
PPP1R16B	Protein phosphatase 1, regulatory subunit 16B	X		
PPP1R8	Protein phosphatase 1, regulatory subunit 8	X		
PPP1Cα	Protein phosphatase 1, catalytic subunit, isozyme α	X		
PPP1Cβ	Protein phosphatase 1, catalytic subunit, isozyme β	X		
PPP1Cγ	Protein phosphatase 1, catalytic subunit, isozyme γ	X		
PPP2R2A	Protein phosphatase 2, regulatory subunit B, α	X	X	X
PPP2R2B	Protein phosphatase 2, regulatory subunit B, β	X	X	X
PPP2R2C	Protein phosphatase 2, regulatory subunit B, γ	X	X	X
PPP2R3B	Protein phosphatase 2, regulatory subunit B'', β	X		
PPP2R3C	Protein phosphatase 2, regulatory subunit B", γ	X	X	
PPP2R5B	Protein phosphatase 2, regulatory subunit B', β	X	X	
PPP2R5C	Protein phosphatase 2, regulatory subunit B', γ	X		
PPP2Cα	Protein phosphatase 2, catalytic subunit α isozyme	X	X	X
PPP2Cβ	Protein phosphatase 2, catalytic subunit β isozyme	X	X	

Table 3.1. - List of the proteins with the greatest effect on LRRK2 phosphorylation at S935 in the siRNA screen and 2nd selection. The subunits of the PP2A complex that were selected for final validation are indicated in the 5th column.

3.2. Follow-up screening using lentiviral vector mediated knockdown

To further narrow down the selection of proteins, we generated 2 shRNA sequences for each of the 39 selected proteins, which were cloned in the pGAE-SFFV-eGFP-P2A-ZeoR-miRNa30 stem loop Esp3I plasmid[310]. A shRNA sequence against firefly luciferase (fLuc) was included as negative control. Next, lentiviral (LV) vectors were produced to transduce HEK293T cells with stable overexpression of 3Flag-LRRK2. Besides studying the effect on S935 phosphorylation after knockdown of candidate proteins in basal conditions (Figure 3.2.A), we also questioned if some phosphatases were specifically involved in the dephosphorylation of LRRK2 induced by LRRK2 kinase inhibition (Figure 3.2.B). Therefore, cells were treated with the LRRK2 kinase inhibitor LRRK2-IN1 before cell lysis. Both LRRK2 levels and LRRK2 phosphorylation levels at S935 were analyzed. Top candidate proteins were chosen based on following selection criteria: 1) presence in the top 10 for at least 3 out of 5 repetition experiments and 2) induction of ≥20% change in S935 phosphorylation in at least 1 repetition experiment in basal conditions (Figure 3.2.A) or induction of ≥50% change in S935 phosphorylation in at least 1 repetition experiment after LRRK2 kinase inhibition (Figure 3.2.B). In total, 12 top hits were selected for further validation (Table 3.1.). Unexpectedly, the selection included only one regulatory protein of the PP1 complex, although we have previously shown the involvement of PPP1Cα in LRRK2 phosphorylation[163]. Yet, several subunits of PP2A were included in the selection (Table 3.1.).

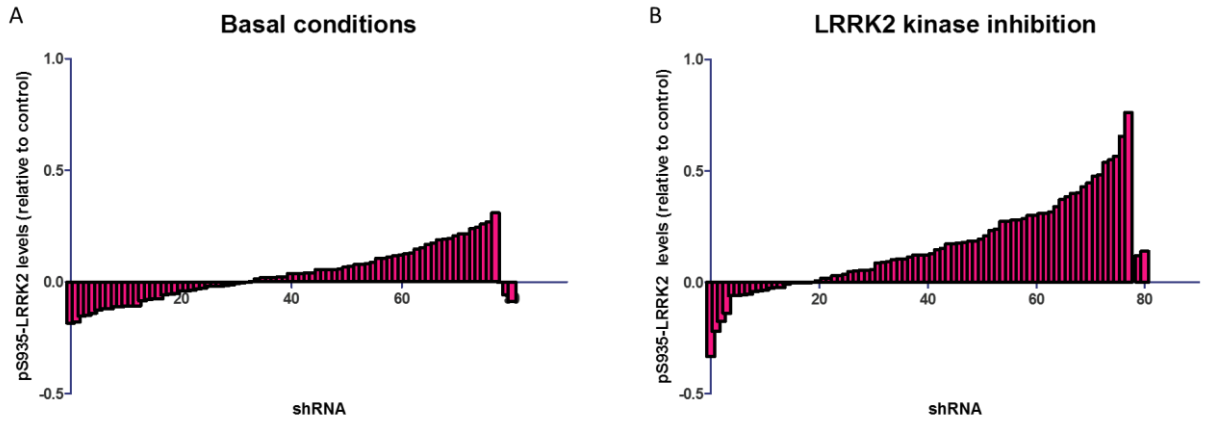


Figure 3.2. – Graphical overview of the results from the lentiviral vector mediated screen from which we selected 12 top hits for further validation. Changes in LRRK2 S935 phosphorylation induced by knockdown of 39 phosphatases and phosphatase regulators (each was tested with 2 shRNA's) in basal conditions (A) or after LRRK2 kinase inhibition with LRRK2-IN1 (B) are depicted in the diagrams.

3.3. Overexpression of PP2A complexes results in decreased phosphorylation of LRRK2

To further validate the results from the screens, the effect on LRRK2 phosphorylation at four heterologous phosphorylation sites (S910, S935, S955 and S973) was examined in HEK293 cell lines with overexpression of LRRK2 and one of the 12 selected phosphatases. We expected that overexpression of phosphatases involved in the LRRK2 dephosphorylation cycle, induces dephosphorylation of heterologous LRRK2 phosphorylation sites. As the specificity of a phosphatase complex is determined by the regulatory subunit, we evaluated both single overexpression of the catalytic subunits and combined overexpression of the catalytic subunit and a regulatory subunit of a

phosphatase complex. We only observed significant dephosphorylation on all four heterologous phosphorylation sites after combined overexpression of a catalytic with a regulatory subunit of the PP2A complex (Figure 3.3.), and not after combined overexpression of subunits of the PP1 complex, nor for the other phosphatases listed in the 2nd selection (Table 3.1.). Therefore, we have only included data on the PP2A complex in this chapter. Overexpression of the catalytic subunit α of the PP2A complex (PPP2Cα) alone did induce significant changes in phosphorylation at three out of four phosphorylation sites tested. However, overexpression of both PPP2Cα and a regulatory subunit from the B (PR55) family (PPP2R2A, PPP2R2B, PPP2R2C or PPP2R2D), induced a more significant decrease in phosphorylation on all four heterologous phosphorylation sites tested. Combined overexpression with a regulatory subunit that belongs to the B' (PR56) family (PPP2R5B and PPP2R5C) or the B'' (PR72) family (PPP2R3B and PPP2R3C)[311] induced a significant decrease in phosphorylation for several, but not all, of the phosphorylation sites tested. Moreover, this decrease was more modest than the decrease observed after combined overexpression with subunits of the B (PR55) family.

3.4. The PP2A complex is involved in LRRK2 kinase inhibitor-induced dephosphorylation of heterologous sites

Combined overexpression of both the catalytic and a regulatory subunit of the PP2A complex resulted in greatest effect on LRRK2 heterologous phosphorylation sites. Therefore, we wanted to further validate the effects of stable double knockdown of these subunits on LRRK2 phosphorylation. We aimed to obtain a stable double knockdown of both the catalytic and a regulatory subunit in SH-SY5Y cells that already stably overexpressed LRRK2. A short hairpin sequence against fLuc was used as a negative control and we used two different sequences against the same target protein, and analyzed the results as one group. Moreover, we only included experiments in which at least 50% knockdown was achieved for both the catalytic and the regulatory subunit (Figure 3.4.B, C, F, G). To identify PP2A complexes that operate on LRRK2 S935 after LRRK2 kinase inhibition, we treated cells with either the LRRK2 kinase inhibitor PF-06447475 or DMSO as control for 2 h (Figure 3.4.A) or 24 h (Figure 3.4E). After 2 h of treatment, combined knockdown of phosphatase complexes could not prevent LRRK2 kinase inhibitor-induced dephosphorylation at S935 compared to control conditions, except for combined knockdown of PPP2Cα and PPP2R2B (Figure 3.4.D). After 24 h of LRRK2 kinase inhibition, knockdown of all three phosphatase complexes prevented dephosphorylation at S935 (Figure 3.4.H). These results suggest that PP2A complexes are involved in dephosphorylation of at least S935 after LRRK2 kinase inhibition.

4. Discussion

In this study, we aimed to identify phosphatase complexes that dephosphorylate heterologous phosphorylation sites of LRRK2 in basal conditions or during LRRK2 kinase inhibition. Previously, we have identified the involvement of the catalytic subunit PPP1Cα, however, the regulatory subunits of the PP1 complex or the role of other phosphatase complexes remain unknown.

We set up a phosphatome-wide siRNA screen that comprised approximately 300 phosphatases and evaluated the effect of knockdown of each candidate protein on LRRK2 phosphorylation at S935, which is the best characterized heterologous phosphorylation site to date. Candidate proteins that met the selection criteria (≥75% change in S935 phosphorylation in at least one of three replicate experiments or ≥60% change in S935 phosphorylation in at least two out of the three replicate experiments) included several subunits of both PP1 and PP2A, which were selected for further validation. Remarkably, also several tyrosine phosphatases were identified, which were no candidates as a direct regulator of the serines involved in the LRRK2 phosphorylation cycle, but might play a crucial role in the regulation of phosphorylation.

Subsequently, we narrowed down the number of proteins using LV-mediated miRNA-based knockdown in HEK293T cells, in which we investigated effects on LRRK2 S935 phosphorylation in both basal and LRRK2 kinase inhibited conditions. 12 candidate proteins met our selection criteria in this setup ((1) presence in the top 10 of effectors for at least 3 out of 5 repetition experiments and (2) induction of ≥20% change in S935 phosphorylation in at least 1 experiment in basal conditions or induction of ≥50% change in S935 phosphorylation in at least 1 experiment after LRRK2 kinase inhibition). Interestingly, both regulatory subunits and the two catalytic subunits of the PP2A complex showed greater effects on LRRK2 phosphorylation than subunits from the PP1 complex, of which only the regulatory subunit PPP1R1A met our selection criteria. Since approximately 90% of all eukaryotic dephosphorylation processes involve either PP1 or PP2A, a potential role of PP2A in dephosphorylation of LRRK2 is not surprising and is in line with a report describing the interaction between PP2A and LRRK2[185]. However, effects of PP2A on heterologous LRRK2 phosphorylation sites have not been reported to date.

Experiments in which we overexpressed both a catalytic and regulatory subunit of the PP2A complex showed that a PP2A complex affects LRRK2 phosphorylation significantly more than overexpression of a catalytic subunit alone. Moreover, regulatory subunits that belong to the B (PR55) family seem to induce greater effects on LRRK2 phosphorylation compared to regulatory subunits from the B' (PR56) or B'' (PR72) families. However, we cannot exclude the involvement of regulatory subunits from these families, as they also induce significant changes on phosphorylation levels of certain heterologous phosphorylation sites of LRRK2.

Our results further indicate that PP2A complexes might be involved in LRRK2 kinase inhibitor-induced dephosphorylation, as we show that the significant dephosphorylation of LRRK2, induced by LRRK2 kinase inhibition, is decreased after knockdown of PP2A complexes. However, we should take into account that these experiments show a rather high variation (Figure 3.4.H), potentially because of the degradation of LRRK2 protein induced by LRRK2 kinase inhibition[169]. We could assume that a potential reason for the moderate effects on LRRK2 dephosphorylation at S935 after knockdown of phosphatase

complexes of LRRK2 kinase inhibition is the fact that other PP2A subunits can still take over the function of the subunits that were knocked down.

In conclusion, we propose PP2A as an additional regulating phosphatase of the LRRK2 phosphorylation cycle, next to PP1. With regard to the development of potential new therapies for PD, it remains to be determined whether dephosphorylation of heterologous LRRK2 phosphorylation sites is an unwanted side-effect of LRRK2 kinase inhibition, as pathogenic LRRK2 variants also show dephosphorylation of heterologous phosphorylation sites in physiological conditions. Further validation is crucial to determine the specific conditions in which each phosphatase operates on phosphorylation sites of LRRK2. Moreover, the identification of the exact composition of the PP2A complex responsible for dephosphorylation in either pathogenic mutants or after LRRK2 kinase inhibition is crucial. If a different composition of the phosphatase complexes can be shown, a potential new therapeutic target might be available. Based on our results, several regulatory subunits of the PP2A complex might act on LRRK2 phosphorylation sites. Consequently, insight in the degree of specificity of the PP2A complex for LRRK2 will be pivotal with potential PD therapies in mind.

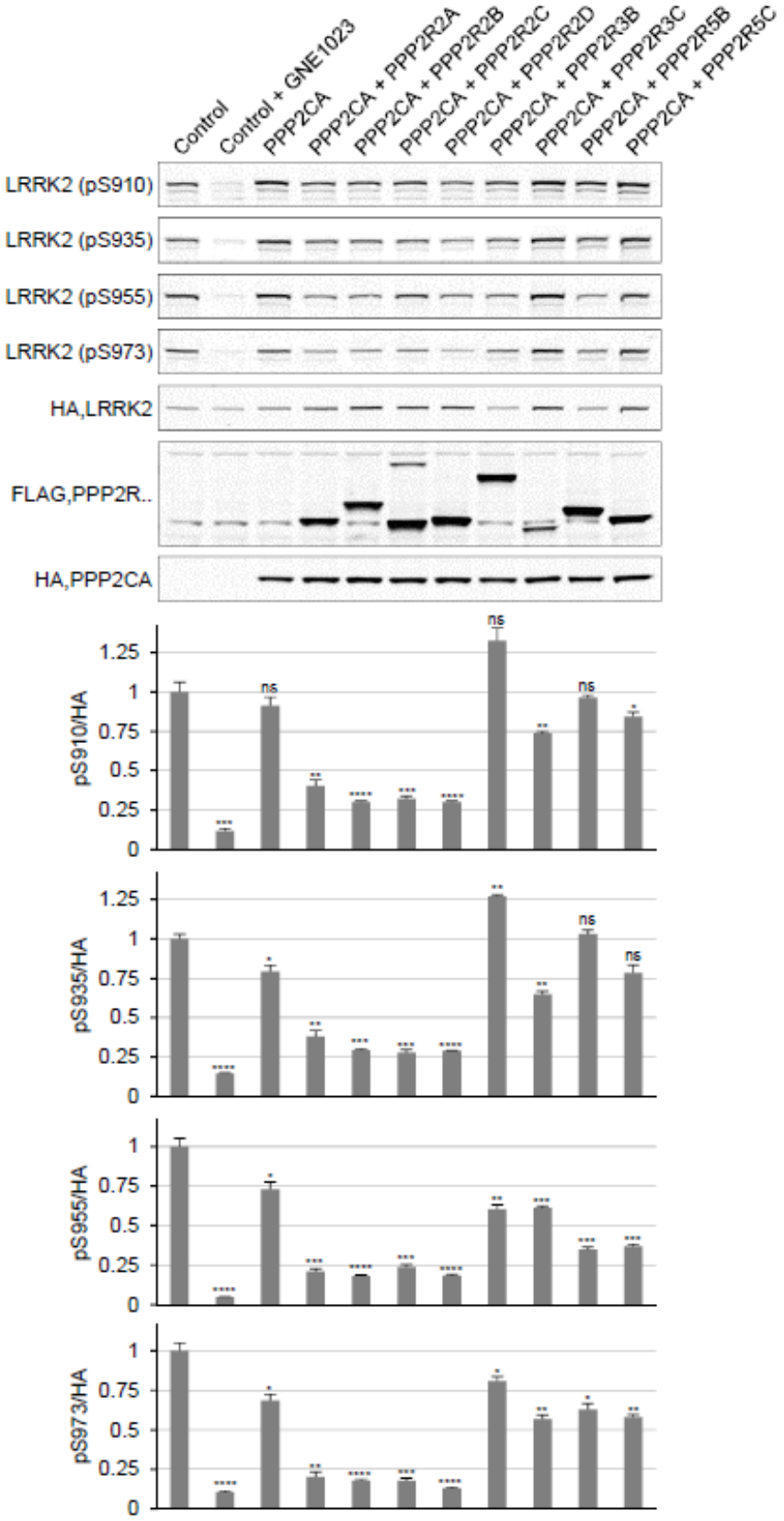


Figure 3.3. – Effect of PP2A overexpression on phosphorylation of LRRK2. HEK293 cells were transiently transfected with cDNAs encoding HA-tagged LRRK2, HA-tagged catalytic PP2A subunits and FLAG-tagged regulatory PP2A subunits, as indicated. Cell lysates were analyzed with immunoblotting using anti-HA for LRRK2 and catalytic PP2A subunit detection, anti-LRRK2 P-S910/S935/S955/S973 for LRRK2 phosphorylation detection and anti-Flag for detection of the regulatory PP2A subunits. A representative blot is shown. Graphs show the quantification of blots representing the ratio of phosphorylated LRRK2 at S910, S935, S9355 or S973 over total LRRK2 signal. N=3. Graphs show mean ± s.e.m.; statistical significance was tested with two-tailed t-test; not significant (ns) P>0.05; * P≤0.05; ** P≤0.01; *** P≤0.001; **** P≤0.0001.

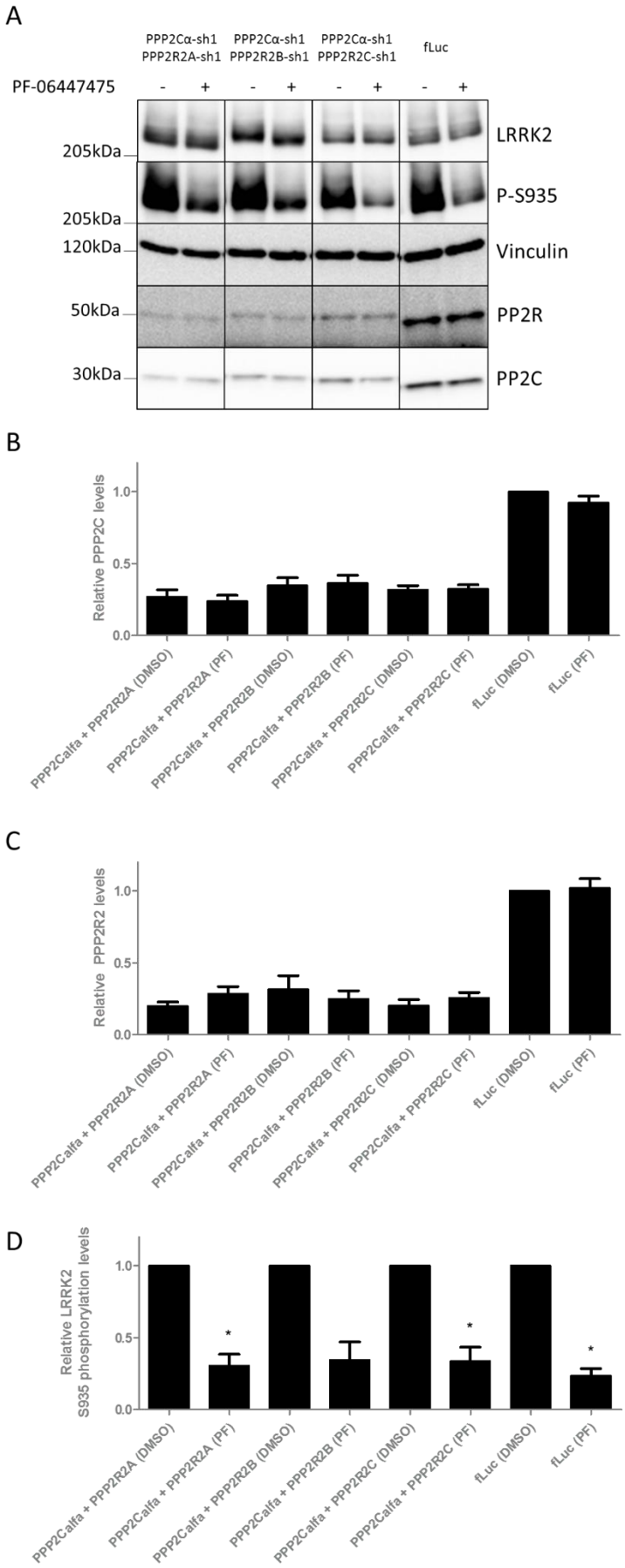
5. Materials & Methods

Initial siRNA screen. U2OS cells were transduced with BacMam LRRK2(WT)-GFP vector and transfected with a mix of Lipofectamine™ RNAiMAX transfection reagent and siRNA oligo's (20 nM). Cells are further assayed using LantaScreen® to determine LRRK2 phosphorylation at S935.

Lentiviral vector mediated miRNA-based knockdown screening. shRNA sequences against the selected proteins were cloned in a pGAE-SFFV-eGFP-P2A-ZeoR-miRNa30 stem loop Esp3I plasmid[310]. LVs were produced and cell transduction was performed as described in [163]. Cells were cultured and treated with DMSO or LRRK2-IN1 (1 µM) for 1 hour and further lysed, prepared for dot blot immunoblotting and analyzed as described in [169].

Overexpression of PP2A complexes. HEK293 cells were transiently transfected with cDNAs encoding HA-tagged LRRK2, HA-tagged catalytic subunits of PP2A and FLAG-tagged regulatory subunits of PP2A and treated with DMSO or GNE1023.

Knockdown of PP2A complexes. SH-SY5Y cells with overexpression of 3Flag-LRRK2 were generated and cultured as described in [169]. Transduction with knockdown constructs was performed as described in [163] and cells were treated with DMSO or PF-06447475 (150 nM) for indicated periods of time. Cell lysates were taken and prepared for immunoblotting as described in [169].


A
PPP2Cα-sh1 PPP2R2A-sh1
PPP2Cα-sh1 PPP2R2B-sh1
PPP2Cα-sh1 PPP2R2C-sh1
fLuc
PF-06447475
- + - + - + - +
205kDa
205kDa
120kDa
50kDa
30kDa
LRRK2
P-S935
Vinculin
PP2R
PP2C
B
Relative PPP2C levels
1.0
0.5
0.0
PPP2Calfa + PPP2R2A (DMSO)
PPP2Calfa + PPP2R2A (PF)
PPP2Calfa + PPP2R2B (DMSO)
PPP2Calfa + PPP2R2B (PF)
PPP2Calfa + PPP2R2C (DMSO)
PPP2Calfa + PPP2R2C (PF)
fLuc (DMSO)
fLuc (PF)
C
Relative PPP2R2 levels
1.0
0.5
0.0
PPP2Calfa + PPP2R2A (DMSO)
PPP2Calfa + PPP2R2A (PF)
PPP2Calfa + PPP2R2B (DMSO)
PPP2Calfa + PPP2R2B (PF)
PPP2Calfa + PPP2R2C (DMSO)
PPP2Calfa + PPP2R2C (PF)
fLuc (DMSO)
fLuc (PF)
D
Relative LRRK2 S935 phosphorylation levels
1.0
0.5
0.0
*
*
*
PPP2Calfa + PPP2R2A (DMSO)
PPP2Calfa + PPP2R2A (PF)
PPP2Calfa + PPP2R2B (DMSO)
PPP2Calfa + PPP2R2B (PF)
PPP2Calfa + PPP2R2C (DMSO)
PPP2Calfa + PPP2R2C (PF)
fLuc (DMSO)
fLuc (PF)

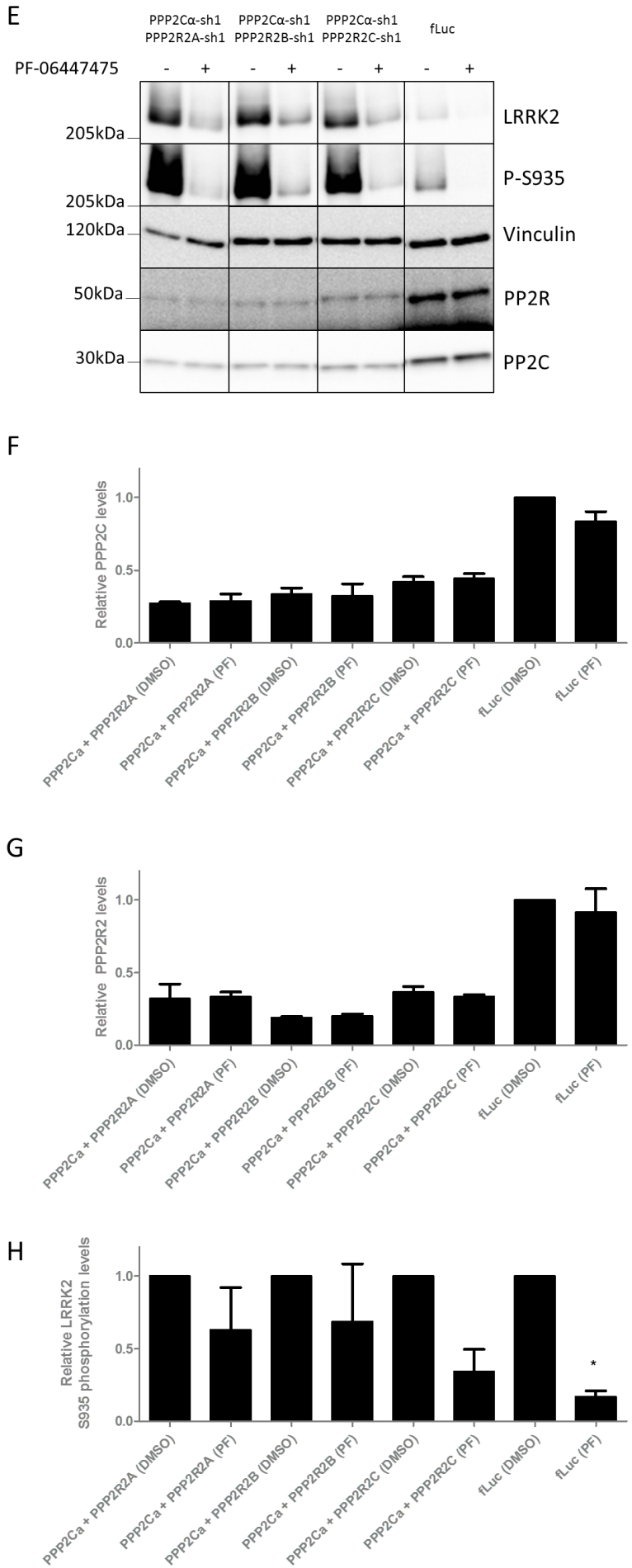


Figure 3.4. – Knockdown of protein phosphatase 2 complexes affects LRRK2 dephosphorylation at S935. SH-SY5Y cells overexpressing 3flag-LRRK2 WT and selected for stable knockdown of protein phosphatase 2 complexes (PPP2Cα with either PPP2R2A, PPP2R2B or PPP2R2C) were treated for 2 h (A, B, C, D) or 24 h (E, F, G, H) with PF-06447475 (PF, 150 nM) or DMSO. Cell lysates were analyzed with immunoblotting using FlagM2 antibody for LRRK2 detection, anti-LRRK2 P-S935 for LRRK2 phosphorylation detection, anti-PPP2R2x, anti-PPP2Cx and vinculin for equal loading. A representative blot is shown. Graphs show the quantification of blots representing the ratio total protein levels of catalytic subunits of protein phosphatase 2 over housekeeping signal (B, F), total protein levels of regulatory subunits of protein phosphatase 2 over housekeeping signal (C, G), phosphorylation at S935 over total LRRK2 signal (D H). N ≥ 3, except for combined knockdown of PPP2Cα with PPP2R2B after 24 h of LRRK2 kinase inhibition. Statistical significance was assessed by column statistics (one-sample t-test) with Bonferroni correction. *p < 0.05.

6. Supplementary material

Table 3.S1. - Overview of the 298 phosphatase proteins tested in the initial siRNA screen

1	ACP1	acid phosphatase 1, soluble
2	ACP2	acid phosphatase 2, lysosomal
3	ACP5	acid phosphatase 5, tartrate resistant
4	ACP6	acid phosphatase 6, lysophosphatidic
5	ACPL2	acid phosphatase-like 2
6	ACPP	acid phosphatase, prostate
7	ACPT	acid phosphatase, testicular
8	ACYP1	acylphosphatase 1, erythrocyte (common) type
9	ACYP2	acylphosphatase 2, muscle type
10	ALPI	alkaline phosphatase, intestinal
11	ALPL	alkaline phosphatase, liver/bone/kidney
12	ALPP	alkaline phosphatase, placental
13	ALPPL2	alkaline phosphatase, placental-like 2
14	ANP32A	acidic (leucine-rich) nuclear phosphoprotein 32 family, member A
15	ANP32B	acidic (leucine-rich) nuclear phosphoprotein 32 family, member B
16	ANP32C	acidic (leucine-rich) nuclear phosphoprotein 32 family, member C
17	ANP32D	acidic (leucine-rich) nuclear phosphoprotein 32 family, member D
18	ANP32E	acidic (leucine-rich) nuclear phosphoprotein 32 family, member E
19	ASNA1	arsA arsenite transporter, ATP-binding, homolog 1 (bacterial)
20	ATP6V0E1	ATPase, H+ transporting, lysosomal 9kDa, V0 subunit e1
21	ATP6V0E2	ATPase, H+ transporting V0 subunit e2
22	BPNT1	3'(2'), 5'-bisphosphate nucleotidase 1
23	C3orf48	chromosome 3 open reading frame 48
24	CABIN1	calcineurin binding protein 1
25	CANT1	calcium activated nucleotidase 1
26	CCDC104	coiled-coil domain containing 104
27	CCDC155	coiled-coil domain containing 155
28	CDC14A	CDC14 cell division cycle 14 homolog A (S. cerevisiae)
29	CDC14B	CDC14 cell division cycle 14 homolog B (S. cerevisiae)
30	CDC14C	CDC14 cell division cycle 14 homolog C (S. cerevisiae)
31	CDC25A	cell division cycle 25 homolog A (S. pombe)
32	CDC25B	cell division cycle 25 homolog B (S. pombe)
33	CDC25C	cell division cycle 25 homolog C (S. pombe)
34	CDKN3	cyclin-dependent kinase inhibitor 3
35	CHP1	calcineurin B homologous protein 1
36	CHP2	calcineurin B homologous protein 2
37	CHTF18	CTF18, chromosome transmission fidelity factor 18 homolog (S. cerevisiae)
38	CIB2	calcium and integrin binding family member 2
39	CIB3	calcium and integrin binding family member 3
40	CTDP1	CTD (carboxy-terminal domain, RNA polymerase II, polypeptide A)
41	CTDSP1	CTD (carboxy-terminal domain, RNA polymerase II, polypeptide A) small phosphatase 1
42	CTDSP2	CTD (carboxy-terminal domain, RNA polymerase II, polypeptide A) small phosphatase 2
43	CTDSPL	CTD (carboxy-terminal domain, RNA polymerase II, polypeptide A) small phosphatase-
44	DOLPP1	dolichyl pyrophosphate phosphatase 1
45	DUPD1	dual specificity phosphatase and pro isomerase domain containing 1
46	DUSP1	dual specificity phosphatase 1
47	DUSP10	dual specificity phosphatase 10
48	DUSP11	dual specificity phosphatase 11 (RNA/RNP complex 1-interacting)
49	DUSP12	dual specificity phosphatase 12
50	DUSP13	dual specificity phosphatase 13
51	DUSP14	dual specificity phosphatase 14
52	DUSP16	dual specificity phosphatase 16
53	DUSP18	dual specificity phosphatase 18
54	DUSP19	dual specificity phosphatase 19
55	DUSP2	dual specificity phosphatase 2
56	DUSP21	dual specificity phosphatase 21
57	DUSP22	dual specificity phosphatase 22

58	DUSP23	dual specificity phosphatase 23
59	DUSP26	dual specificity phosphatase 26 (putative)
60	DUSP28	dual specificity phosphatase 28
61	DUSP3	dual specificity phosphatase 3
62	DUSP4	dual specificity phosphatase 4
63	DUSP5	dual specificity phosphatase 5
64	DUSP6	dual specificity phosphatase 6
65	DUSP7	dual specificity phosphatase 7
66	DUSP8	dual specificity phosphatase 8
67	DUSP9	dual specificity phosphatase 9
68	DUT	deoxyuridine triphosphatase
69	ENOPH1	enolase-phosphatase 1
70	ENTPD1	ectonucleoside triphosphate diphosphohydrolase 1
71	ENTPD2	ectonucleoside triphosphate diphosphohydrolase 2
72	ENTPD3	ectonucleoside triphosphate diphosphohydrolase 3
73	ENTPD4	ectonucleoside triphosphate diphosphohydrolase 4
74	ENTPD5	ectonucleoside triphosphate diphosphohydrolase 5
75	ENTPD6	ectonucleoside triphosphate diphosphohydrolase 6 (putative function)
76	ENTPD7	ectonucleoside triphosphate diphosphohydrolase 7
77	ENTPD8	ectonucleoside triphosphate diphosphohydrolase 8
78	EPM2A	epilepsy, progressive myoclonus type 2A, Lafora disease (laforin)
79	FBP1	fructose-1,6-bisphosphatase 1
80	FBP2	fructose-1,6-bisphosphatase 2
81	FHIT	fragile histidine triad gene
82	FIG4	FIG4 homolog (S. cerevisiae)
83	G6PC	glucose-6-phosphatase, catalytic subunit
84	G6PC2	glucose-6-phosphatase, catalytic, 2
85	G6PC3	glucose 6 phosphatase, catalytic, 3
86	HDDC3	HD domain containing 3
87	HDHD1A	haloacid dehalogenase-like hydrolase domain containing 1A
88	HDHD2	haloacid dehalogenase-like hydrolase domain containing 2
89	HINT1	histidine triad nucleotide binding protein 1
90	HINT2	histidine triad nucleotide binding protein 2
91	ILKAP	integrin-linked kinase-associated serine/threonine phosphatase
92	IMPA1	inositol(myo)-1(or 4)-monophosphatase 1
93	IMPA2	inositol(myo)-1(or 4)-monophosphatase 2
94	IMPAD1	inositol monophosphatase domain containing 1
95	INPP1	inositol polyphosphate-1-phosphatase
96	INPP5A	inositol polyphosphate-5-phosphatase, 40kDa
97	INPP5B	inositol polyphosphate-5-phosphatase, 75kDa
98	INPP5D	inositol polyphosphate-5-phosphatase, 145kDa
99	INPP5E	inositol polyphosphate-5-phosphatase, 72 kDa
100	INPP5F	inositol polyphosphate-5-phosphatase F
101	INPP5J	inositol polyphosphate-5-phosphatase J
102	INPP5K	inositol polyphosphate-5-phosphatase K
103	INPPL1	inositol polyphosphate phosphatase-like 1
104	ITPA	inosine triphosphatase (nucleoside triphosphate pyrophosphatase)
105	KIAA1274	KIAA1274
106	LHPP	phospholysine phosphohistidine inorganic pyrophosphate phosphatase
107	LOC441868	similar to cSH-PTP2
108	LPPR1	lipid phosphate phosphatase-related protein type 1
109	LPPR2	lipid phosphate phosphatase-related protein type 2
110	LPPR3	lipid phosphate phosphatase-related protein type 3
111	LPPR5	lipid phosphate phosphatase-related protein type 5
112	LRGUK	leucine-rich repeats and guanylate kinase domain containing
113	MFN1	mitofusin 1
114	MFN2	mitofusin 2
115	MINPP1	multiple inositol polyphosphate histidine phosphatase, 1
116	MTM1	myotubularin 1
117	MTMR1	myotubularin related protein 1
118	MTMR10	myotubularin related protein 10

119	MTMR11	myotubularin related protein 11
120	MTMR12	myotubularin related protein 12
121	MTMR14	myotubularin related protein 14
122	MTMR2	myotubularin related protein 2
123	MTMR3	myotubularin related protein 3
124	MTMR4	myotubularin related protein 4
125	MTMR6	myotubularin related protein 6
126	MTMR7	myotubularin related protein 7
127	MTMR8	myotubularin related protein 8
128	MTMR9	myotubularin related protein 9
129	NAP1L1	nucleosome assembly protein 1-like 1
130	NAP1L2	nucleosome assembly protein 1-like 2
131	NAP1L3	nucleosome assembly protein 1-like 3
132	NAP1L4	nucleosome assembly protein 1-like 4
133	NAP1L5	nucleosome assembly protein 1-like 5
134	NT5C2	5'-nucleotidase, cytosolic II
135	NT5E	5'-nucleotidase, ecto (CD73)
136	NUDT10	nudix (nucleoside diphosphate linked moiety X)-type motif 10
137	NUDT11	nudix (nucleoside diphosphate linked moiety X)-type motif 11
138	NUDT14	nudix (nucleoside diphosphate linked moiety X)-type motif 14
139	NUDT3	nudix (nucleoside diphosphate linked moiety X)-type motif 3
140	NUDT4	nudix (nucleoside diphosphate linked moiety X)-type motif 4
141	NUDT5	nudix (nucleoside diphosphate linked moiety X)-type motif 5
142	NUDT6	nudix (nucleoside diphosphate linked moiety X)-type motif 6
143	NUDT8	nudix (nucleoside diphosphate linked moiety X)-type motif 8
144	OCRL	oculocerebrorenal syndrome of Lowe
145	PAPL	purple acid phosphatase long form
146	PDP2	pyruvate dehyrogenase phosphatase catalytic subunit 2
147	PDXP	pyridoxal (pyridoxine, vitamin B6) phosphatase
148	PFKFB1	6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 1
149	PFKFB2	6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 2
150	PFKFB3	6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3
151	PFKFB4	6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 4
152	PGP	phosphoglycolate phosphatase
153	PHACTR1	phosphatase and actin regulator 1
154	PHACTR2	phosphatase and actin regulator 2
155	PHACTR3	phosphatase and actin regulator 3
156	PHACTR4	phosphatase and actin regulator 4
157	PNKP	polynucleotide kinase 3'-phosphatase
158	PPA1	pyrophosphatase (inorganic) 1
159	PPA2	pyrophosphatase (inorganic) 2
160	PPAP2A	phosphatidic acid phosphatase type 2A
161	PPAP2B	phosphatidic acid phosphatase type 2B
162	PPAP2C	phosphatidic acid phosphatase type 2C
163	PPAPDC1A	phosphatidic acid phosphatase type 2 domain containing 1A
164	PPAPDC1B	phosphatidic acid phosphatase type 2 domain containing 1B
165	PPAPDC2	phosphatidic acid phosphatase type 2 domain containing 2
166	PPAPDC3	phosphatidic acid phosphatase type 2 domain containing 3
167	PPEF1	protein phosphatase, EF-hand calcium binding domain 1
168	PPEF2	protein phosphatase, EF-hand calcium binding domain 2
169	PPM1A	protein phosphatase 1A (formerly 2C), magnesium-dependent, alpha isoform
170	PPM1B	protein phosphatase, Mg2+/Mn2+ dependent, 1B
171	PPM1D	protein phosphatase, Mg2+/Mn2+ dependent, 1D
172	PPM1E	protein phosphatase, Mg2+/Mn2+ dependent, 1E
173	PPM1F	protein phosphatase, Mg2+/Mn2+ dependent, 1F
174	PPM1G	protein phosphatase, Mg2+/Mn2+ dependent, 1G
175	PPM1H	protein phosphatase 1H (PP2C domain containing)
176	PPM1J	protein phosphatase, Mg2+/Mn2+ dependent, 1J
177	PPM1K	protein phosphatase 1K (PP2C domain containing)
178	PPM1L	protein phosphatase, Mg2+/Mn2+ dependent, 1L
179	PPM1M	protein phosphatase 1M (PP2C domain containing)

180	PPM1N	protein phosphatase, Mg2+/Mn2+ dependent, 1N (putative)
181	PPM2C	protein phosphatase 2C, magnesium-dependent, catalytic subunit
182	PPP1CA	protein phosphatase 1, catalytic subunit, alpha isozyme
183	PPP1CB	protein phosphatase 1, catalytic subunit, beta isozyme
184	PPP1CC	protein phosphatase 1, catalytic subunit, gamma isoform
185	PPP1R11	protein phosphatase 1, regulatory (inhibitor) subunit 11
186	PPP1R12A	protein phosphatase 1, regulatory (inhibitor) subunit 12A
187	PPP1R12B	protein phosphatase 1, regulatory (inhibitor) subunit 12B
188	PPP1R12C	protein phosphatase 1, regulatory (inhibitor) subunit 12C
189	PPP1R14A	protein phosphatase 1, regulatory (inhibitor) subunit 14A
190	PPP1R14B	protein phosphatase 1, regulatory (inhibitor) subunit 14B
191	PPP1R14C	protein phosphatase 1, regulatory (inhibitor) subunit 14C
192	PPP1R14D	protein phosphatase 1, regulatory (inhibitor) subunit 14D
193	PPP1R16A	protein phosphatase 1, regulatory (inhibitor) subunit 16A
194	PPP1R16B	protein phosphatase 1, regulatory (inhibitor) subunit 16B
195	PPP1R1A	protein phosphatase 1, regulatory (inhibitor) subunit 1A
196	PPP1R1B	protein phosphatase 1, regulatory (inhibitor) subunit 1B
197	PPP1R1C	protein phosphatase 1, regulatory (inhibitor) subunit 1C
198	PPP1R2	protein phosphatase 1, regulatory (inhibitor) subunit 2
199	PPP1R3A	protein phosphatase 1, regulatory (inhibitor) subunit 3A
200	PPP1R3B	protein phosphatase 1, regulatory (inhibitor) subunit 3B
201	PPP1R3C	protein phosphatase 1, regulatory (inhibitor) subunit 3C
202	PPP1R3D	protein phosphatase 1, regulatory (inhibitor) subunit 3D
203	PPP1R3F	protein phosphatase 1, regulatory (inhibitor) subunit 3F
204	PPP1R7	protein phosphatase 1, regulatory (inhibitor) subunit 7
205	PPP1R8	protein phosphatase 1, regulatory (inhibitor) subunit 8
206	PPP2CA	protein phosphatase 2, catalytic subunit, alpha isozyme
207	PPP2CB	protein phosphatase 2, catalytic subunit, beta isozyme
208	PPP2R1A	protein phosphatase 2 (formerly 2A), regulatory subunit A, alpha isoform
209	PPP2R1B	protein phosphatase 2, regulatory subunit A, beta
210	PPP2R2A	protein phosphatase 2 (formerly 2A), regulatory subunit B, alpha isoform
211	PPP2R2B	protein phosphatase 2, regulatory subunit B, beta
212	PPP2R2C	protein phosphatase 2, regulatory subunit B, gamma
213	PPP2R2D	protein phosphatase 2, regulatory subunit B, delta isoform
214	PPP2R3A	protein phosphatase 2 (formerly 2A), regulatory subunit B'', alpha
215	PPP2R3B	protein phosphatase 2 (formerly 2A), regulatory subunit B'', beta
216	PPP2R3C	protein phosphatase 2, regulatory subunit B'', gamma
217	PPP2R4	protein phosphatase 2A activator, regulatory subunit 4
218	PPP2R5A	protein phosphatase 2, regulatory subunit B', alpha isoform
219	PPP2R5B	protein phosphatase 2, regulatory subunit B', beta isoform
220	PPP2R5C	protein phosphatase 2, regulatory subunit B', gamma isoform
221	PPP2R5D	protein phosphatase 2, regulatory subunit B', delta
222	PPP2R5E	protein phosphatase 2, regulatory subunit B', epsilon isoform
223	PPP3CA	protein phosphatase 3 (formerly 2B), catalytic subunit, alpha isoform
224	PPP3CB	protein phosphatase 3 (formerly 2B), catalytic subunit, beta isoform
225	PPP3CC	protein phosphatase 3, catalytic subunit, gamma isozyme
226	PPP3R1	protein phosphatase 3, regulatory subunit B, alpha
227	PPP3R2	protein phosphatase 3, regulatory subunit B, beta
228	PPP4C	protein phosphatase 4, catalytic subunit
229	PPP4R1	protein phosphatase 4, regulatory subunit 1
230	PPP5C	protein phosphatase 5, catalytic subunit
231	PPP6C	protein phosphatase 6, catalytic subunit
232	PPP6R2	protein phosphatase 6, regulatory subunit 2
233	PPTC7	PTC7 protein phosphatase homolog (S. cerevisiae)
234	PRG-1	plasticity related gene 1
235	PSPH	phosphoserine phosphatase
236	PTEN	phosphatase and tensin homolog
237	PTP4A1	protein tyrosine phosphatase type IVA, member 1
238	PTP4A2	protein tyrosine phosphatase type IVA, member 2
239	PTP4A3	protein tyrosine phosphatase type IVA, member 3
240	PTPDC1	protein tyrosine phosphatase domain containing 1

241	PTPN1	protein tyrosine phosphatase, non-receptor type 1
242	PTPN11	protein tyrosine phosphatase, non-receptor type 11
243	PTPN12	protein tyrosine phosphatase, non-receptor type 12
244	PTPN13	protein tyrosine phosphatase, non-receptor type 13
245	PTPN14	protein tyrosine phosphatase, non-receptor type 14
246	PTPN18	protein tyrosine phosphatase, non-receptor type 18 (brain-derived)
247	PTPN2	protein tyrosine phosphatase, non-receptor type 2
248	PTPN20A	protein tyrosine phosphatase, non-receptor type 20A
249	PTPN20B	protein tyrosine phosphatase, non-receptor type 20B
250	PTPN21	protein tyrosine phosphatase, non-receptor type 21
251	PTPN22	protein tyrosine phosphatase, non-receptor type 22 (lymphoid)
252	PTPN23	protein tyrosine phosphatase, non-receptor type 23
253	PTPN3	protein tyrosine phosphatase, non-receptor type 3
254	PTPN5	protein tyrosine phosphatase, non-receptor type 5 (striatum-enriched)
255	PTPN6	protein tyrosine phosphatase, non-receptor type 6
256	PTPN7	protein tyrosine phosphatase, non-receptor type 7
257	PTPN9	protein tyrosine phosphatase, non-receptor type 9
258	PTPRA	protein tyrosine phosphatase, receptor type, A
259	PTPRC	protein tyrosine phosphatase, receptor type, C
260	PTPRD	protein tyrosine phosphatase, receptor type, D
261	PTPRE	protein tyrosine phosphatase, receptor type, E
262	PTPRF	protein tyrosine phosphatase, receptor type, F
263	PTPRG	protein tyrosine phosphatase, receptor type, G
264	PTPRH	protein tyrosine phosphatase, receptor type, H
265	PTPRJ	protein tyrosine phosphatase, receptor type, J
266	PTPRK	protein tyrosine phosphatase, receptor type, K
267	PTPRM	protein tyrosine phosphatase, receptor type, M
268	PTPRN	protein tyrosine phosphatase, receptor type, N
269	PTPRN2	protein tyrosine phosphatase, receptor type, N polypeptide 2
270	PTPRO	protein tyrosine phosphatase, receptor type, O
271	PTPRR	protein tyrosine phosphatase, receptor type, R
272	PTPRS	protein tyrosine phosphatase, receptor type, S
273	PTPRT	protein tyrosine phosphatase, receptor type, T
274	PTPRU	protein tyrosine phosphatase, receptor type, U
275	PTPRZ1	protein tyrosine phosphatase, receptor-type, Z polypeptide 1
276	RNGTT	RNA guanylyltransferase and 5'-phosphatase
277	RWDD2A	RWD domain containing 2A
278	RWDD2B	RWD domain containing 2B
279	SACM1L	SAC1 suppressor of actin mutations 1-like (yeast)
280	SAPS1	SAPS domain family, member 1
281	SAPS3	SAPS domain family, member 3
282	SBF1	SET binding factor 1
283	SBF2	SET binding factor 2
284	SET	SET nuclear oncogene
285	SGPP1	sphingosine-1-phosphate phosphatase 1
286	SGPP2	sphingosine-1-phosphate phosphatase 2
287	SSH1	slingshot homolog 1 (Drosophila)
288	SSH2	slingshot homolog 2 (Drosophila)
289	SSH3	slingshot homolog 3 (Drosophila)
290	STYXL1	serine/threonine/tyrosine interacting-like 1
291	SYNJ1	synaptojanin 1
292	SYNJ2	synaptojanin 2
293	TNS1	tensin 1
294	TNS3	tensin 3
295	TPTE	transmembrane phosphatase with tensin homology
296	TPTE2	transmembrane phosphoinositide 3-phosphatase and tensin homolog 2
297		similar to template acyivating factor-I alpha
298		similar to mitofusin 1

Chapter 4 – Pharmacological LRRK2 kinase inhibition induces LRRK2 protein destabilization and proteasomal degradation

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Scientific acknowledgement & personal contribution

Evy Lobbestael, Jean-Marc Taymans and Veerle Baekelandt designed the research. Evy Lobbestael and I performed the research for Figures 4.1, 4.2 and 4.3, analyzed the data and made the final figures. More specifically, together with Evy Lobbestael, Stephanie Deman and Sophie Croes, I performed the experiments in which the effect of different LRRK2 kinase inhibitors on LRRK2 protein stability of several LRRK2 mutants was tested in cell culture (Figure 4.1). I optimized and performed the experiments in which the effect of chloroquine on LRRK2 protein stability was tested (Figure 4.2.c). Next, I optimized and executed the *in vivo* experiments (Figure 4.3). Laura Civiero and Elisa Greggio designed, performed and analyzed the research on primary astrocytes (Figure 4.4). Evy Lobbestael and Veerle Baekelandt wrote the paper. All authors read and approved the paper**.** We thank Stephanie Deman, Sophie Croes and Laura Carrera Carballes for technical assistance and the Leuven Viral Vector Core for the production of lentiviral vectors (https://gbiomed.kuleuven.be/english/ research/50000715/laboratory-of-molecular-virology-and-gene-therapy/lvvc). This work was supported by the MJFox Foundation and the IWT SBO/100042 project.

Conflict of interest statements

The authors declare no competing financial interests.

1. Abstract

Leucine-rich repeat kinase 2 (LRRK2) kinase activity is increased in several pathogenic mutations, including the most common mutation, G2019S, and is known to play a role in Parkinson's disease (PD) pathobiology. This has stimulated the development of potent, selective LRRK2 kinase inhibitors as one of the most prevailing disease-modifying therapeutic PD strategies. Although several lines of evidence support beneficial effects of LRRK2 kinase inhibitors, many questions need to be answered before clinical applications can be envisaged. Using six different LRRK2 kinase inhibitors, we show that LRRK2 kinase inhibition induces LRRK2 dephosphorylation and can reduce LRRK2 protein levels of overexpressed wild type and G2019S, but not A2016T or K1906M, LRRK2 as well as endogenous LRRK2 in mouse brain, lung and kidney. The inhibitor-induced reduction in LRRK2 levels could be reversed by proteasomal inhibition, but not by lysosomal inhibition, while mRNA levels remained unaffected. In addition, using LRRK2 S910A and S935A phosphorylation mutants, we show that dephosphorylation of these sites is not required for LRRK2 degradation. Increasing our insight in the

molecular and cellular consequences of LRRK2 kinase inhibition will be crucial in the further development of LRRK2-based PD therapies.

2. Introduction

Leucine-rich repeat kinase 2 (LRRK2) kinase inhibition is currently one of the prevailing disease-modifying therapeutic strategies for Parkinson's disease (PD)[312]. LRRK2 is a very attractive target since pathogenic LRRK2 mutations are a common cause of inherited forms of PD[313,314] and genetic variations in the LRRK2 locus are associated with an increased risk to develop sporadic PD[55,68–70]. The most common pathogenic mutation, G2019S, increases kinase activity[135,136,315], which plays a crucial role in mutant LRRK2-induced toxicity[74,142,144] and can be reversed by LRRK2 kinase inhibition[144,145,151,315]. This has stimulated academic and industrial efforts on the development of potent and selective LRRK2 kinase inhibitors[152,153].

LRRK2 is phosphorylated at multiple serines including S910, S935, S955 and S973[96,134,154]. Although these sites are most likely phosphorylated by other kinases[96,134,154,155,162,168,170,173,174,195], the LRRK2 kinase domain appears to play a regulatory role in this phosphorylation event since all LRRK2 kinase inhibitors also induce LRRK2 dephosphorylation at S935[161,165,195,316]. Therefore, LRRK2 dephosphorylation at S935 is widely used as a surrogate readout for LRRK2 kinase inhibition in a cellular context[161,165,166,171]. Before clinical applications can be envisaged, more insight in the molecular and cellular consequences of LRRK2 kinase inhibition will be needed since there might be (side) effects we do not fully understand to date. We have previously shown that LRRK2 kinase inhibition induces PP1-mediated LRRK2 dephosphorylation[163]. Although not proven to be a pathogenic mechanism, the fact that PP1-mediated dephosphorylation is also observed in most pathogenic mutants[134,163,195,317], calls for caution when considering LRRK2 kinase inhibitors in the clinic. In addition, LRRK2 kinase inhibition can induce LRRK2 ubiquitination[184] and a reduction of protein levels[184,198,203,205], which may explain the cellular changes in the lung of non-human primates[205] or mice[204] treated with LRRK2 kinase inhibitor, given the close resemblance with the lung phenotype observed in LRRK2 knockout animals[203,205,211,318]. One of the important outstanding questions is whether this reduction of LRRK2 protein level is purely an unwanted effect or whether this might (partially) account for the beneficial effects of LRRK2 kinase inhibition. LRRK2 ablation was shown to protect against α-synuclein- and LPS-induced toxicity[228,233] and a recent study postulated that reduced LRRK2 protein levels, rather than kinase inhibition, explains the beneficial effects on LRRK2-induced toxicity[198]. Together, these findings underline the importance of understanding LRRK2 kinase inhibitor-induced dephosphorylation and destabilization as a crucial step in the development of LRRK2 kinase inhibition as a PD therapy.

Given the fast LRRK2 dephosphorylation after LRRK2 kinase inhibition[161], most published reports use kinase inhibitor treatment for a short period of time (minutes to hours). Here, we aimed to assess the effects of sustained LRRK2 kinase inhibition (hours to days) on heterologous LRRK2 phosphorylation sites and protein stability as well as their relationship using phosphorylation mutants. With a view on therapeutic applications, we examined inhibition effects in neuronal and non-neuronal cells using wild type (WT) and pathogenic LRRK2 and different LRRK2 kinase inhibitors.

3. Results and Discussion

3.1. Pharmacological LRRK2 kinase inhibition reduces LRRK2 protein levels in overexpressing cells

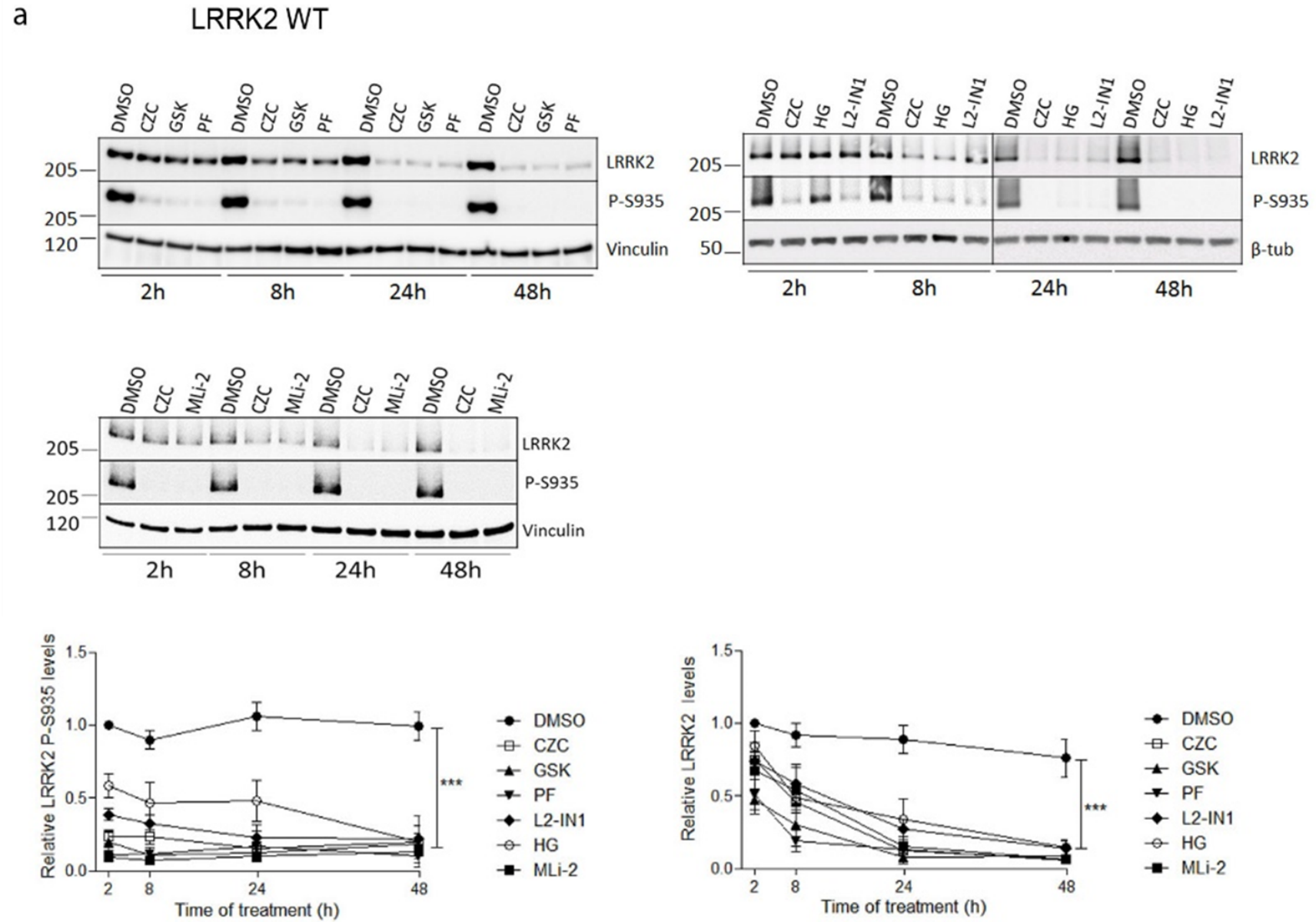
To investigate the effects of pharmacological LRRK2 kinase inhibition on LRRK2, SH-SY5Y cells with stable lentiviral vector-mediated overexpression of LRRK2 were treated with six different LRRK2 kinase inhibitors: MLi-2[204], PF-06447475[206], GSK2578215A[319], LRRK2-IN1[164], HG 10-102-01[316] and CZC-25146[320] (for more information see [312]). As expected, treatment of cells induced a rapid dephosphorylation at S935. In addition, treatment with each of the inhibitors resulted in a gradual decrease in LRRK2 protein levels, starting from 8 h of treatment (Figure 4.1.a).

The observation that six different LRRK2 kinase inhibitors, each with different selectivity profiles, reduce LRRK2 protein levels, points to a LRRK2-specific effect. This was further corroborated by the lack of LRRK2 dephosphorylation and protein reduction after treatment of the inhibitor insensitive mutant A2016T[158,164,319,321] with LRRK2-IN1, CZC-25146 or HG 10-102-01 (Figure 4.1.b). Interestingly, a trend towards LRRK2 protein destabilization was observed for the HG 10-102-01 compound, which is reported to show strongly reduced activity against LRRK2 A2016T[316]. In addition, also the kinase dead LRRK2 variant K1906M, which was reported to display reduced sensitivity to several LRRK2 kinase inhibitors[162], did not display S935 dephosphorylation and destabilization upon inhibitor treatment (Figure 4.1.c).

To examine whether the inhibitor-induced destabilization also holds true for the most common pathogenic mutation G2019S, we performed the same experiment in SH-SY5Y cells stably overexpressing LRRK2 G2019S. As for LRRK2 WT, LRRK2 S935 dephosphorylation was observed starting from the 2 h time point, while LRRK2 levels were decreased from 8 h treatment on (Figure 4.1.d).

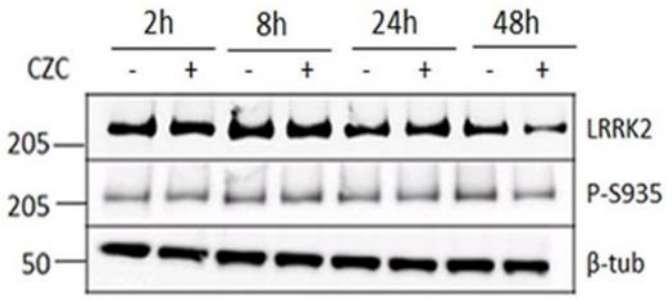
Our results show that LRRK2 kinase inhibition induces LRRK2 dephosphorylation at S935 prior to a reduction in LRRK2 levels. To examine whether dephosphorylation of the heterologous LRRK2 phosphorylation sites S910 and S935 is crucial for the reduction of LRRK2 levels, we treated SH-SY5Y cells with stable overexpression of the LRRK2 phosphorylation mutants S910A or S935A (Figure 4.1.e,f). Interestingly, inhibitor-induced destabilization also occurred in these mutants that cannot be further dephosphorylated at S910 and S935, which indicates that LRRK2 dephosphorylation at S910 and S935 is not required for the reduction of LRRK2 levels. This is in line with the findings of Zhao et al.[184] showing increased ubiquitination of the double S910A/S935A mutant upon inhibitor treatment. Future studies will need to elucidate whether LRRK2 dephosphorylation at other (auto)phosphorylation sites induces the reduction in protein levels or whether other factors such as conformational changes induced by binding of the inhibitor or the LRRK2 kinase inhibition itself, regulate LRRK2 protein levels.

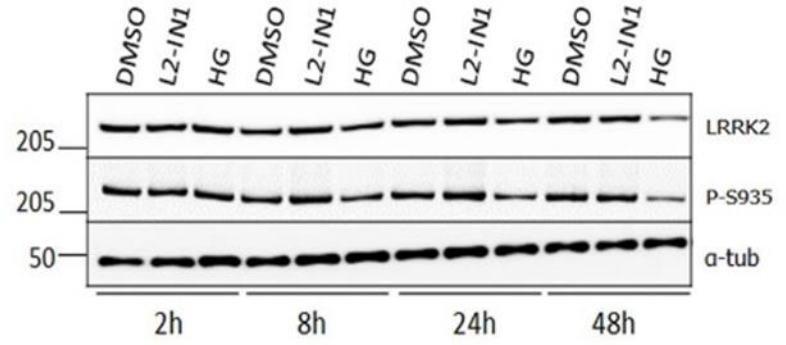
a

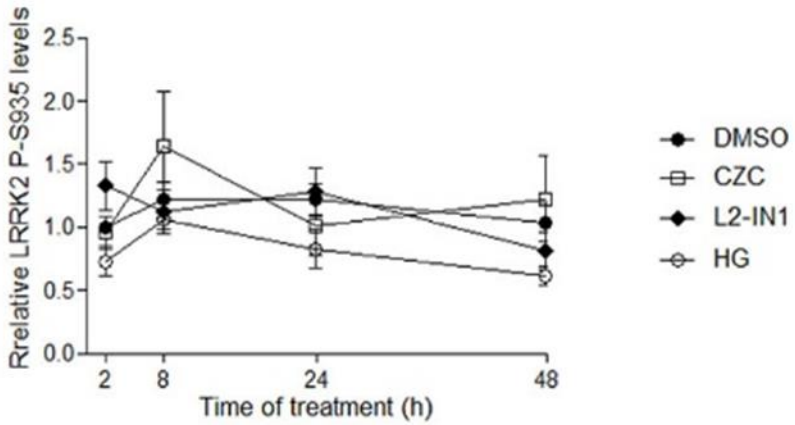

LRRK2 WT
DMSO
CZC
GSK
PF
HG
L2-IN1
MLi-2
205
120
50
LRRK2
P-S935
Vinculin
β-tub
2h
8h
24h
48h
Relative LRRK2 P-S935 levels
Relative LRRK2 levels
Time of treatment (h)
***

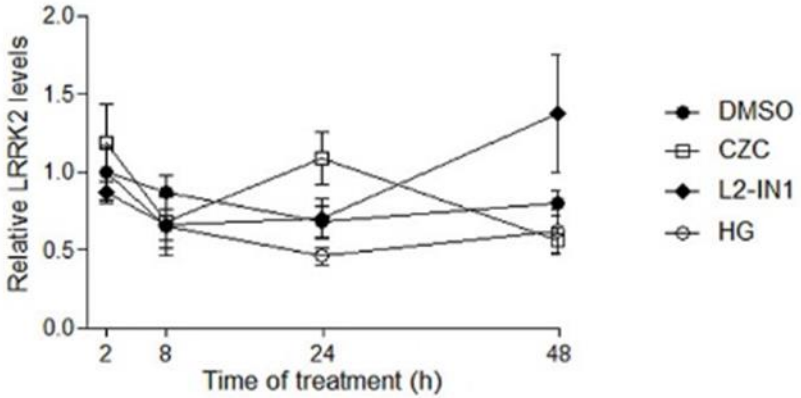
b

LRRK2 A2016T









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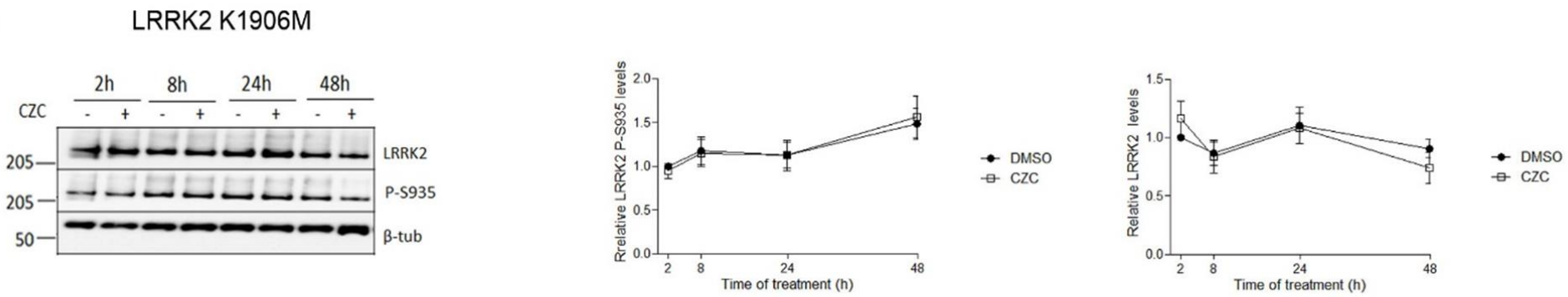
LRRK2 K1906M
2h
8h
24h
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CZC
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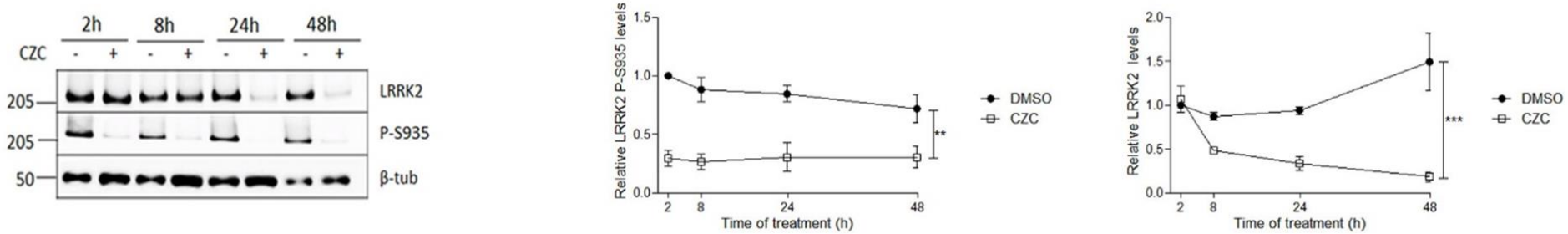
205
205
50
LRRK2
P-S935
β-tub
Rrelative LRRK2 P-S935 levels
Time of treatment (h)
DMSO
CZC
Relative LRRK2 levels
Time of treatment (h)
DMSO
CZC

d


LRRK2 G2019S
2h
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205
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LRRK2
P-S935
β-tub
Relative LRRK2 P-S935 levels
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Time of treatment (h)
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Relative LRRK2 levels
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Time of treatment (h)
DMSO
CZC

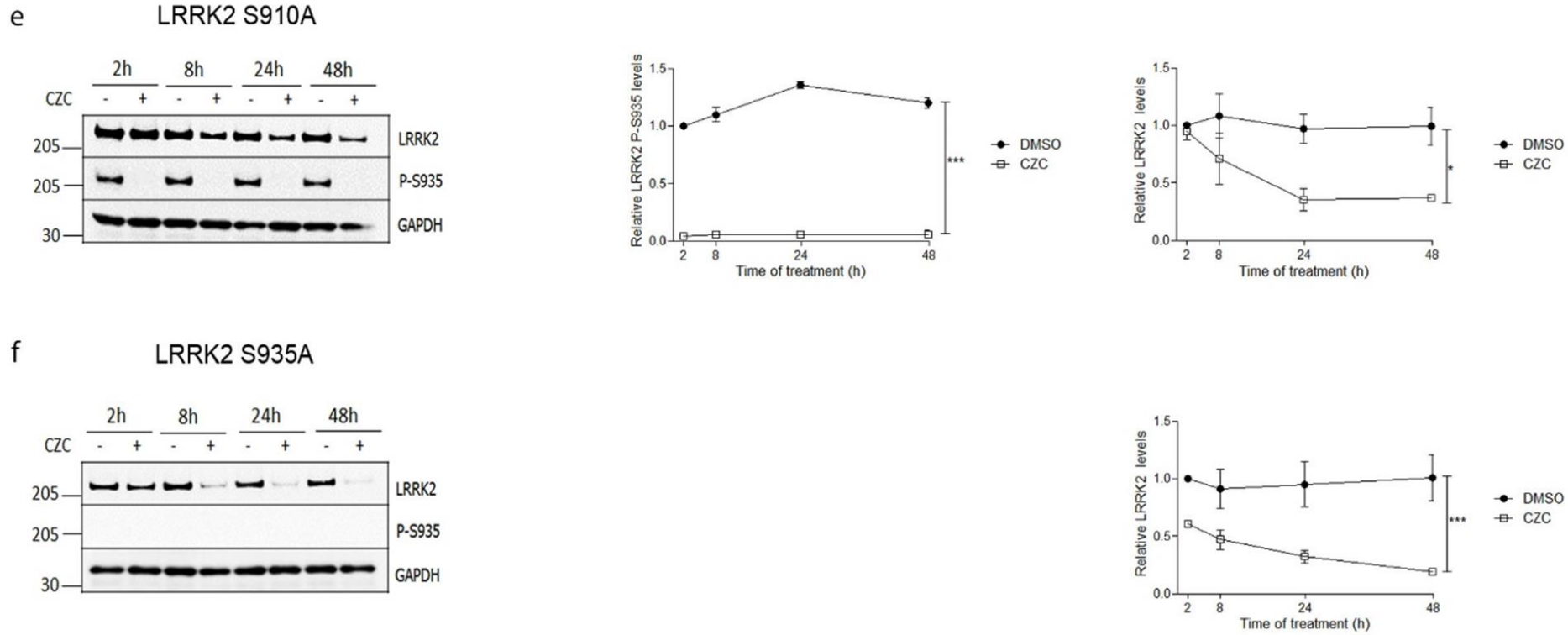


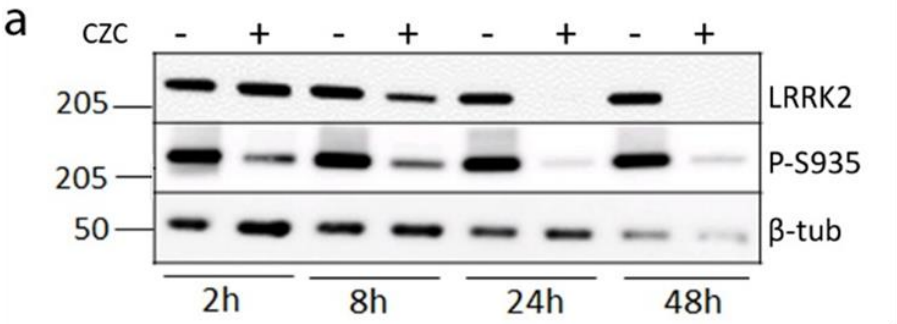
Figure 4.1. – LRRK2 kinase inhibition reduces LRRK2 protein levels. SH-SY5Y overexpressing 3flag-LRRK2 WT (**a**) A2016T (**b**) K1906M (**c**) G2019S (**d**) S910A (**e**) or S935A (**f**) were treated according to different time schedules with LRRK2-IN1 (L2-IN1, 1 µM), CZC-25146 (CZC, 200 nM), PF-06447475 (PF, 150 nM), GSK2578215A (GSK, 1 µM), MLi-2 (10 nM) or HG 10-102-01 (HG, 1 µM) or DMSO. Cell lysates were analyzed with immunoblotting using FlagM2 antibody for LRRK2 detection, anti-LRRK2 P-S935 and anti-α - or β -tubulin, anti-GAPDH or anti-vinculin for equal loading. Shown are representative blots. Graphs show the quantification of blots representing the ratio of phosphorylation at S935 over total LRRK2 signal or total LRRK2 over housekeeping protein signal. Error bars indicate s.e.m. with N ≥ 3. Statistical significance was tested using a two-way ANOVA test with Bonferroni post-tests. ***p < 0.001, **p < 0.01, *p < 0.05.

3.2. Reduced LRRK2 protein levels result from increased proteasomal protein degradation

Next, we set out to gain mechanistic insight in the LRRK2 kinase inhibitor-induced reduction of LRRK2 protein levels. SH-SY5Y cells, overexpressing LRRK2, were treated for different periods of time with CZC-25146 followed by total RNA isolation and QPCR analysis. In parallel, cells were treated with inhibitor for subsequent immunoblotting analysis to confirm LRRK2 dephosphorylation and protein level reduction. QPCR analysis revealed that, although LRRK2 protein levels were dramatically decreased, LRRK2 mRNA levels remained constant during the entire experiment (Figure 4.2.a), pointing to a regulation at the protein level rather than a transcriptional event. Since WT LRRK2 has been mainly reported to be degraded by the ubiquitin proteasome system[133,208,209,322], we treated LRRK2 overexpressing cells with LRRK2 kinase inhibitor together with the proteasomal inhibitor, MG132. Although LRRK2 kinase inhibition still induced LRRK2 dephosphorylation, no decrease in LRRK2 protein levels was observed when proteasomal activity was blocked (Figure 4.2.b). Conversely, chloroquine-mediated lysosomal inhibition was not able to rescue the LRRK2 kinase inhibitor-induced LRRK2 protein destabilization (Figure 4.2.c). Together, these findings strongly suggest that the inhibitor-induced reduction of LRRK2 protein levels can be mainly explained by proteasome-mediated LRRK2 degradation. This is in line with a recent report showing increased LRRK2 ubiquitination and degradation after LRRK2 kinase inhibition[184].

3.3. LRRK2 kinase inhibition reduces endogenous LRRK2 protein levels *in vivo*

To further investigate the *in vivo* relevance of our findings, we examined the effect of LRRK2 kinase inhibition on endogenous LRRK2 in mouse. Mice were injected four times with 10 mg/kg of the highly potent and selective LRRK2 kinase inhibitor MLi-2 or with DMSO. A significant decrease in LRRK2 phosphorylation at S935 and total protein levels was observed in mouse brain (Figure 4.3.). Given the reported abnormalities in LRRK2 KO mice[203,205,291,292] and in LRRK2 kinase inhibitor-treated animals[204,205], we also analyzed LRRK2 levels in lung and kidney of mice injected with MLi-2. As in brain, a significant decrease in P-S935 as well as total LRRK2 protein levels was observed in lung and kidney (Figure 4.3.). A reduction of LRRK2 protein levels in lung and kidney of mice was also reported after in-diet dosing with MLi-2, but no effect on LRRK2 levels in mouse cortex could be observed[204]. Still, a reduction in striatal LRRK2 protein levels has been reported in non-human primates treated with GNE-087756. It will be interesting to further investigate whether this discrepancy can be explained by differences between brain regions, or rather to technical aspects such as buffer composition. Interestingly, in contrast to full length LRRK2, protein stability of the truncated form of LRRK2 in kidney (~170 kDa) was not affected upon LRRK2 kinase inhibition, although S935 dephosphorylation was evident (Figure 4.3.). This is in line with the reported reduction in full length, but not the truncated, LRRK2 protein levels in the kidney of mice treated with a Novartis LRRK2 kinase inhibitor[203].


a
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LRRK2
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β-tub
2h
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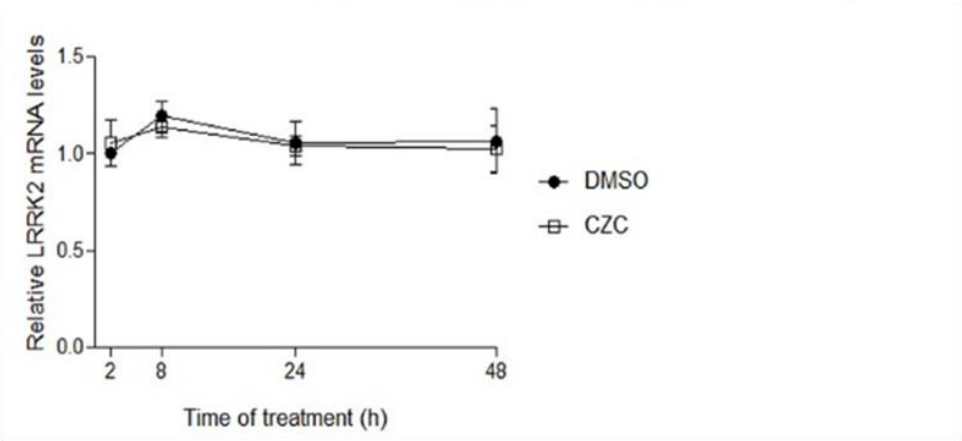
Relative LRRK2 mRNA levels
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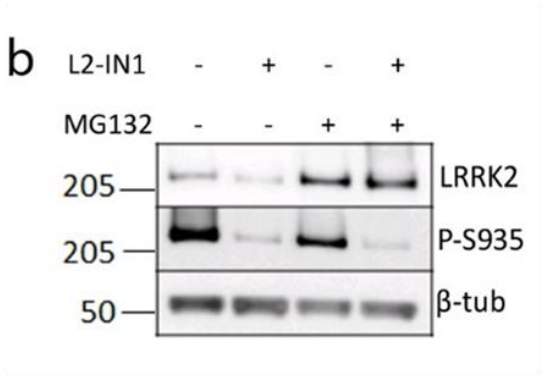
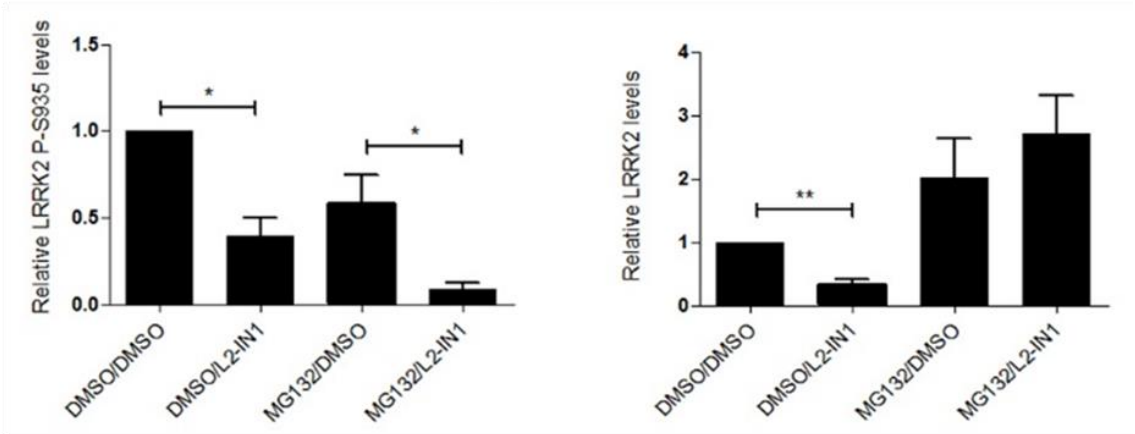
1.0
0.5
0.0
2
8
24
48
Time of treatment (h)
DMSO
CZC


b
L2-IN1 - + - +
MG132 - - + +
205
LRRK2
205
P-S935
50
β-tub


Relative LRRK2 P-S935 levels
1.5
1.0
0.5
0.0
*
*
DMSO/DMSO
DMSO/L2-IN1
MG132/DMSO
MG132/L2-IN1
Relative LRRK2 levels
4
3
2
1
0
**
DMSO/DMSO
DMSO/L2-IN1
MG132/DMSO
MG132/L2-IN1

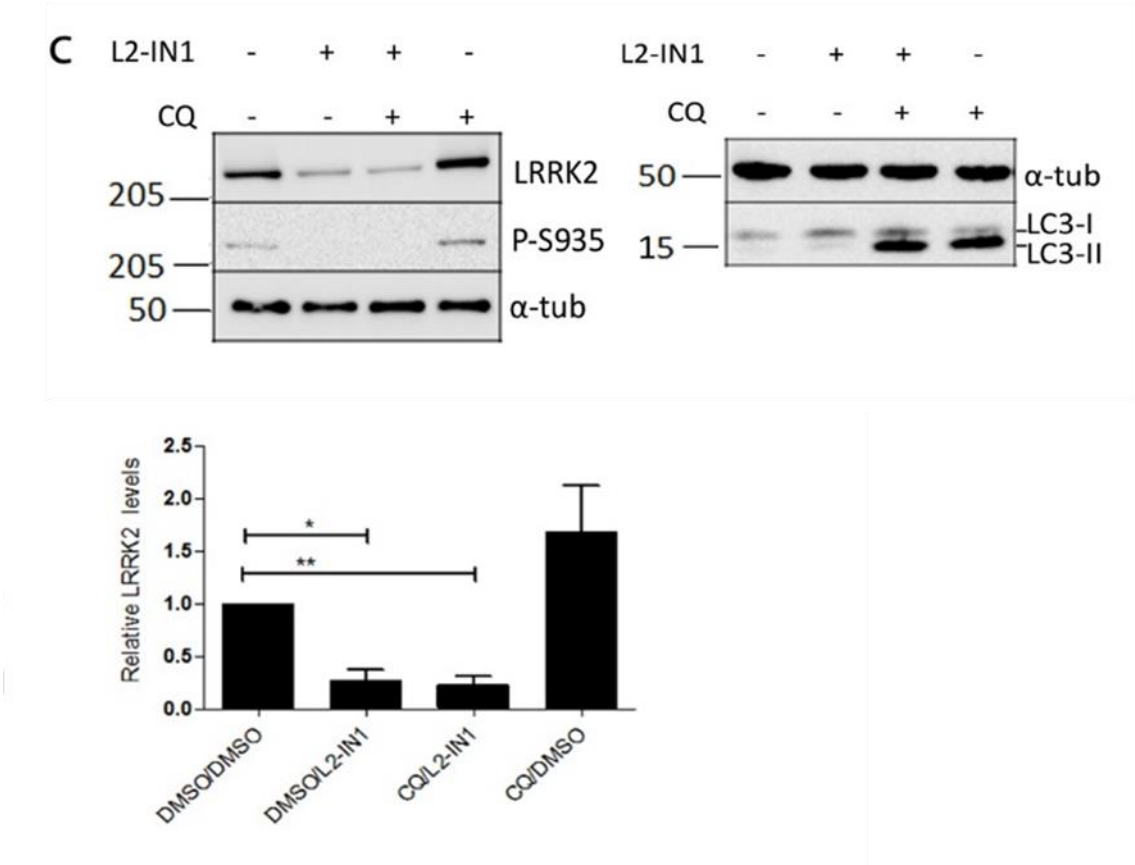


Figure 4.2. - LRRK2 kinase inhibitor-induced reduction of LRRK2 protein levels is caused by proteasomal degradation. (**a**) QPCR analysis was performed on mRNA derived from SH-SY5Y cells overexpressing 3flag- LRRK2 WT, treated with CZC-25146 (CZC, 200 nM) or DMSO for different periods of time. Immunoblotting analysis was performed on cells cultured and treated in parallel with cells for QPCR analysis, using FlagM2 antibody for LRRK2 detection, anti-LRRK2 P-S935 and anti-β -tubulin for equal loading. Shown are representative blots. Quantification of LRRK2 mRNA shows LRRK2 mRNA levels normalized to β -actin mRNA levels with error bars indicating s.e.m., N = 3. (**b**) SH-SY5Y cells overexpressing 3flag-LRRK2 WT were treated for 17 h with LRRK2-IN1 (L2-IN1, 1 μM) and/or MG132 (1 μM), DMSO was used as negative control. Cell lysates were analyzed with immunoblotting using FlagM2 antibody for LRRK2 detection, anti-LRRK2 P-S935 and anti-β -tubulin for equal loading, N ≥ 4. Statistical significance was assessed by column statistics (one-sample t-test) or a nonparametric t-test (Mann-Whitney). **$p < 0.01$; *$p < 0.05$. (**c**) SH-SY5Y cells overexpressing 3flag-LRRK2 WT were treated for 48 h with LRRK2-IN1 (L2-IN1, 1 μM) and/or chloroquine (CQ) (10 μM), DMSO was used as negative control. Cell lysates were analyzed with immunoblotting using FlagM2 antibody for LRRK2 detection, anti-LRRK2 P-S935, anti-LC3 antibody and anti-α tubulin for equal loading, N ≥ 4. Statistical significance was assessed by column statistics (one-sample t-test) with Bonferroni correction or a nonparametric t-test (Mann-Whitney). **$p < 0.01$; *$p < 0.05$.

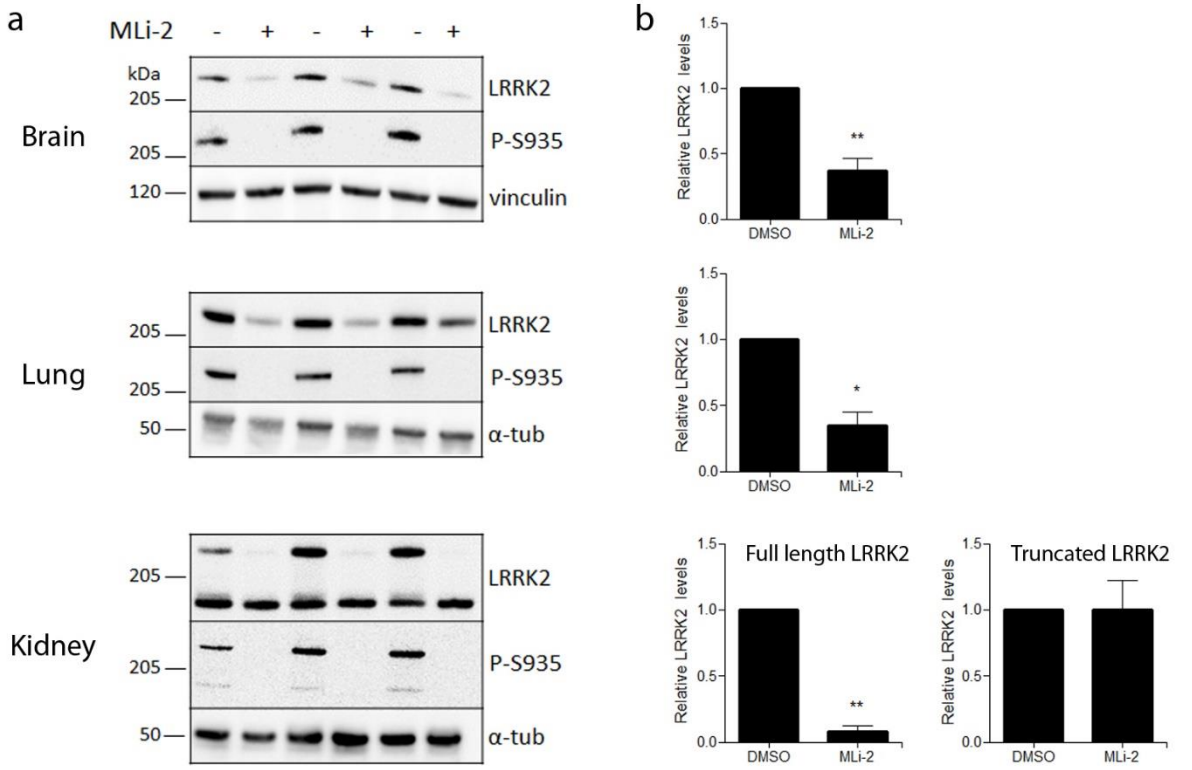


Figure 4.3. - LRRK2 kinase inhibition in vivo induces LRRK2 protein destabilization in brain, lung and kidney. C57BL/6J mice received four intraperitoneal injections with 10 mg/kg MLi-2 or DMSO over 30 h. Brain, lung and kidney extracts were taken 2 h after the last injection and analyzed with immunoblotting using MJFF-2 anti-LRRK2 antibody, anti-LRRK2 P-S935 and anti-vinculin or α -tubulin for equal loading. Shown are representative blots with each lane representing a separate animal. Quantification of the blots is represented as a histogram and shows the ratio of total LRRK2 over vinculin or α -tubulin signal, error bars indicate s.e.m. with N ≥ 3, statistical significance was assessed using column statistics. **p < 0.01; *p < 0.05.

3.4. LRRK2 kinase inhibition in primary astrocytes

To investigate the effect of LRRK2 kinase inhibition on endogenous LRRK2 in brain cells other than neurons, we used primary astrocyte cultures. Treatment of primary astrocytes with the kinase inhibitors GSK2578215A, CZC-25146, HG 10-102-01 or PF-06447475 for up to 48 h did induce significant S935 dephosphorylation but not destabilization of endogenous LRRK2 (Figure 4.4.). Also sustained LRRK2 kinase inhibition for several days did not induce destabilization, except for the GSK2578215A (Figure 4.4.). A more in-depth study in primary astrocytes is needed to explain why not all inhibitors where able to induce LRRK2 destabilization. In addition, it will be interesting to investigate the effects of kinase inhibition in other cell types such as microglial cells. By using six compounds with different selectivity profiles, the A2016T mutant and different test systems we provide robust evidence that pharmacological LRRK2 kinase inhibition can induce proteasomal LRRK2 protein degradation in different cell types in a LRRK2 inhibitor-specific manner. This is in line with previous studies reporting reduced LRRK2 protein levels after kinase inhibitor treatment of cells overexpressing LRRK2[184,198], and in kidney[184,203,205] or lung[184] of WT mice. Given the fast effects of LRRK2 kinase inhibition (LRRK2 dephosphorylation within 30 min[161]), most studies have not applied LRRK2 kinase inhibitor treatment for a time span longer than 2 h. This most likely explains why the 'delayed' degradation of LRRK2 (more than 2 h after inhibition) in cell lines overexpressing LRRK2 has not been observed in most reports. Interestingly, LRRK2 kinase

inhibition *in vivo* does not induce destabilization in all conditions. PF-06447475 treatment in mice for 90 minutes[206] and in rats for 14 days[143] does not induce reduced LRRK2 levels in the brain, while we observed a significant reduction of LRRK2 protein levels upon PF-06447475 treatment of LRRK2 overexpressing cell lines. As mentioned before, in-diet MLi-2 treatment of mice caused LRRK2 protein destabilization in lung and kidney, but not in the cortex[204], while we also observed clear destabilization in full brain extracts. Identification of the key variables in LRRK2 kinase inhibitor-induced LRRK2 protein destabilization will be crucial to explain the observed differences. Inhibitor-induced LRRK2 protein degradation is an important 'side' effect, which needs to be considered in view of clinical applications. Future studies should therefore be directed at understanding how LRRK2 destabilization is triggered and how it takes place. Understanding whether reduced LRRK2 levels are beneficial or rather an unwanted effect of LRRK2 kinase inhibition will be crucial to pursue or redirect current pharmacological LRRK2 therapeutic strategies.

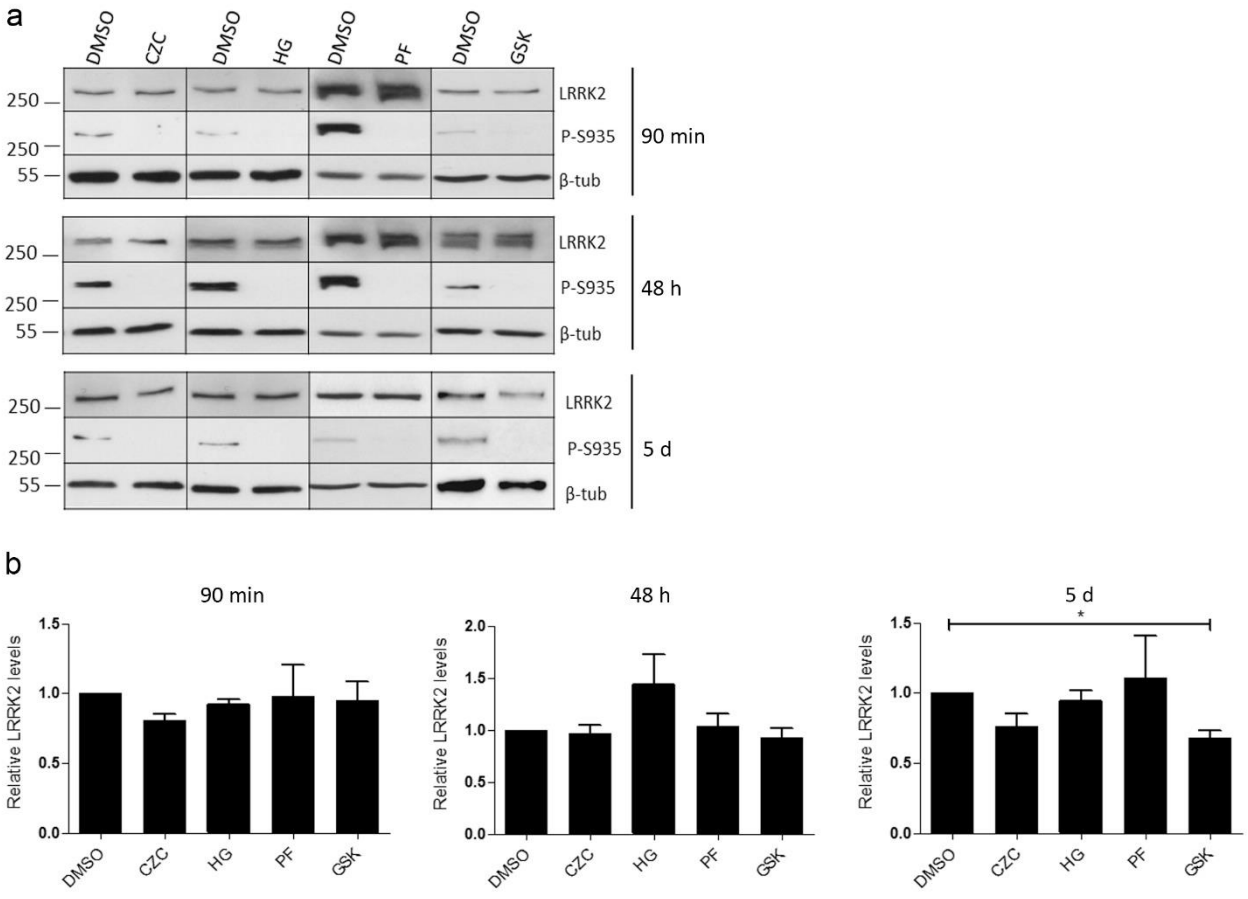


Figure 4.4. - LRRK2 kinase inhibition in primary astrocytes. (**a**) Primary astrocytes were treated with GSK2578215A (GSK, 2 µM), CZC-25146 (CZC, 200 nM), HG 10-102-01 (HG, 1 µM) or PF-06447475 (PF, 150 nM) for 90 min or 48 h or with a tenfold dilution of the concentrations mentioned for 5 or 10 days with fresh compound every two days. Immunoblotting was done with MJFF-2 anti-LRRK2 antibody, anti-LRRK2 P-S935 and anti-β -tubulin for equal loading. Representative blots are shown. (**b**) Quantifications of the blots represent total LRRK2 signal over β -tubulin. Statistical significance was assessed by column statistics (one-sample t-test) with Bonferroni correction for multiple comparisons. *p < 0.05.

4. Materials & Methods

Antibodies and reagents. LRRK2 kinase inhibitor-1 (L2-IN1), HG 10-102-01 and CZC-25146 were purchased from Calbiochem, GSK2578215A from Tocris and PF-06447475, MG132 and chloroquine from Sigma- Aldrich. MLi-2 was kindly provided by Dr. D. Alessi (Division of Signal Transduction Therapy, University of Dundee). Antibodies used are as follows: anti-flagM2, anti-vinculin and anti-α or β -tubulin (Sigma-Aldrich), anti-LRRK2 MJFF-2, anti-LRRK2 P-S935, anti-LC3 (Novus Biologicals) and anti-GAPDH (Abcam). QPCR probes were from Sigma-Aldrich, QPCR primers from IDT. Lentiviral vectors encoding full length LRRK2 were produced by the Leuven viral vector core (https://gbiomed.kuleuven.be/english/research/50000715/ laboratory-of-molecular- virology-and-gene-therapy/lvvc).

QPCR. LRRK2 mRNA levels were determined using RT-QPCR. Total RNA was reverse transcribed using the High-Capacity cDNA Archive kit (Applied Biosystems). Samples corresponding to 5 µg RNA were subsequently used for QPCR analysis with the iQ5 Multicolor RT-PCR detection system (BioRad). LRRK2 primer/probe set: LRRK2 Fw: 5′ -ACGCAGCGAGCATTGTACCTT-3′, LRRK2 Rev: 5′ -GGCTTCATGGCATCAACTTCA-3′ , LRRK2 probe: 5′ -GCTGTCTATGACCTCAGCAAGGGAC AGG C-3′ . LRRK2 mRNA levels were normalized to β -actin mRNA levels.

Cell lines. SH-SY5Y cells stably overexpressing 3flag-LRRK2 (WT or mutants) were generated as described in [165]. Cells were maintained in Dulbecco's modified Eagle's medium (Gibco-life technologies) supplemented with 15% fetal calf serum (Gibco), 1x non-essential amino acids (Gibco), 200 µg/ml hygromycin and 50 µg/ml gentamycin at 37°C in a humidified atmosphere containing 5% CO_2. All cultures were mycoplasma-free. For compound treatment, cells were treated in a 24-well cell culture vessel for the indicated period of time with the compound indicated or DMSO as negative control. Compound concentrations were selected based on the respective cellular IC50 values[312]. To obtain proteasomal or lysosomal inhibition, cells were treated with 1 µM MG132 for 17 h or 10 µM chloroquine for 48 h respectively, DMSO was used as negative control. For cell lysis, cells were rinsed in PBS and lysed in lysis buffer (Tris 20 mM pH 7.5, NaCl 150 mM, EDTA 1 mM, Triton 1%, glycerol 10%, protease inhibitor cocktail (Roche) and phosphatase inhibitor (PhosStop, Roche)). Cell lysates were cleared by centrifugation at 14,000 g for 10 min and further analyzed via immunoblot.

Primary cortical astrocyte cultures. Mixed cortical cell isolation for astrocyte cultures were performed using P1 to P4 mouse pups. Isolated cortex was mechanically dissociated and cells were cultured in BME supplemented with 10% FBS. After 10 days in culture, astrocyte purity was examined by immunofluorescence with anti-GFAP antibody (Dako).

Brain extracts. All animal experiments were performed in accordance with the European Communities Council Directive of November 24, 1986 (86/609/EEC) and approved by the Bioethical Committee of the KU Leuven (Belgium). C57BL/6J mice were injected i.p. with 10 mg/kg MLi-2 or DMSO in hydroxypropyl-β -cyclodextrin (Sigma-Aldrich) and PBS. Four injections were given in total over 30 h and animals were

sacrificed 2 h after the last injection. Whole brain, lung and kidney extracts were lysed in sucrose buffer (10 mM TrisHCl, 1 mM EDTA, 0.25 mM sucrose, protease inhibitor cocktail and phosphatase inhibitor) using a dounce homogenizer. Brain extracts were cleared by 10 min centrifugation at 3000 g followed by centrifugation of the supernatant for 30 min at 20000 g.

Immunoblotting. Protein content of cell lysates was determined using the bicinchoninic acid (BCA) protein determination assay (Pierce Biotechnology). Cell lysates were resolved by electrophoresis on an in-house 12.5% polyacrylamide gel or a NuPage 3–8% tris-acetate gradient gel. Separated proteins were transferred to a polyvinylidene fluoride membrane (Bio-Rad) and non-specific binding sites were blocked for 30 min in PBS with 0.1% Triton X-100 (PBS-T) and 5% non-fat milk. After overnight incubation at 4°C with primary antibodies, blots were washed 3 times with PBS-T, incubated with horseradish peroxidase-conjugated secondary antibody (Dako, Glostrup) for 1 h and washed again. Bands were visualized using enhanced chemiluminescence (Amersham Pharmacia Biotech). To normalize the signal of phospho-specific antibodies to LRRK2 expression levels, blots were stripped after detection of the LRRK2 signal and reprobed with anti-phospho-LRRK2 antibody by incubating the blot with stripping buffer (62.5 mM Tris-HCl pH 6.8, 2% SDS and 100 mM β-mercaptoethanol) for 30 min at 70°C, followed by 2 10 min wash steps with PBS-T. Densitometric analysis of the bands on the blot autoradiograms was performed with Aida analyzer v1.0 (Raytest).

Statistics. Figures shown are representative of at least three independent experiments. Phosphorylation levels were normalized for expression levels and experimental test conditions for control conditions. Statistical analysis was performed with a t-test, two-way ANOVA test or column statistics (one-sample t-test) comparing test values to the hypothetical value of 1 with Bonferroni correction for multiple comparisons. Statistical significance was set at $p < 0.05$.

Chapter 5 – Inhibition of LRRK2 or casein kinase 1 results in LRRK2 protein destabilization

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Scientific acknowledgement & personal contribution

Evy Lobbestael, Veerle Baekelandt and I designed the research. Evy Lobbestael and I performed the research and analyzed the data. More specifically, I prepared the lysates of the kidney tissue of WT and LRRK2$^{-/-}$ mice, in order to perform the antibody-detection experiments (Figure 5.S1). Cloning of truncated and phospho LRRK2 variants was performed by Joris Van Asselberghs and myself. I optimized and executed the experiments in cell culture, in which the effect of LRRK2 kinase inhibition or CK1 inhibition on LRRK2 protein stability was tested for several LRRK2 mutants (Figure 5.2, 5.3, 5.4, 5.5, 5.S2, 5.S3 and 5.S4). I optimized and executed the *in vivo* experiments (Figure 5.6). I prepared the final figures. Evy Lobbestael and I wrote the paper. All authors read and approved the paper. Financial support from the Michael J. Fox Foundation (grant ID 11472), KU Leuven, Flanders Innovation & Entrepreneurship (VLAIO) (doctoral fellowship TDW IWT.141396) and Research Foundation Flanders (FWO, projects G0E1917N and SBOS006617N) is gratefully acknowledged. LRRK2 kinase inhibitor MLi-2 was kindly provided by Dr. D. Alessi (Division of Signal Transduction Therapy, University of Dundee). We thank the Leuven Viral Vector Core for generation of lentiviral vectors and Sophie Croes and Joris Van Asselberghs for technical assistance.

Conflict of interest statements

The authors declare that they have no conflict of interest.

Keywords

Parkinson's disease, LRRK2, LRRK2 kinase inhibitor, phosphorylation, protein stability, casein kinase 1

1. Abstract

Mutations and variations in the leucine-rich repeat kinase 2 (LRRK2) gene are strongly associated with an increased risk to develop Parkinson's disease (PD). Most pathogenic LRRK2 mutations display increased kinase activity, which is believed to underlie LRRK2-mediated toxicity. Therefore, major efforts have been invested in the development of potent and selective LRRK2 kinase inhibitors. Several of these compounds have proven beneficial in cells and *in vivo*, even in a LRRK2 wild type background. Therefore, LRRK2 kinase inhibition holds great promise as disease-modifying PD therapy, and is currently tested in preclinical and early clinical studies. One of the safety concerns is the development

of lung pathology in mice and non-human primates, which is most likely related to the strongly reduced LRRK2 protein levels after LRRK2 kinase inhibition. In this study, we aimed to better understand the molecular consequences of chronic LRRK2 kinase inhibition, which may be pivotal in the further development of a LRRK2 kinase inhibitor-based PD therapy. We found that LRRK2 protein levels are not restored during long-term LRRK2 kinase inhibition, but are recovered upon inhibitor withdrawal. Interestingly, LRRK2 kinase inhibitor-induced destabilization does not occur in all pathogenic LRRK2 variants and the N-terminal part of LRRK2 appears to play a crucial role in this process. In addition, we identified CK1, an upstream kinase of LRRK2, as a regulator of LRRK2 protein stability in cell culture and *in vivo*. We propose that pharmacological LRRK2 kinase inhibition triggers a cascade that results in reduced CK1-mediated phosphorylation of yet unidentified LRRK2 phosphorylation sites. This process involves the N-terminus of LRRK2 and ultimately leads to LRRK2 protein degradation.

2. Introduction

Leucine-rich repeat kinase 2 (LRRK2) is a very attractive target in the development of disease-modifying strategies for Parkinson's disease (PD). Pathogenic mutations in the LRRK2 gene are the most common cause of inherited forms of PD and genetic variations in the LRRK2 locus contribute to the risk of developing sporadic forms of the disease[43–45,68–71,323,324]. All pathogenic mutations (N1437H, R1441C/G/H/S, Y1699C, G2019S and I2020T)[51,52] cluster in the catalytic core of the protein that consists of the GTPase domain ROC (Ras of complex proteins), which is linked via the COR domain (C-terminal of ROC) to a kinase domain[94]. This suggests that altered LRRK2 activity is involved in PD pathogenesis, which is supported by the fact that most pathogenic mutations (N1437H, R1441C/G/H, Y1699C, G2019S and I2020T) display increased LRRK2 kinase activity in cells and *in vivo*[132,136–138]. LRRK2-related toxicity in cell culture[74,142] and in rodent models[143,144] has been related to augmented LRRK2 kinase activity and can be rescued by LRRK2 kinase inhibitors[132,143–145,151]. Interestingly, LRRK2 kinase inhibition is also believed to be beneficial in PD cases without genetic LRRK2 modifications given the increased autophosphorylation at S1292 observed in urinary exosomes[139,140] and substantia nigra dopaminergic neurons in postmortem brain tissue from patients with idiopathic PD[141]. Moreover, LRRK2 kinase inhibition was shown to protect against α-synuclein-induced toxicity in rodent brain[143]. Consequently, specific and potent LRRK2 kinase inhibitors[152,153], are considered one of the prevalent disease-modifying therapeutic agents for PD and are currently tested in preclinical studies and phase I clinical trials.

Of particular interest are the molecular consequences of pharmacological LRRK2 kinase inhibition. We and others have shown that LRRK2 becomes ubiquitinated[184] and directed for proteasomal degradation upon LRRK2 kinase inhibition[169], resulting in reduced LRRK2 protein levels[169,184,198,203–205]. How pharmacological inhibition can induce LRRK2 protein degradation is not completely understood. LRRK2 kinase activity has been proposed as a regulator of LRRK2 stability, which is supported by the finding that transgenic knock-in mice expressing a kinase-dead form display decreased LRRK2 protein levels[203]. This is consistent with the recent observations that functional mutants L728D and L729D in the ankyrin domain of LRRK2 show both decreased kinase activity and decreased LRRK2 protein stability[121]. Moreover, preclinical safety studies revealed that LRRK2 kinase inhibitors induce

morphological changes in the lungs of mice[204] and non-human primates[205], that resemble the lung phenotype in LRRK2 knock-out mice[211]. However, destabilization upon LRRK2 kinase inhibition is not observed in all conditions[143,169,184,204,205] and not all animals with affected lungs display decreased LRRK2 levels[205], which indicates that inhibition of LRRK2 kinase activity alone is not sufficient to initiate protein destabilization.

Dephosphorylation at S935 has been suggested to regulate LRRK2 destabilization[184], given that dephosphorylation of heterologous phosphorylation sites, mediated by protein phosphatase 1 (PP1)[163], is a well-validated consequence of LRRK2 kinase inhibition[164–166,200,303]. However, we observed that mutating S935 to an alanine residue does not affect inhibitor-induced destabilization[169], which points to a more complex, yet unidentified, relation between LRRK2 S935 dephosphorylation and LRRK2 protein degradation.

Understanding how LRRK2 protein degradation is regulated may be crucial for understanding potential side effects of LRRK2 kinase inhibitors and at the same time reveal new therapeutic clues. In the present study, we aimed to gain better insight in the mechanism of LRRK2 kinase inhibitor-induced destabilization. We show that LRRK2 protein levels are not restored during sustained inhibition, but that this effect is reversible after inhibitor withdrawal. We provide strong evidence that dephosphorylation of the well-characterized heterologous phosphorylation sites is not crucial for inhibition-induced LRRK2 protein destabilization, and that the N-terminus of LRRK2 is presumably involved. We identified casein kinase 1 (CK1) as a regulator of LRRK2 protein stability in cell culture and *in vivo*. In addition, we show that several pathogenic mutants do not destabilize upon inhibition of LRRK2 kinase activity, but retain sensitivity to CK1 inhibition. Together, our results suggest that LRRK2 homeostasis in cell culture and *in vivo* relies on a complex, multifactorial mechanism that involves phosphorylation of yet unidentified phosphorylation sites regulated by CK1.

3. Results

3.1. LRRK2 S935 phosphorylation and protein levels do not recover during chronic pharmacological LRRK2 kinase inhibition

As LRRK2 kinase inhibitor treatment is considered one of the prevailing disease-modifying strategies for PD, insight in the consequences of chronic treatment will be crucial. We have previously shown that LRRK2 kinase inhibition induces degradation of LRRK2 protein starting after 8 h until at least 48 h of treatment[169]. To investigate whether LRRK2 protein levels and S935 phosphorylation remain low during sustained LRRK2 kinase inhibition, we treated SH-SY5Y cells overexpressing LRRK2 WT with the LRRK2 kinase inhibitors CZC-25146 (200 nM) or PF-06447475 (150 nM) over a period of six weeks. LRRK2 kinase inhibition was confirmed by dephosphorylation at S935 and S1292. No compensatory effects were observed as both phosphorylation levels at S935, S1292 and total LRRK2 protein levels remained strongly decreased (Figure 5.1.a).

To test whether the observed effects of LRRK2 kinase inhibition are reversible, cells were treated for different time periods with CZC-25146 or PF-06447475, followed by a washout period of 24 h or 72 h (Figure 5.1.b, c). Phosphorylation at S935 was completely restored during the washout period. Compared to inhibitor treatment without washout, incomplete recovery of LRRK2 protein levels was

observed 24 h after removal of the inhibitor, while LRRK2 levels were fully restored after three days of inhibitor withdrawal. This is in line with the relatively long half-life reported for LRRK2[184,208,213,216] as the reduction in LRRK2 protein levels is caused by proteasomal degradation[169] and hence, recovery relies on *de novo* protein synthesis.

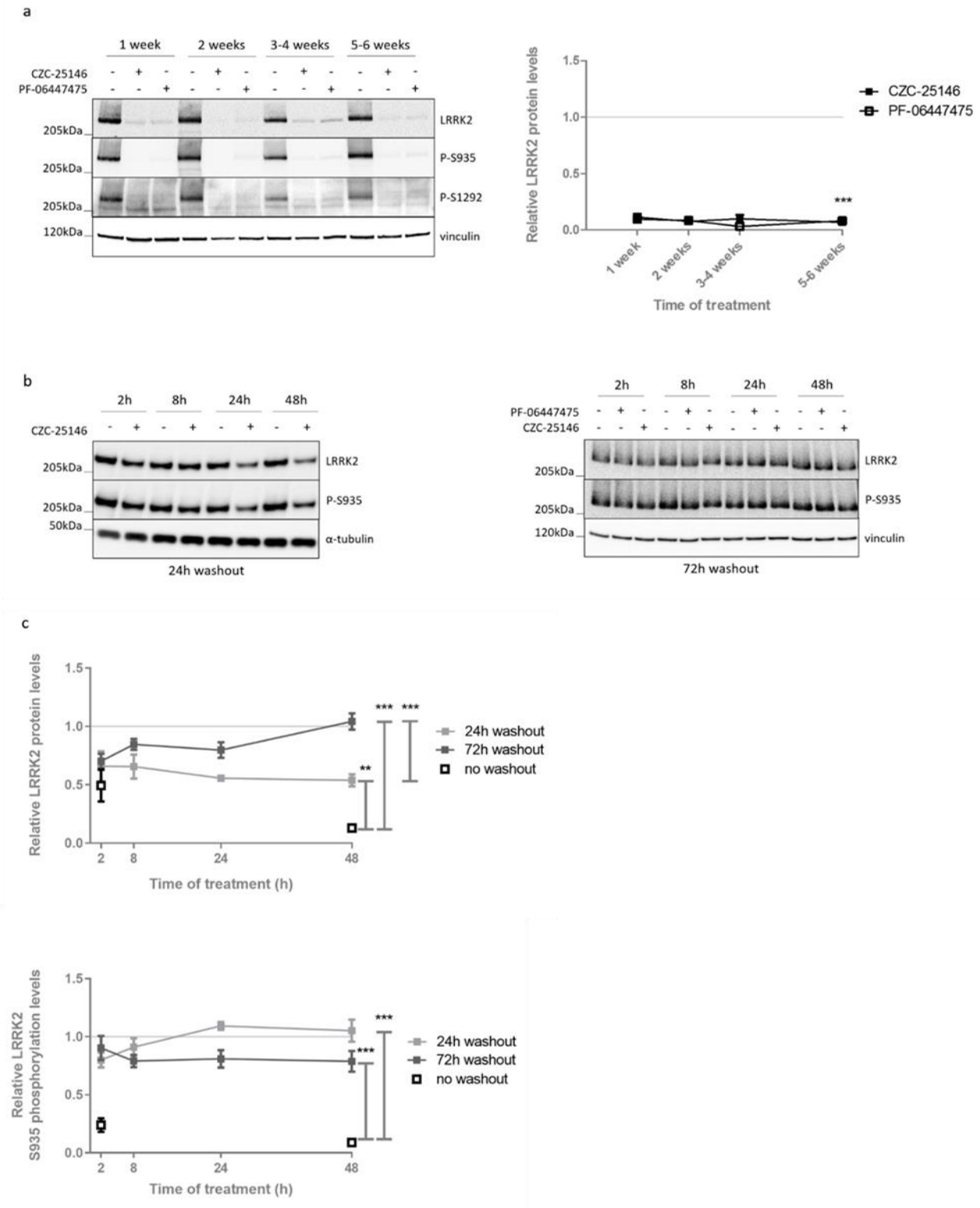


Figure 5.1. - Long-term LRRK2 kinase inhibitor treatment induces a sustained reduction in LRRK2 protein and phosphorylation levels, which can be completely restored after inhibitor removal. SH-SY5Y cells overexpressing 3Flag-LRRK2 WT were treated for indicated terms with CZC-25146 (200 nM), PF-06447475 (150 nM) or DMSO as control. Cell lysates were taken (a) on different days during several weeks of treatment or (b) 24 h or 72 h after removal of the LRRK2 kinase inhibitor and analyzed with immunoblotting using the FlagM2 antibody for LRRK2 detection, anti-LRRK2 P-S935, anti-LRRK2 P-S1292, anti-α-tubulin or anti-vinculin for checking equal loading. Representative blots are shown. (a, c) Graphs show the quantification of blots representing the ratio of total LRRK2 over housekeeping proteins or the ratio of phosphorylation at S935 over total LRRK2 signal. Error bars indicate S.E.M. with N≥3. Statistical significance was tested using (a) a 2-way ANOVA test with Bonferroni post-tests or (c) a Mann-Whitney U-test. *** p<0.001, ** p<0.01.

3.2. The N-terminus of LRRK2 is involved in inhibitor-induced LRRK2 destabilization

We have previously reported that, in contrast to full length LRRK2, a ~170 kDa truncated form of LRRK2 in mouse kidney is dephosphorylated at S935, but not destabilized upon LRRK2 kinase inhibition[169]. Based on previous reports[203,207] and the use of antibodies raised against different regions of LRRK2 (Figure 5.S1), we propose that this truncated form of LRRK2 lacks a part of the N-terminus. Therefore, we hypothesized that the N-terminal part of LRRK2 is involved in the regulation of LRRK2 protein degradation during kinase inhibition. We generated SH-SY5Y cells with stable overexpression of two truncated forms of LRRK2. The first one lacks the armadillo and ankyrin domain, but still contains the linker sequence N-terminal of the LRR that is highly phosphorylated in the full length protein (LRRK2$^{823-2527}$ or PLRCKW, predicted molecular weight of 193.8 kDa) (Figure 5.2.a). To investigate the importance of the ankyrin domain, we included a truncated LRRK2 variant only missing the armadillo domain (LRRK2$^{702-2527}$ or APLRCKW, with predicted molecular weight of 206.9 kDa) (Figure 5.2.a). Remarkably, we were not able to detect basal phosphorylation at S935 in these truncated forms (Figure 5.2.b), suggesting that the N-terminal part of LRRK2 might be crucially involved in the regulation of LRRK2 phosphorylation at S935. Interestingly, treatment up to 48 h with PF-06447475 or MLi-2 did not induce a decrease in LRRK2 protein levels for both N-terminal truncated forms (Figure 5.2.c, Figure 5.S2.b), while S1292 dephosphorylation confirmed inhibition of LRRK2 kinase activity (Figure 5.2.c). As an additional control for inhibitor activity, inhibition of LRRK2 kinase activity was assessed by S935 and S1292 dephosphorylation in parallel in a cell line expressing full length LRRK2 WT (Figure 5.S2).

3.3. Several pathogenic LRRK2 mutants do not destabilize upon LRRK2 kinase inhibition.

Several pathogenic LRRK2 mutations display strongly reduced expression levels and reduced basal phosphorylation at S935 (and other serines between the ankyrin and LRR domain)[134] (Figure 5.3.a). Therefore, we wondered whether these LRRK2 variants are still sensitive to LRRK2 kinase inhibitor-induced destabilization. Treatment of SH-SY5Y cells with stable overexpression of LRRK2 R1441G, Y1699C or I2020T with two LRRK2 kinase inhibitors revealed that no LRRK2 protein degradation is induced in those variants with strongly reduced basal S935 phosphorylation levels (Figure 5.3.a, c-e). In contrast, LRRK2 R1441C, which only displays modest, non-significant, S935 dephosphorylation compared to LRRK2 WT under basal conditions (Figure 5.3.a), still destabilizes upon kinase inhibitor treatment (Figure 5.3.b). Again, WT LRRK2 was included in each of the experiments to confirm inhibition of LRRK2 kinase activity, as we could only confirm robust dephosphorylation at S1292 for LRRK2 R1441C and Y1699C (Figure 5.S3).

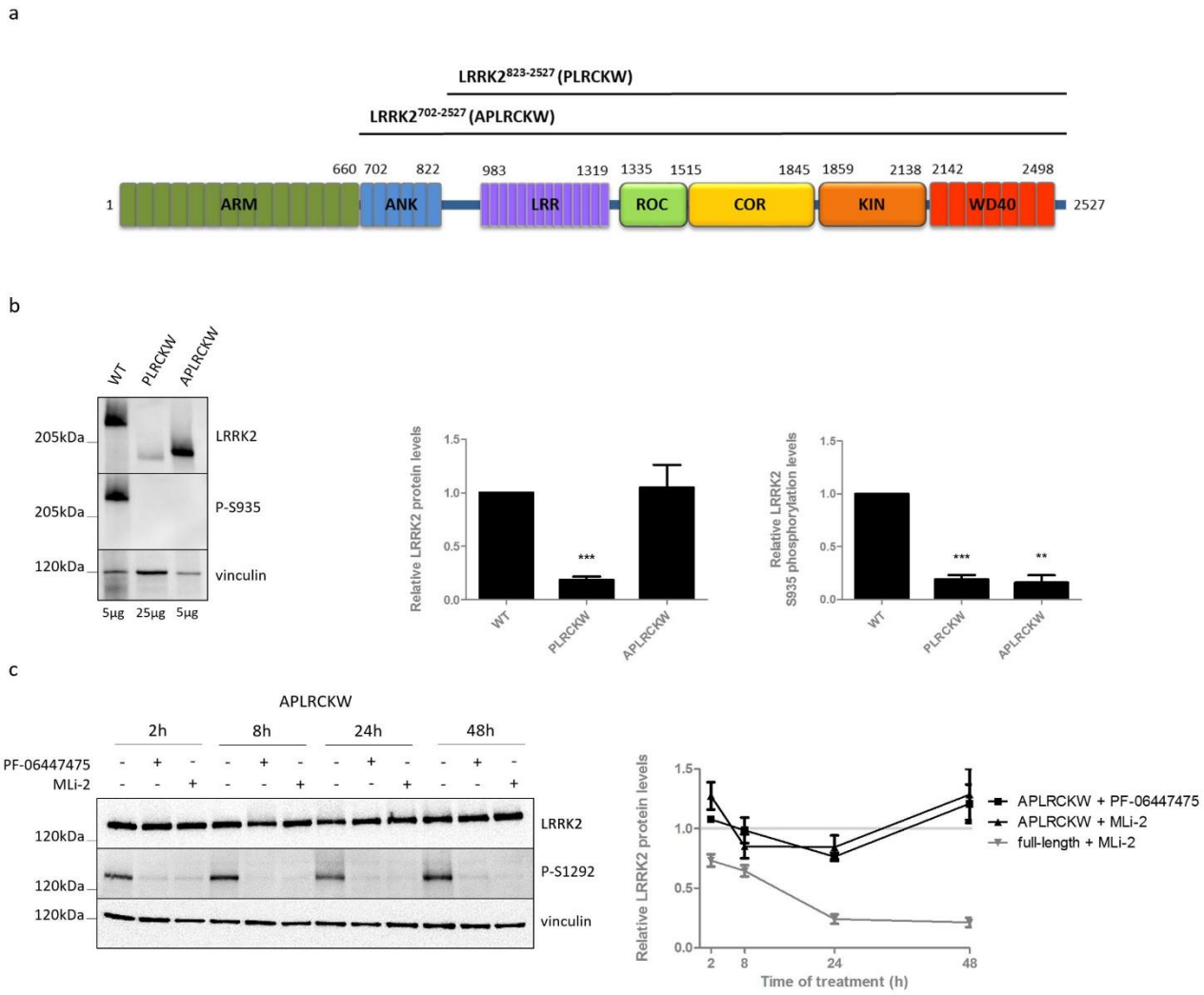


Figure 5.2. - Protein levels of truncated forms of LRRK2 are not reduced upon LRRK2 kinase inhibitor treatment. We generated SH-SY5Y cells overexpressing 3Flag-truncated forms of LRRK2 (a): LRRK2$^{823-2527}$ or PLRCKW, LRRK2$^{702-2527}$ or APLRCKW. Cell lines were either not treated (b) or treated for different periods of time with PF-06447475 (150 nM), MLi-2 (10 nM) or DMSO as control (c). Cell lysates were analyzed with immunoblotting using FlagM2 antibody for LRRK2 detection, anti-LRRK2 P-S935, anti-LRRK2 P-S1292 or anti-vinculin for equal loading. Representative blots are shown. Graphs show the quantification of blots representing the ratio of total LRRK2 over housekeeping protein signal or the ratio of phosphorylation at S935 over total LRRK2 signal. Error bars indicate S.E.M. with N≥3. Statistical significance was tested using a 2-way ANOVA test with Bonferroni post-tests or column statistics with Bonferroni correction. *** p<0.001, ** p<0.01.

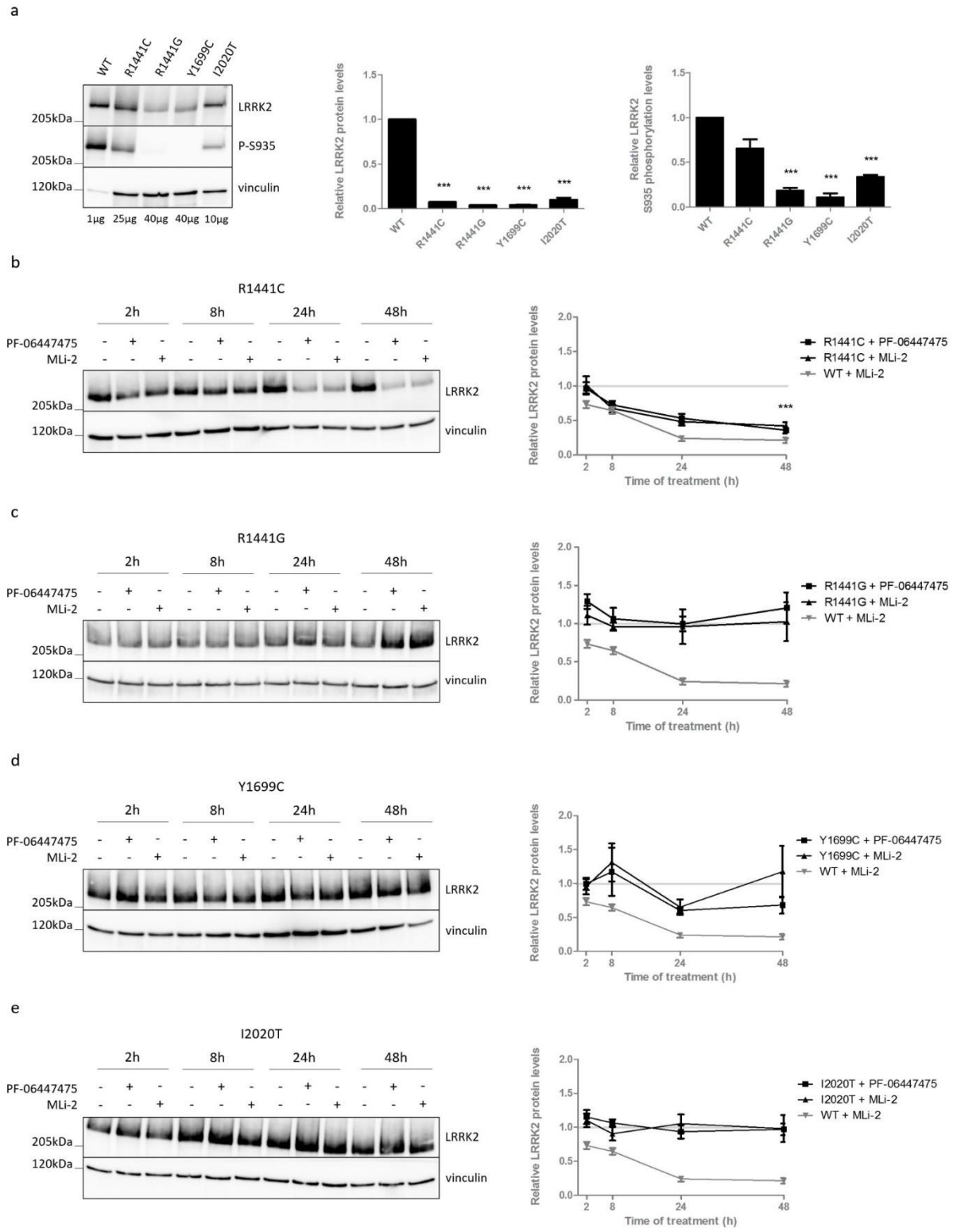


Figure 5.3.- LRRK2 kinase inhibition does not induce destabilization in dephosphorylated pathogenic mutants. SH-SY5Y cells overexpressing LRRK2 R1441C, R1441G, Y1699C or I2020T, were not treated (a) or treated for different periods of time with PF-06447475 (150 nM), MLi-2 (10 nM) or DMSO as control (b-e). Cell lysates were analyzed with immunoblotting using FlagM2 antibody for LRRK2 detection, anti-LRRK2 P-S935 or anti-vinculin. Representative blots are shown. Graphs show the quantification of blots representing the ratio of total LRRK2 over housekeeping protein signal or the ratio of phosphorylation at S935 over total LRRK2 signal. Error bars indicate S.E.M. with N≥3. Statistical significance was tested using a 2-way ANOVA test with Bonferroni post-tests or column statistics with Bonferroni correction. *** p<0.001..

3.4. Inhibition of casein kinase 1 induces LRRK2 destabilization

The results with the clinical LRRK2 mutants suggest that heterologous phosphorylation levels rather than kinase activity of LRRK2 determine the inhibitor-induced destabilization effect. Chia et al. (2014) identified casein kinase 1α (CK1α) as the kinase responsible for phosphorylation of S935[168]. Therefore, we decided to inhibit the upstream LRRK2 kinase CK1α in cells expressing LRRK2 WT and examine the effect on LRRK2 protein stability (Figure 5.4.a). Six hours of treatment with the CK1 inhibitor IC261 induced LRRK2 S935 dephosphorylation, as described previously[168]. This was accompanied by a strong reduction in total LRRK2 protein levels, which was even more pronounced than after MLi-2 treatment (Figure 5.4.a). This might indicate that the LRRK2 phosphorylation levels and not the kinase activity per se are important for the destabilization effect. Next, we examined the effect of CK1 inhibition on LRRK2 variants that are not destabilized after LRRK2 kinase inhibition. Interestingly, protein levels of pathogenic mutants R1441G (Figure 5.4.b), Y1699C (Figure 5.4.c) and I2020T (Figure 5.4.d), as well as the two truncated LRRK2 variants (lacking the armadillo domain or/and the ankyrin domain) (Figure 5.4.e, f), were significantly decreased after treatment with CK1 inhibitor, but not with LRRK2 kinase inhibitor.

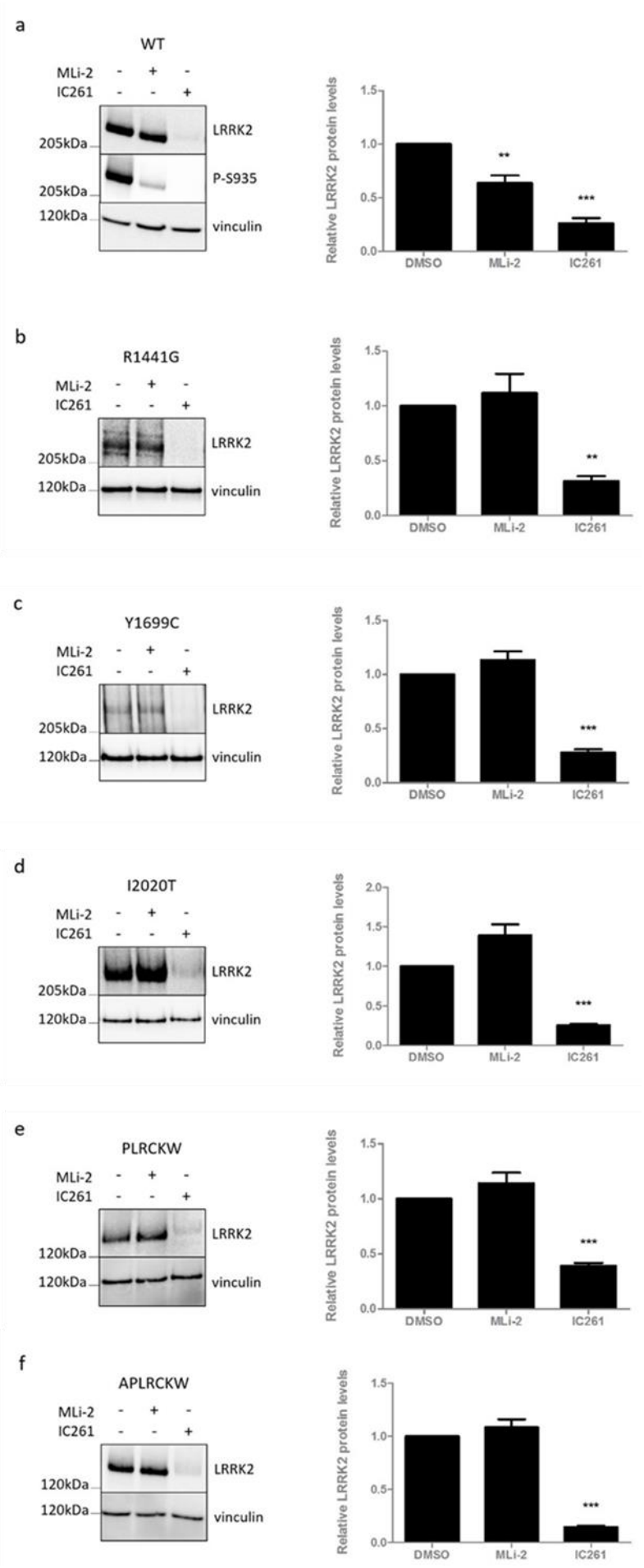


Figure 5.4. - LRRK2 variants that do not destabilize upon LRRK2 kinase inhibition, do destabilize upon CK1 inhibition. SH-SY5Y cells overexpressing LRRK2 (a) WT, pathogenic: (b) R1441G, (c) Y1699C or (d) I2020T, truncated: (e) PLRCKW or (f) APLRCKW were treated between 6 h and 8 h with MLi-2 (10 nM), IC261 (300 µM) or DMSO as control. Cell lysates were analyzed with immunoblotting using the FlagM2 antibody for LRRK2 detection, anti-LRRK2 P-S935 or anti-vinculin for equal loading. Representative blots are shown. Graphs shown the quantification of blots representing the ratio of total LRRK2 over housekeeping proteins signal. Error bars indicate S.E.M. with N≥3. Statistical significance was tested using column statistics with Bonferroni correction. *** p<0.001, ** p<0.01.

3.5. Known heterologous phosphorylation sites do not determine LRRK2 destabilization

The results obtained so far might indicate that the basal phosphorylation at S935 is crucial for the inhibitor-induced LRRK2 destabilization, as was suggested previously[184]. However, we previously reported that a truncated form of LRRK2 in mouse kidney could be dephosphorylated at S935, but not destabilized[169]. In addition, substitution to an alanine at S935 or S910 could not prevent LRRK2 from degradation during kinase inhibition[169], indicating that S935 dephosphorylation is not sufficient for inhibitor-induced LRRK2 destabilization. In fact, S935 is part of a cluster of heterologous phosphorylation sites N-terminal of the LRR domain, and CK1 has been reported to phosphorylate not only S935, but also the S910, S955, S973 sites, in addition to two extra residues, S908 and S976. Therefore, we hypothesized that dephosphorylation of one of the other serines might be involved in the destabilization effects induced by pharmacological LRRK2 kinase inhibition. To test this, we generated SH-SY5Y cells stably overexpressing a mutant form of LRRK2, in which the six serines are mutated to an alanine (phosphodeficient) or glutamic acid (phosphomimetic). Treatment of these cell lines with PF-06447475 or MLi-2 revealed that the 6x phosphodeficient and phosphomimetic mutant LRRK2 still destabilized upon LRRK2 kinase inhibition to the same extent as WT LRRK2 (Figure 5.5.a, b). Remarkably, the phosphodeficient LRRK2 variant displays increased autophosphorylation levels at S1292 after LRRK2 kinase inhibition, while effects on autophosphorylation levels after LRRK2 kinase inhibition of the phosphomimetic LRRK2 variant are elusive (Figure 5.S4). Also in this set of experiments, cell lines overexpressing WT LRRK2 were treated in parallel and confirmed inhibitor-induced dephosphorylation at S1292 and S935, as expected.

Treatment of these mutants with CK1 inhibitor resulted in a stronger reduction in total LRRK2 protein levels compared to LRRK2 kinase inhibition, indicating that dephosphorylation at the six phosphorylation sites is not essential for inhibitor-induced degradation of LRRK2 (Figure 5.5c, d).

3.6. CK1 inhibition induces LRRK2 protein destabilization in the lung

Next, we aimed to investigate the effect of CK1 inhibition in a more physiological model. Remarkably, LRRK2 kinase inhibitor or CK1 inhibitor treatment of primary cortical neurons did not induce significant changes in LRRK2 protein levels (Figure 5.S5). It should be noted that IC261 treatment induced cellular toxicity upon 8 h of treatment, which might explain the lack of effect on LRRK2. Next, we examined the effect of CK1 inhibition *in vivo.* Since little information is available on brain permeability and differences in potency and isoform-specificity have been ascribed to different CK1 inhibitors[325], we compared two CK1 inhibitors, IC261 and PF-670462. PF-670462 induced the strongest LRRK2 protein destabilization and this compound has been reported to have a greater potency to inhibit CK1 compared to IC261[325], PF-670462 was selected for further *in vivo* experiments.

We treated wild type mice with the LRRK2 kinase inhibitor MLi-2 (10 mg/kg), CK1 inhibitor PF-670462 (50 mg/kg) or with DMSO and analyzed brain, lung and kidney tissue. As shown previously[169], LRRK2 kinase inhibition induced a significant decrease in LRRK2 phosphorylation at S935 and total full-length LRRK2 protein levels in the brain, lung and kidney (Figure 5.6.a-c). In contrast, protein stability of the truncated LRRK2 variant in the kidney was not affected, despite a significant dephosphorylation at S935 (Figure 5.6.c). CK1 inhibition induced LRRK2 S935 dephosphorylation in lung and kidney, however no

S935 dephosphorylation could be observed in brain extracts. In line with the cellular experiments, CK1 inhibition induced a significant reduction in total LRRK2 protein levels in the lung (Figure 5.6.b).

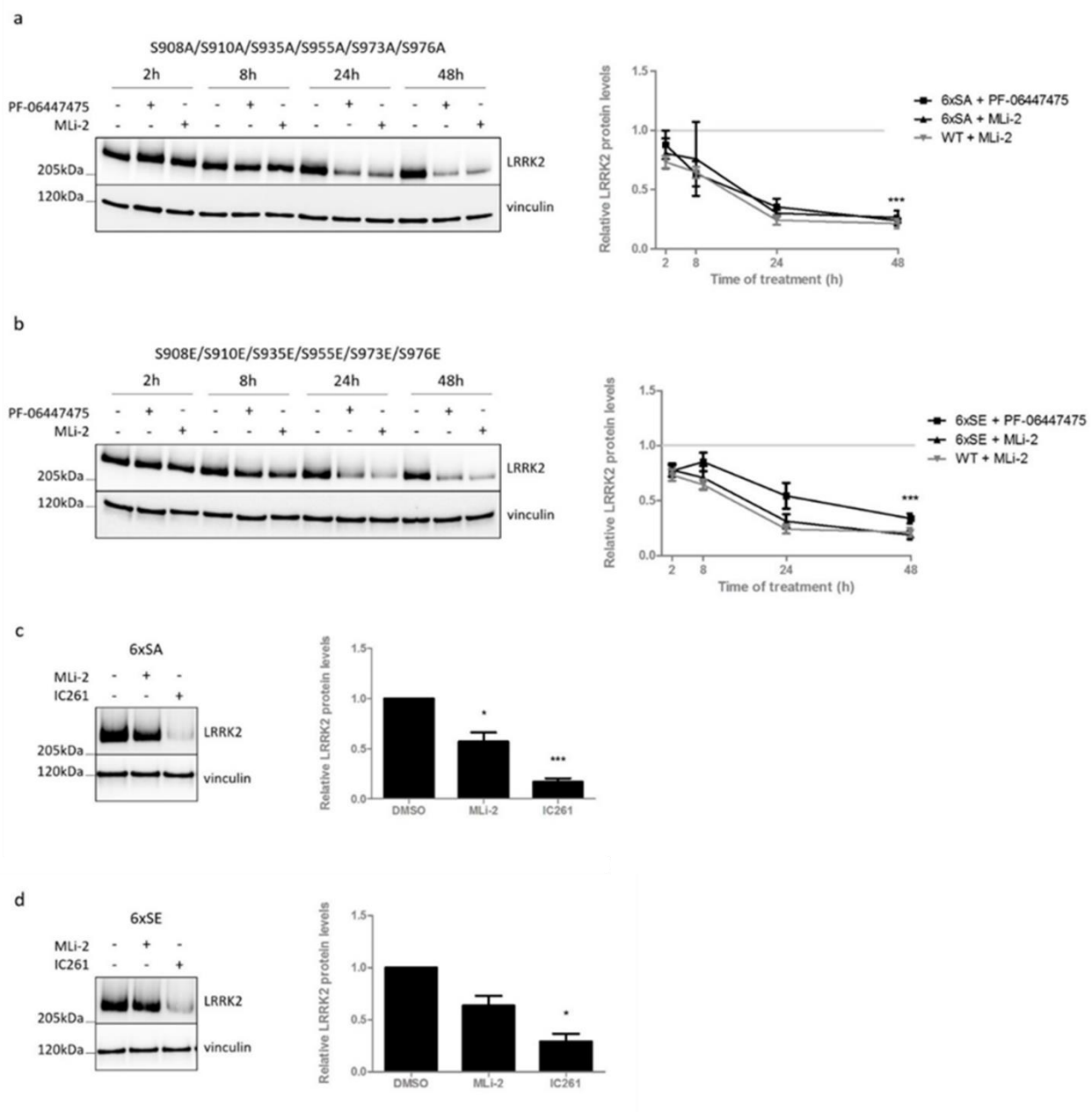


Figure 5.5. - Dephosphorylation at residues S908, S910, S935, S955, S973 or S976 is not crucial for LRRK2 kinase inhibitor-induced destabilization. SH-SY5Y cells overexpressing LRRK2 with (a, c) 6xSA (S908A/S910A/S935A/S955A/S973A/S976A) or (b, d) 6xSE (S908E/S910E/S935E/S955E/S973E/S976E) were treated (a, b) for different periods of time with PF-06447475 (150 nM), MLi-2 (10 nM) or DMSO as control or (c, d) between 6 h and 8 h with MLi-2 (10 nM), IC261 (300 µM) or DMSO as control. Cell lysates were analyzed with immunoblotting using FlagM2 antibody for LRRK2 detection or anti-vinculin for equal loading. Representative blots are shown. Graphs show the quantification of blots representing the ratio of total LRRK2 over housekeeping protein signal. Error bars indicate S.E.M. with N≥3. Statistical significance was tested using a 2-way ANOVA test with Bonferroni post-tests or column statistics with Bonferroni correction. *** p<0.001, *p<0.05.

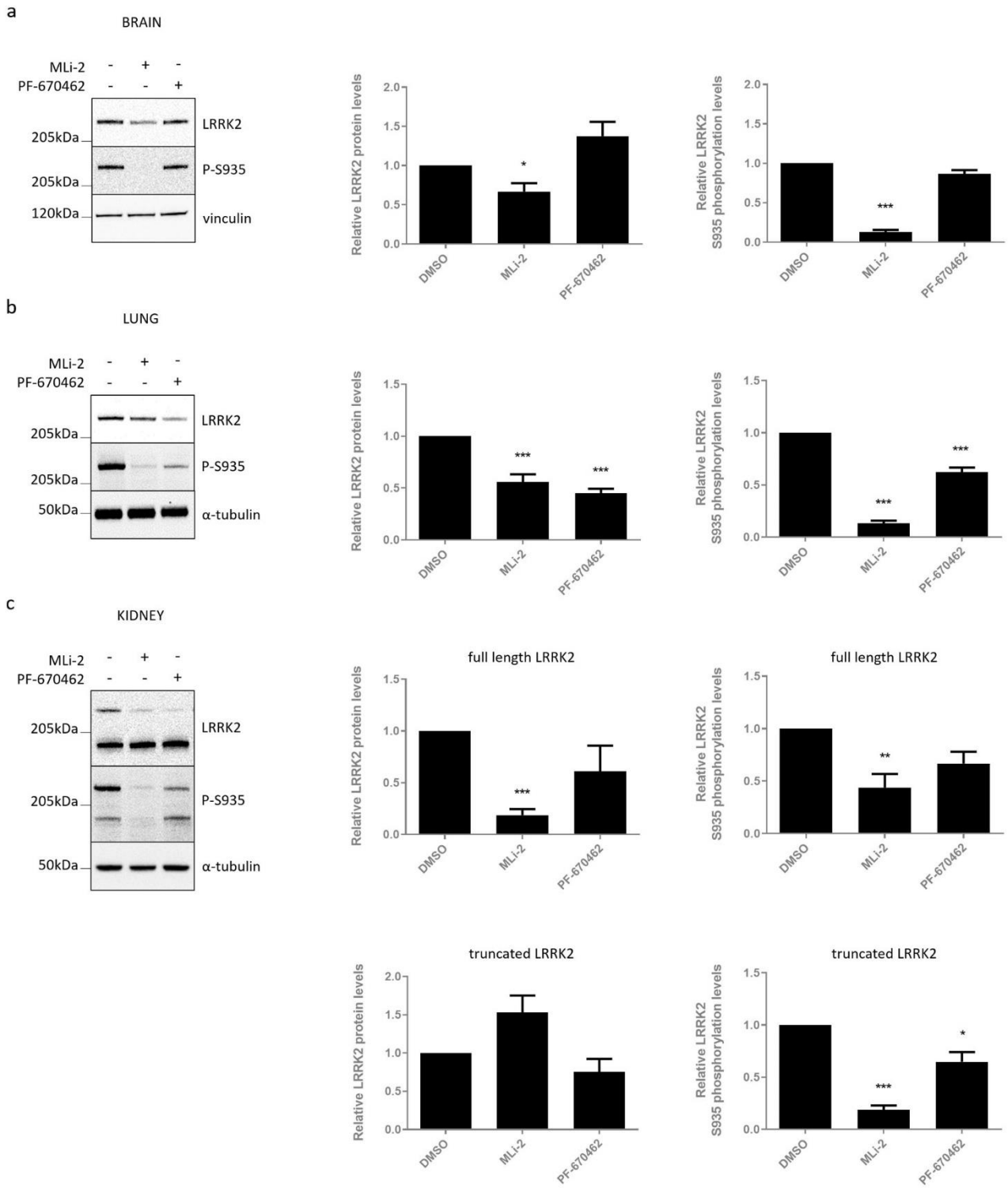


Figure 5.6. - Casein kinase 1 inhibition-induced destabilization of LRRK2 protein also occurs *in vivo*. C57Bl/6J mice were injected four times intraperitoneally with the LRRK2 kinase inhibitor MLi-2 (10 mg/kg), the casein kinase 1 inhibitor PF-670462 (50 mg/kg) or DMSO as a negative control over a period of 30 h. Brain (**a**), lung (**b**) and kidney (**c**) extracts were taken 2 h after the last injection and analyzed with immunoblotting using MJFF-2 anti-LRRK2 antibody, anti-LRRK2 P-S935 or anti-α-tubulin for equal loading. Representative blots are shown. Graphs show the quantification of blots representing the ratio of total LRRK2 signal over housekeeping proteins signal or the ratio of phosphorylation at S935 over total LRRK2 signal. Error bars indicate S.E.M. with N≥3. Statistical significance was tested using column statistics with Bonferroni correction. *** p<0.001, **p<0.01, *p<0.05.

4. Discussion

How LRRK2 proteostasis is regulated in basal conditions and upon pharmacological kinase inhibition is not completely understood. Since clinical applications will most likely require long-term administration and thus a chronic suppression of LRRK2 protein levels, we have investigated the effects of sustained LRRK2 kinase inhibition in the present study. We found that LRRK2 S935 dephosphorylation and total protein destabilization are maintained during chronic LRRK2 kinase inhibition, but are reversible when the inhibitor is withdrawn. This is in line with the finding that the lung phenotype, observed in non-human primates treated with different LRRK2 kinase inhibitors, is normalized upon cessation of compound administration [326]. Therefore, clinical studies with long-term dosing, ideally in a Parkinson's disease context, will have to reveal to what extent the observed phenotypes are a real safety issue.

Here we confirm our previously reported finding that a physiologically occurring truncated LRRK2 form in mouse kidney, which still contains the S935, the LRR, COR and kinase domain (Figure 5.S1 and [169,203]), does not destabilize upon LRRK2 kinase inhibition in contrast to full length LRRK2 (Figure 5.6 and [169]). Using truncated forms of LRRK2, we here show that the presence of the armadillo domain, or N-terminus of LRRK2 is crucial for inhibitor-induced LRRK2 protein degradation. As these LRRK2 variants are artificial mutants, we cannot rule out potential effects on protein folding and hence induction of secondary effects. For example, the epitope for the P-S935 antibody might be inaccessible in these truncated variants, explaining the lack of phosphorylated S935 detection. Still, our findings are in line with the observations on the truncated form in the kidney. A ~170 kDa truncated form of LRRK2 was also observed in neutrophils and could be destabilized by 30 minutes of MLi-2 treatment, which was not the case for its full length counterpart[207]. These apparently contradicting results might be related to cell type-specific effects[169], different sequences of this truncation or an alternative mechanism, as high doses of another specific inhibitor, PF-06447475, could not induce a similar effect in neutrophils.

Dephosphorylation of S935 is induced by every LRRK2 kinase inhibitor reported to date and was previously suggested to be sufficient for LRRK2 degradation[184]. However, our findings point to a much more complex mechanism as the truncated form of LRRK2 in mouse kidney can still be dephosphorylated at S935, but is not destabilized upon LRRK2 kinase inhibition[169]. By extension, using a phosphodeficient and phosphomimetic mutant, we show that none of the six well-characterized heterologous phosphorylation sites (i.e. S908, S910, S935, S955, S973 and S976) are crucially involved in inhibitor-induced LRRK2 protein degradation. To our knowledge, these functional LRRK2 mutants have not been reported before. The intriguing observation that the phosphodeficient LRRK2 variant displays increased autophosphorylation at S1292 after LRRK2 kinase inhibition might point to an interesting regulatory mechanism and will need additional investigation. Still, we should take into account that the introduction of artificial phosphorylation site mutations might affect the conformation, resulting in secondary molecular changes.

In contrast to LRRK2 G2019S, which is destabilized by kinase inhibition to the same extent as LRRK2 WT [169], pathogenic mutations R1441G, Y1699C and I2020T are not degraded when inhibited. This is in line with the reported lack of increased ubiquitination upon LRRK2 kinase inhibition for LRRK2 mutants R1441G, Y1699C and I2020T[184]. Of particular interest is the finding that the R1441C variant behaves similarly to LRRK2 WT in terms of inhibitor-induced destabilization. Remarkably, LRRK2 R1441C only

displays a modest reduction in basal phosphorylation compared to LRRK2 WT, in contrast to the pathogenic variants that are not destabilized upon inhibition and show strongly reduced heterologous phosphorylation levels[134]. Also truncated LRRK2 (i.e. both the artificial constructs used here and the physiologically occurring LRRK2 truncation in the kidney) does not destabilize and displays strongly reduced heterologous phosphorylation levels (at least at S935)[169]. These data suggest that pharmacological LRRK2 kinase inhibition is not sufficient for LRRK2 protein destabilization and point to dephosphorylation of LRRK2 as a potential mechanism underlying inhibitor-induced destabilization, albeit not at S908, S910, S935, S955, S973 or S976. Although future studies should confirm these findings on endogenous (knock-in) LRRK2, the observation that several pathogenic mutants are not degraded after LRRK2 kinase inhibition is intriguing and should be taken into consideration when stratifying clinical studies. Indeed, potential protective effects of kinase inhibitors might be mutation-dependent and not only rely on the decrease in kinase activity, but also on the degree of protein degradation, as shown in cell lines[198].

To further investigate the hypothesis that phosphorylation levels rather than kinase activity of LRRK2 determines the inhibitor-induced destabilization effect, we assessed the effects of inhibition of CK1, which was reported as an upstream LRRK2 kinase[168]. Interestingly, pharmacological CK1 inhibition led to a strong and fast reduction in LRRK2 protein levels. Therefore, we here identify CK1 as a new regulator of LRRK2 homeostasis in cell culture and *in vivo*. As CK1 inhibition can induce protein destabilization in LRRK2 variants that are insensitive to LRRK2 kinase inhibitor-induced destabilization, we reasoned that CK1 might act downstream of LRRK2 kinase inhibition in the pathway that ultimately leads to LRRK2 degradation. Somewhat surprisingly, using a six-fold phosphorylation mutant of LRRK2, none of the reported LRRK2 target sites of CK1α appeared to be crucially involved in LRRK2 kinase inhibitor-induced destabilization. This corresponds to our observation that protein levels of these phosphorylation deficient/mimetic variants are still reduced upon CK1 inhibition. Since the CK1 inhibitors used here are not specific for the CK1α isoform, which was proposed as a LRRK2 interactor[168], we cannot rule out that other CK1 isoforms are involved in the regulation of LRRK2 protein stability in different tissues. Indeed, the CK1δ and ε isoform preference of CK1 inhibitor PF-670462 might explain the lack of LRRK2 S935 dephosphorylation in brain tissue, although low brain permeability of the compound cannot be excluded either.

In the kidney, we observed a trend towards decreased LRRK2 protein levels for full length LRRK2 and truncated LRRK2, although S935 dephosphorylation of full length LRRK2 did not reach significance (Figure 5.6.c). In contrast, we have observed strong destabilization of artificially truncated LRRK2 variants after CK1 inhibition in cell culture. An important consideration is the similarity between our artificial truncated variant and the one found in the kidney as this might help to explain our observations. Although more research is required to exclude indirect or kinase-independent effects of CK1, we postulate that inhibition of CK1 induces LRRK2 dephosphorylation at yet unidentified sites, which ultimately induces proteasomal degradation. The hypothesis of dephosphorylation-induced destabilization of LRRK2 is in line with the findings of Zhao et al. that inhibition of 14-3-3 binding by difopein treatment reduces LRRK2 protein levels, taking into account that 14-3-3 proteins interact with LRRK2 in a phosphorylation-dependent manner. In addition, they showed that inhibition of protein

phosphatase 1 and 2A prevents increased ubiquitination induced by pharmacological kinase inhibition[184]. Given the evidence that phosphorylation of LRRK2 is involved in inhibitor-induced LRRK2 protein destabilization, a potential role for other upstream LRRK2 phosphorylation regulators such as PKA, PAK6 and IKKα/β (summarized in [155], reviewed in [293]) should be considered in future research in addition to CK1.

Taken together, we hypothesize that pharmacological LRRK2 kinase inhibition induces N-terminal changes that lead to reduced CK1 interaction, resulting in reduced heterologous phosphorylation levels and ultimately LRRK2 protein degradation. This is in line with the reported close interactions between the N-terminal part of LRRK2 (mainly the ankyrin domain) and its kinase domain[327], as well as with the reduced LRRK2-CK1 interaction after LRRK2 kinase inhibition[168]. To date, it is not clear which phosphorylation sites are involved in this process, although they probably interact with 14-3-3 proteins, and are located outside the N-terminal part of LRRK2, given that also truncated LRRK2 variants destabilized upon CK1 inhibition.

Finally, the clinical LRRK2 mutants R1441G, Y1699C and I2020T as well as the artificial N-terminal LRRK2 truncations display reduced expression levels in all stable cell lines generated (Figure 5.2.b and 5.3.a), which might explain why phosphorylation levels at S1292 were under the detection limit for some of these LRRK2 variants. The reduced expression levels might be explained by the reported reduction in half-life of pathogenic LRRK2 mutants compared to LRRK2 WT[128,213,214]. As these LRRK2 variants also display reduced phosphorylation levels at S935, a similar pathway as hypothesized above may underlie the reduced protein stability in basal conditions. This would involve decreased interaction of pathogenic mutants or N-terminal truncations with CK1, leading to basally dephosphorylated residues, thereby marking LRRK2 for protein degradation.

In conclusion, we show that chronic treatment with LRRK2 kinase inhibitors induces sustained LRRK2 S935 dephosphorylation and LRRK2 degradation, which can be reversed after inhibitor withdrawal. We identify the N-terminus as a crucial mediator of inhibitor-induced protein degradation. We show that S935 dephosphorylated pathogenic mutations R1441G, Y1699C and I2020T do not destabilize upon LRRK2 kinase inhibition, in contrast to LRRK2 WT or R1441C. Lastly, inhibition of CK1 induces fast and strong destabilization of LRRK2, also in conditions where LRRK2 kinase inhibitor cannot. The involvement of CK1 in the regulation of endogenous LRRK2 protein levels was further confirmed *in vivo*. Altogether, we postulate that LRRK2 kinase inhibition induces N-terminal changes that disrupt the LRRK2-CK1 interaction, leading to reduced heterologous phosphorylation levels and LRRK2 protein degradation. Although several questions remain, our study provides important new insights in LRRK2 protein homeostasis.

5. Materials & Methods

Antibodies, plasmids and reagents. LRRK2 kinase inhibitors CZC-25146 and PF-06447475 and casein kinase 1 inhibitor IC261 were purchased from Sigma-Aldrich. Casein kinase 1 inhibitor PF-670462 was purchased from Abcam. LRRK2 kinase inhibitor MLi-2 was kindly provided by Dr. D. Alessi (Division of Signal Transduction Therapy, University of Dundee). pCHMWS_3Flag_LRRK2_Ires_Hygro constructs of pathogenic (R1441C/G, Y1699C and I2020T) LRRK2 variants were cloned as described in [104]. The pCHMWS_3Flag_LRRK2_Ires_Hygro constructs encoding truncated variants PLRCKW and APLRCKW, as well as the LRRK2 S908A/S910A/S935A/S955A/S973A/S976A or S908E/S910E/S935E/S955E/S973E/S976E were generated using gBlock® Gene Fragments (IDT) and as described in [104]. Following antibodies were used: mouse anti-FlagM2 (Sigma-Aldrich, F1804), mouse anti-vinculin (Sigma-Aldrich, V9131), mouse anti-α-tubulin (Sigma-Aldrich, T5168), rabbit anti-LRRK2 P-S935 (Abcam, ab133450), rabbit anti-LRRK2 P-S1292 (Abcam, ab203181), rabbit anti-LRRK2 MJFF-2 antibody (Abcam, ab133474), mouse anti-LRRK2/Dardarin, N-terminus N138/6 (Neuromab 75-188), mouse anti-LRRK2/Dardarin, C-terminus N241A/34 (Neuromab 75-253), mouse anti-LRRK2 MC.028.83.76.242 (ab130277).

Viral vector production. Lentiviral (LV) vectors encoding human 3Flag-LRRK2 variants under control of the cytomegalovirus (CMV)-promotor were produced as described previously [328] by our in-house Leuven viral vector core (https://gbiomed.kuleuven.be/english/research/50000715/laboratory-of-molecular-virology-and-gene-therapy/lvvc).

Cell culture. SH-SY5Y cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Gibco-life technologies), supplemented with 15% fetal calf serum (Gibco), 1x non-essential amino acids (Gibco) and 50 µg/mL gentamycin at 37°C in a humidified atmosphere containing 5% CO_2. All cultures were mycoplasma-free. To generate SH-SY5Y cells stably overexpressing 3Flag-LRRK2 variants, we transduced SH-SY5Y cells with LV vectors and selected them in DMEM supplemented with 200 µg/mL hygromycin. For compound treatment, cells were treated in a 24-well plate for the indicated period of time with the compound indicated or DMSO as negative control. To obtain LRRK2 kinase inhibition, cells were treated with either 200 nM CZC-25146, 150 nM PF-06447475 or 10 nM MLi-2. To obtain inhibition of casein kinase 1α, cells were treated with 300 µM IC261 [168]. For washout experiments, cells were rinsed twice with PBS before they were given fresh medium without compound. For cell lysis, cells were rinsed with PBS and lysed on ice in lysis buffer composed of Tris 20 mM pH 7.5, 400 mM NaCl, 1 mM EDTA, 1% Triton, 10% glycerol, protease inhibitor cocktail (Roche) and phosphatase inhibitors (PhosStop, Roche). Cell lysates were cleared by centrifugation at 14 000 g for 10 min and further analyzed via immunoblotting.

Tissue extraction. All animal experiments were performed in accordance with the European Communities Council Directive of November 24, 1986 (86/609/EEC) and approved by the Bioethical Committee of the KU Leuven (Belgium). Whole brain, lung and kidney extracts of C57Bl/6J mice (WT or LRRK2$^{-/-}$) were lysed in sucrose buffer (10 mM Tris-HCl, 1 mM EDTA, 0.25 mM sucrose, protease

inhibitor cocktail and phosphatase inhibitor) using a dounce homogenizer. Extracts were cleared by 10 min centrifugation at 3000 g, followed by centrifugation of the supernatant for 30 min at 20000 g. For experiments using LRRK2 kinase inhibition or CK1 inhibition, C57Bl/6J WT mice were injected i.p. with 10 mg/kg MLi-2, 50 mg/kg PF-670462 or DMSO in 20% hydroxypropyl-β-cyclodextrin (Sigma-Aldrich) and PBS. Four injections were given over a time interval of 30 h and animals were sacrificed 2 h after the last injection.

Immunoblotting. Protein concentration of cell lysates was determined using the bicinchoninic acid (BCA) protein determination assay (Pierce Biotechnology). Cell lysates were resolved by electrophoresis on 3-8% Criterion™ XT Tris-Acetate protein gels (Bio-Rad). Separated proteins were transferred to a polyvinylidene fluoride membrane (Bio-Rad) and non-specific binding sites were blocked for 15 min in PBS with 0.1% Triton X-100 (PBS-T) and 5% non-fat milk. After overnight incubation at 4°C with primary antibodies, blots were washed 3 times with PBS-T and incubated with horseradish peroxidase-conjugated secondary antibody (Dako, Glostrup) for 1 h and washed again 3 times. Bands were visualized using enhanced chemiluminescence (Amersham Pharmacia Biotech). To normalize the signal of phospho-specific antibodies to LRRK2 expression levels, blots were stripped after detection of the LRRK2 signal and reprobed with anti-phospho-LRRK2 antibody by incubating the blot with stripping buffer (62.5 mM Tris-HCl pH 6.8, 2% SDS and 100 mM β -mercaptoethanol) for 30 min at 70°C, followed by 2x 10 min wash steps with PBS-T.

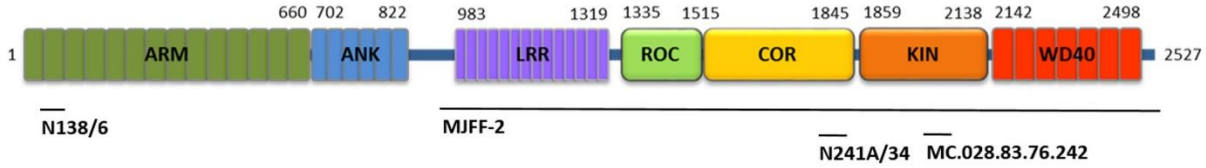
Statistics. Blots shown are representative of at least three independent experiments. LRRK2 phosphorylation levels were normalized to LRRK2 expression levels, LRRK2 protein levels to housekeeping proteins and experimental test conditions to control conditions. Statistical analysis was performed with a 2-way ANOVA test with Bonferroni post-test or column statistics (one-sample t-test) comparing test values to the hypothetical value of 1. If different treatment terms were applied, significance is only shown for the 48 h time point. Statistical significance: *** $p<0.001$, ** $p<0.01$, *$p<0.05$.

6. Supplementary material

6.1. Supplemantary material & methods

Isolation and culture of primary neurons. All animal experiments were performed in accordance with the European Communities Council Directive of November 24, 1986 (86/609/EEC) and approved by the Bioethical Committee of the KU Leuven (Belgium). Primary cortical neurons were prepared from newborn (P0) FVB pups. Cells were dissociated by 15 min incubation at 37°C using trypsin (Gibco), followed by physical dissociation by pipetting. Cells were plated in a 6-well plate precoated with poly-D-lysine (Sigma) in Neurobasal medium (Gibco) supplemented with B27 and 2 mM L-glutamine. Seven days later, cells were treated for 48 h with 10 nM MLi-2 or DMSO or for 8 h with 300 µM IC261. Cells were lysed with 1% SDS supplemented with protease and phosphatase inhibitors (Roche) and subjected to immunoblot analysis.

6.2. Supplementary figures



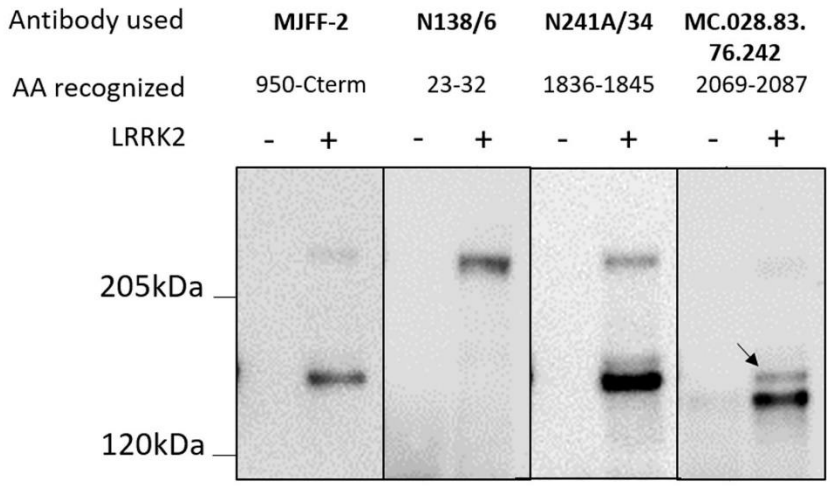


Figure 5.S1. - The ~170 kDa truncated LRRK2 variant in the kidney lacks an N-terminal part. Cell lysates from kidney tissue (C57Bl/6J WT or LRRK2$^{-/-}$ mice) were analyzed with immunoblotting using anti-LRRK2 MJFF-2 (ab133474), anti-LRRK2/Dardarin, N-terminus N138/6 (Neuromab 75-188), anti-LRRK2/Dardarin, C-terminus N241A/34 (Neuromab 75-253), anti-LRRK2 MC.028.83.76.242 (ab130277). The arrow indicates the presumed truncated LRRK2 variant.

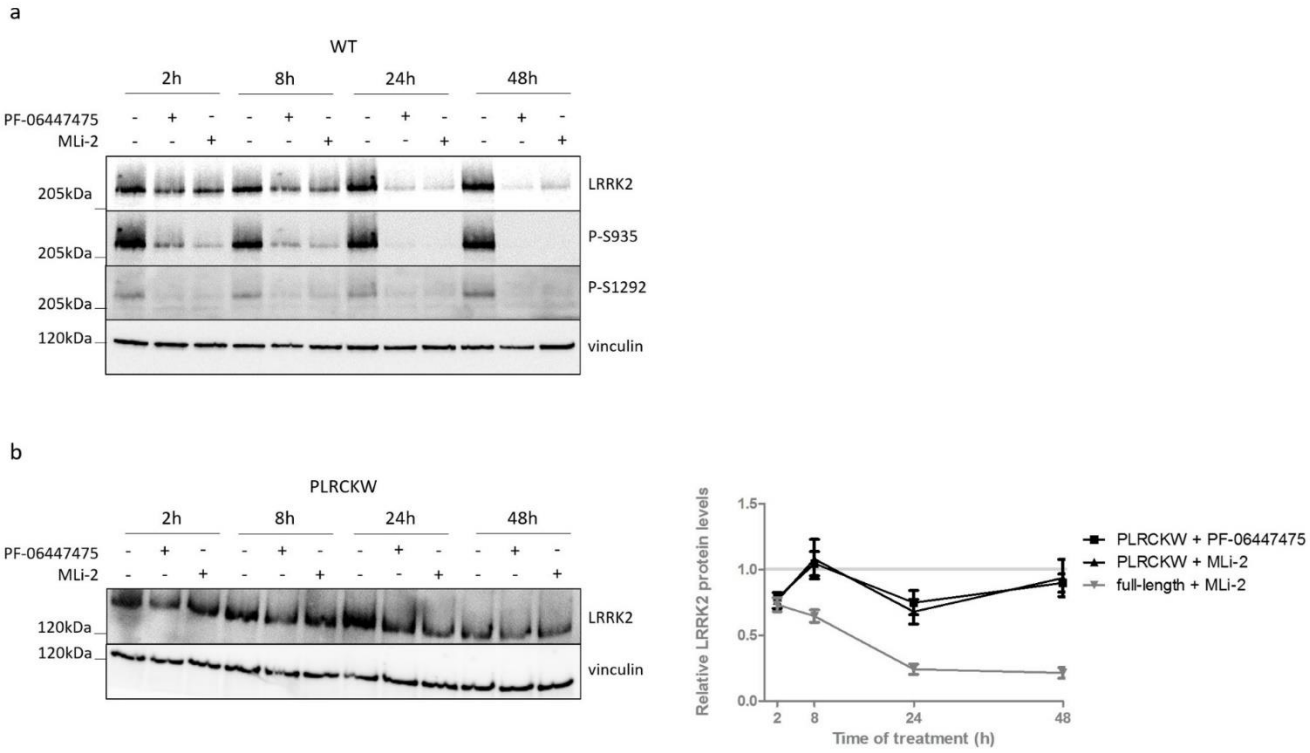


Figure 5.S2 - SH-SY5Y cells with overexpression of LRRK2 WT (**a**) or PLRCKW (**b**) were treated with LRRK2 kinase inhibitors PF-06447475 (150 nM), MLi-2 (10 nM) or DMSO as control for several periods of time. Cell lysates were analyzed with immunoblotting using FlagM2 antibody for LRRK2 detection, anti-LRRK2 P-S935, anti-LRRK2 P-S1292 or anti-vinculin for equal loading. Representative blots are shown. The graph show the quantification of blots representing the ratio of total LRRK2 over housekeeping protein signal. Error bars indicate S.E.M. with N≥3. Statistical significance was tested using a 2-way ANOVA test with a Bonferroni post-test.

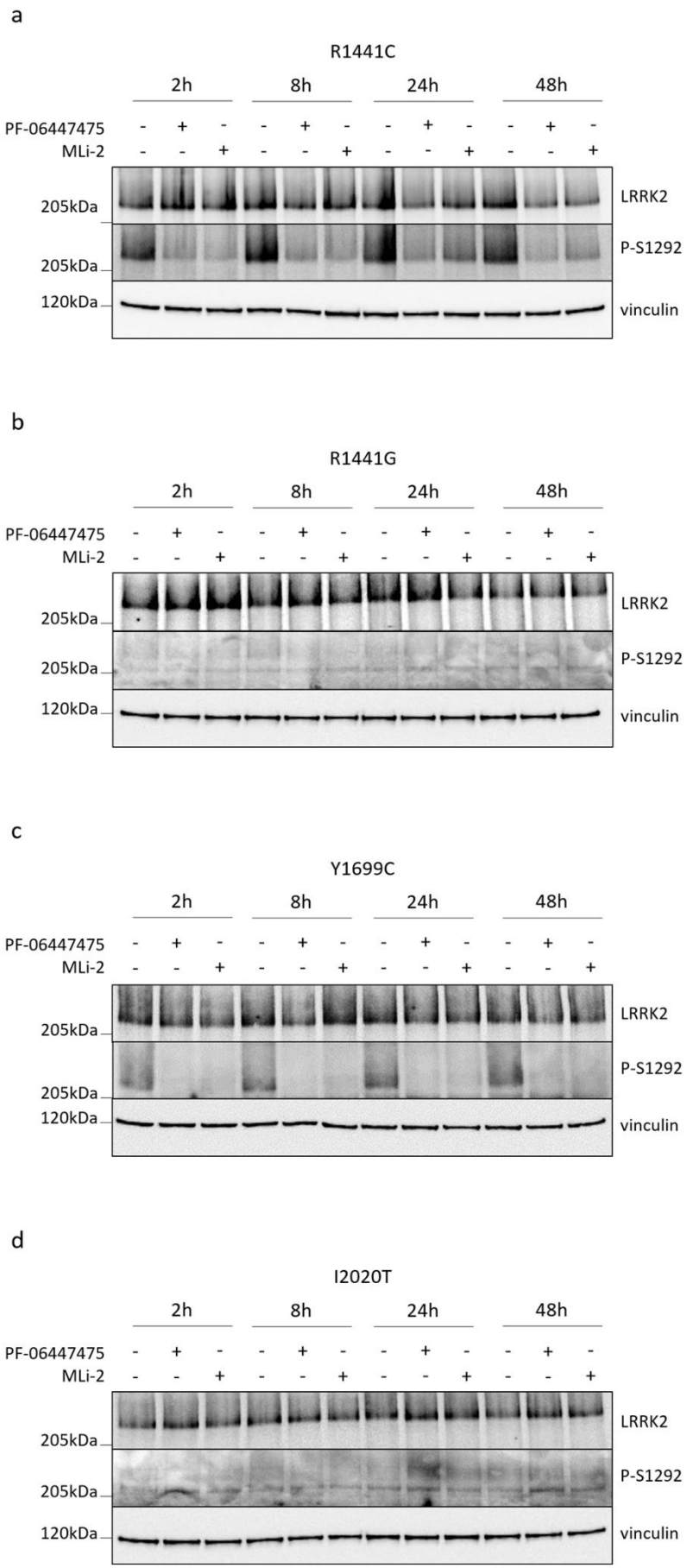


Figure 5.S3 – SH-SY5Y cells with overexpression of LRRK2 R1441C (**a**), R1441G (**b**), Y1699C (**c**) or I2020T (**d**) were treated with LRRK2 kinase inhibitors PF-06447475 (150 nM), MLi-2 (10 nM) or DMSO as control for several periods of time. Cell lysates were analyzed with immunoblotting using FlagM2 antibody for LRRK2 detection, anti-LRRK2 P-S1292 or anti-vinculin.

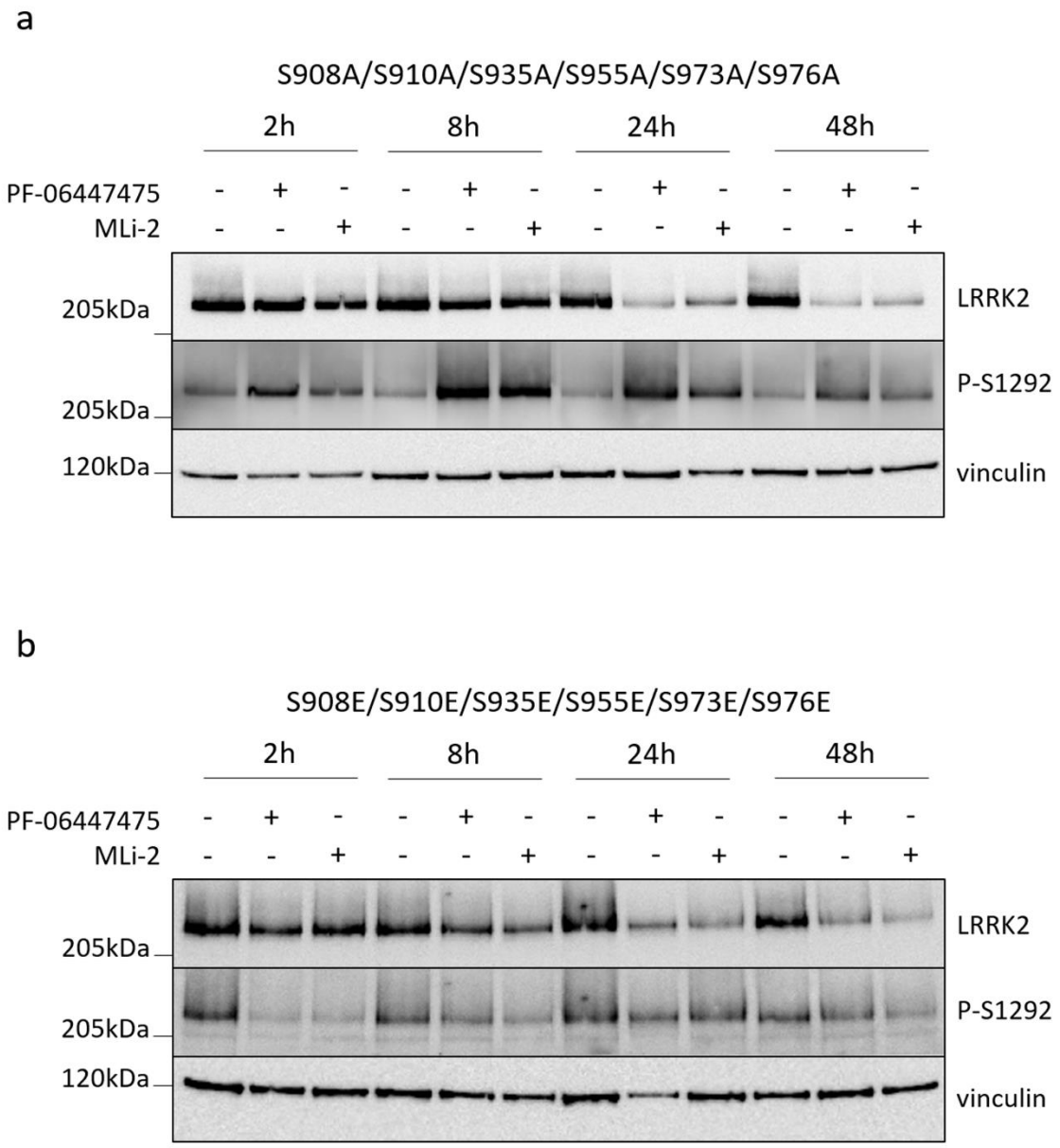


Figure 5.S4 - SH-SY5Y cells with overexpression of phosphomutant LRRK2 S908A/S910A/S935A/S955A/S973A/S976A (**a**) or S908E/S910E/S935E/S955E/S973E/S976E (**b**) were treated with LRRK2 kinase inhibitors PF-06447475 (150 nM), MLi-2 (10 nM) or DMSO as control for several periods of time. Cell lysates were analyzed with immunoblotting using FlagM2 antibody for LRRK2 detection, anti-LRRK2 P-S1292 or anti-vinculin.

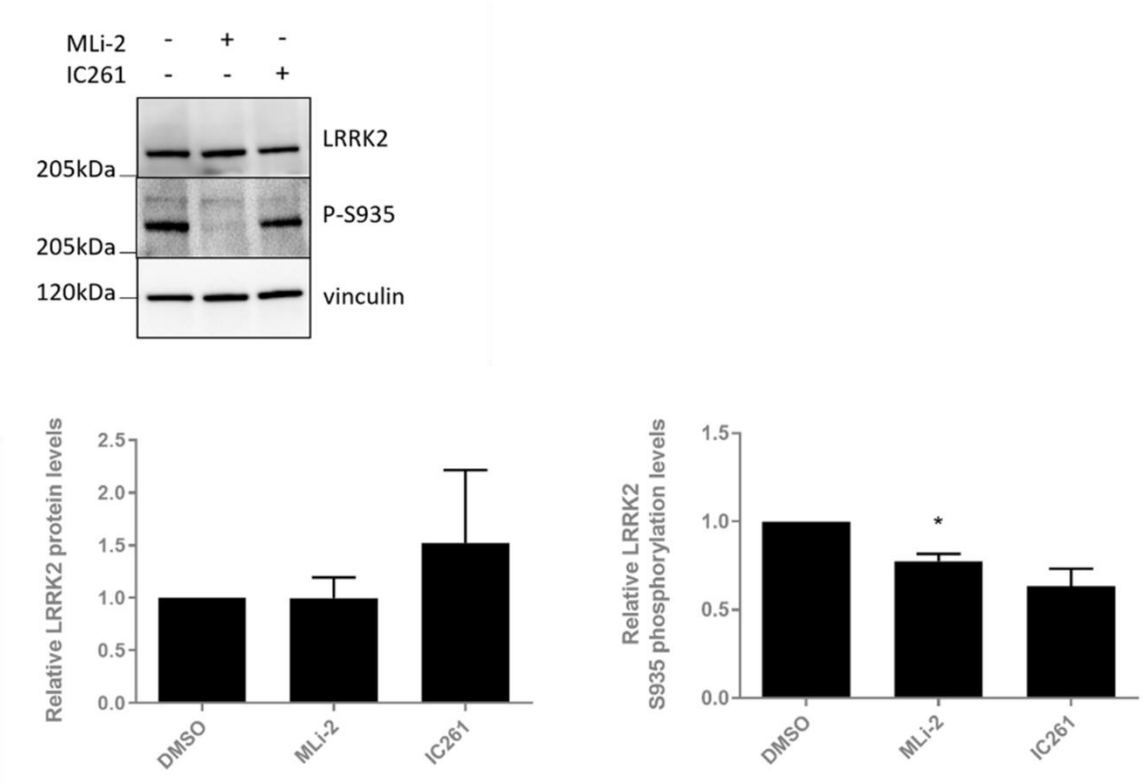


Figure 5.S5 - Primary cortical neurons were treated for 48 h with MLi-2 (10 nM) or 8 h with IC261 (300 µM). DMSO was used as negative control. Cell lysates were analyzed with immunoblotting using MJF-2 antibody for LRRK2 detection, anti-LRRK2 P-S935 or anti-vinculin for equal loading. Graphs show the quantification of blots representing the ratio of total LRRK2 over housekeeping proteins or the ratio of phosphorylation at S935 over total LRRK2 signal. Error bars indicate S.E.M. with N≥3. Statistical significance was tested using column statistics with Bonferroni correction. * $p<0.05$.

Chapter 6 – General discussion and future perspectives

Parkinson's disease (PD) is the second most common neurodegenerative disorder, and affects more than 7 million people worldwide. The degeneration of dopaminergic neurons in the SN leads to dopamine depletion in the striatum and motor dysfunction. Unfortunately, current treatment options are purely symptomatic and thus not able to slow down, stop or cure the disease. The exact etiology of PD remains poorly understood. However, a better understanding of PD etiology and pathology will be crucial to improve the quality of life of PD patients (eg. with regard to potential new disease-modifying therapies) and to lower the economic burden. The general ageing of the population, together with the fact that PD primarily affects people older than 60 years of age, will only further increase the socio-economic impact of PD.

In 10% of the PD-patients a clear genetic cause underlies the disease[34]. Mutations in more than 40 loci (the so-called 'PARK loci') have already been linked to monogenetic familial forms of PD. Almost 15 years ago, LRRK2 was identified as a PARK gene[43,44]. Missense mutations in the LRRK2 gene were later identified as the most common known cause of familial PD[43–45]. Besides, GWAS have identified LRRK2 as a risk factor for sporadic PD[69–71,324,329]. Clinicopathologically, the majority of LRRK2 patients is indistinguishable from idiopathic PD patients, therefore suggesting a possible common mechanism of disease. Consequently, studying both the physiological and pathological role of LRRK2 will be of major importance for the development of LRRK2-based PD disease-modifying therapies, which might be beneficial for both sporadic and familial PD patients. Moreover, LRRK2 has been implicated in other diseases like IBD, leprosy and cancer[46–50]. Although, it is unclear whether the same pathogenic mechanisms give rise to different diseases, studying the function of LRRK2 in PD might hold valuable information on the role of LRRK2 in other diseases as well. Over the years, the amount of information on LRRK2-(dys)function has increased enormously. Still, several questions remain unanswered, of which the most crucial one possibly is: "How does LRRK2 cause disease?". One possibility is that LRRK2 plays a role in the same cellular processes in both physiological and pathological conditions, where mutant LRRK2 would disturb this process in such a way that it eventually leads to cell death. On the other hand, it is possible that pathogenic LRRK2 affects an additional cellular function compared to wild type LRRK2. The fact that LRRK2 has already been associated with a multitude of cellular functions (discussed in Chapter 1.2.3.), only makes it more challenging to answer this question.

Remarkably, all pathogenic mutations of LRRK2 cluster in the catalytic core of the protein, which exists of two enzymatic functions: a GTPase and a kinase function. This suggests that an aberrant enzymatic LRRK2 function contributes to LRRK2 pathogenesis, which is further supported by the observation that most pathogenic mutations increase the LRRK2 kinase activity, both in cell culture and *in vivo*[132,135–138,315]. Moreover, studies have reported increased LRRK2 autophosphorylation in both urinary exosomes and in dopaminergic neurons of idiopathic PD patients[139–141]. Consequently, inhibition of this toxic gain-of-function was proposed as a potential disease-modifying therapy[152,153] and this has stimulated academic and industrial efforts to develop potent and selective LRRK2 kinase inhibitors. To

date, several independent studies have reported beneficial effects of LRRK2 kinase inhibition, *in vitro* and *in vivo*[132,143–145,151,315], resulting in a current phase I clinical trial. Moreover, LRRK2 kinase inhibition was also shown to be protective in a non-LRRK2-related PD model as it showed protection against α-synuclein toxicity in rodent brain[143]. Altogether, LRRK2 kinase inhibition is believed to offer great promises as a potential PD-disease-modifying therapy, yet some caution is warranted, as we might not yet fully comprehend all the consequences of LRRK2 kinase inhibition.

This PhD project has focused on two features that characterize LRRK2 kinase inhibition. First, LRRK2 kinase inhibition induces rapid dephosphorylation of LRRK2 autophosphorylation sites and remarkably, of LRRK2 heterologous phosphorylation sites as well[164,170]. Heterologous phosphorylation sites are sites that are exclusively phosphorylated by kinases other than LRRK2[134,163,293,303]. The potential relevance of dephosphorylated heterologous sites in PD pathogenesis is suggested by the fact that most pathogenic mutations display decreased heterologous phosphorylation levels[134,163,195,317], on the one hand. On the other hand, a study of Dzamko et al. showed that heterologous phosphorylation sites of LRRK2 are dephosphorylated in different brain regions, including the SNpc, of idiopathic PD patients[167]. Remarkably, dephosphorylation of heterologous phosphorylation sites can be observed both in pathogenic conditions and after application of a potential disease-modifying therapy. To date, it is not clear if this dephosphorylation might contribute to PD pathogenesis. Consequently, insight in the molecular regulation of (de)phosphorylation events will be crucial with regard to the further development of LRRK2 kinase inhibitors as a potential PD-therapy. Several kinases have already been proposed to phosphorylate the heterologous phosphorylation sites in LRRK2[168,170,172] (discussed in Chapter 1.2.6.2.). However, only one phosphatase has been linked to LRRK2 dephosphorylation so far. Our group has previously shown that dephosphorylation of heterologous phosphorylation sites during LRRK2 kinase inhibition or in the presence of pathogenic mutations is mediated by the catalytic subunit α of protein phosphatase 1 (PPP1Cα)[163]. Besides a catalytic subunit, specificity and activity of the PP1 complex is further determined by a regulatory subunit. As the regulatory subunit involved in dephosphorylation of heterologous LRRK2 phosphorylation sites remains unknown, we here aimed to identify the regulatory subunits of PP1 or other phosphatases important in the LRRK2 dephosphorylation cycle.

To identify these phosphatases, an siRNA screen was outsourced to Invitrogen. The effect of knockdown of phosphatases on LRRK2 phosphorylation at S935 was assessed. From the initial siRNA screen, we selected 39 proteins, with the greatest effect on LRRK2 phosphorylation at S935, for further validation. Interestingly, next to PP1, also PP2A related subunits showed great effects on LRRK2 phosphorylation as well as some tyrosine phosphatases. In order to further narrow down the selection of proteins, we set up a shRNA screen. First, we generated two shRNAs per protein of interest and cloned these in a miRNA-based plasmid, of which we produced LV vectors to transduce HEK293T cell with stable overexpression of LRRK2. We studied the effect of knockdown of the candidate proteins on LRRK2 heterologous phosphorylation at S935, both in basal conditions and after LRRK2 kinase inhibition and selected 12 proteins out of the original 39 (selection criteria are specified in Chapter

3.3.2.). Remarkably, our selection mainly included proteins of the PP2A complex, instead of regulatory subunits from the PP1 complex. It might not be surprising that both the PP1 complex and the PP2A complex seem to be involved in the LRRK2 dephosphorylation cycle, as both protein complexes are responsible for approximately 90% of all dephosphorylation processes in eukaryotic cells. However, the ubiquity of both PP1 and PP2A in the eukaryotic cell can also be considered a major drawback in studies using protein knockdown to determine phosphatase interactors for a protein of interest. First, knockdown of a common phosphatase might disturb the cellular processes in such a way that toxicity is induced rather quickly. On the other hand, other PP1 or PP2A subunits might take over the function of the lacking subunit, potentially masking the effect. This phenomenon should be kept in mind especially when working with stable cell lines, where cells are kept in culture for several weeks. However, it might be avoided when acutely transducing cells and studying them immediately afterwards. Yet, this might give rise to higher variation in the results, as each repetition experiment depends on an individual transduction event. Furthermore, both PP1 and PP2A have a large array of subunits available to determine specificity of the phosphatase complex. This highlights even more the importance of the correct identification of each subunit of the phosphatase complexes that are involved in the dephosphorylation of LRRK2 in physiological conditions, after LRRK2 kinase inhibition or in disease conditions. As the regulatory subunits determine the specificity and activity of the phosphatase complex, it will be of major importance to determine whether different regulatory subunits are involved in these different conditions.

We further confirmed the importance of the regulatory subunit family B of the PP2A complex in LRRK2 S935 dephosphorylation. We only observed significant effects on LRRK2 S935 phosphorylation after combined overexpression of PPP2Cα and PPP2R2A, PPP2R2B, PPP2R2C or PPP2R2D (four regulatory subunits from the B family) in HEK293 cells. Moreover, we generated SH-SY5Y cells with stable overexpression of LRRK2 and stable combined knockdown of one of these phosphatase complexes. To determine which phosphatase complexes could be involved in S935 dephosphorylation of LRRK2 during LRRK2 kinase inhibition, we treated these cells with the LRRK2 kinase inhibitor PF-06447475 and evaluated if knockdown could prevent LRRK2 kinase inhibitor-induced dephosphorylation. After short term LRRK2 kinase inhibition of 2 h, combined knockdown of the PP2A complexes tested (PPP2Cα and PPP2R2A, PPP2Cα and PPP2R2B, PPP2Cα and PPP2R2C) did not prevent LRRK2 kinase inhibitor-induced LRRK2 dephosphorylation at S935. It is possible that the PP1 complex is involved in early dephosphorylation of LRRK2, while the PP2A complex is recruited later. This is supported by data from our group showing the involvement of PP1 in LRRK2 dephosphorylation after 30-90 minutes of LRRK2 kinase inhibition. Moreover, we have now shown that knockdown of the three phosphatase complexes can prevent the dephosphorylation at S935 after 24 h of LRRK2 kinase inhibition. However, it should be noted that the high variation observed in this experiment might be partly explained by the second effect of LRRK2 kinase inhibition; LRRK2 protein degradation (and will be discussed further on).

Combined, our results in the first part of this project indicate the importance of the PP2A complex, next to the PP1 complex, in LRRK2 kinase inhibitor-induced dephosphorylation of heterologous phosphorylation sites. With regard to the development of potential new therapies for PD, it remains to be determined whether dephosphorylation of heterologous LRRK2 phosphorylation sites are an unwanted effect of LRRK2 kinase inhibition. If so, identification of the exact composition of the PP2A complex responsible for dephosphorylation after LRRK2 kinase inhibition is crucial. However, based on our results, several regulatory subunits of the B family of the PP2A complex might act on LRRK2 phosphorylation sites, especially after LRRK2 kinase inhibition. Consequently, insight in the degree of specificity of the PP2A complex for LRRK2 will be pivotal with potential PD therapies in mind. **In conclusion, we propose PP2A as an additional regulating phosphatase of the LRRK2 phosphorylation cycle, next to PP1** (Figure 6.1.)**.** Yet, further validation is crucial to determine the specific conditions in which each phosphatase operates on phosphorylation sites of LRRK2.

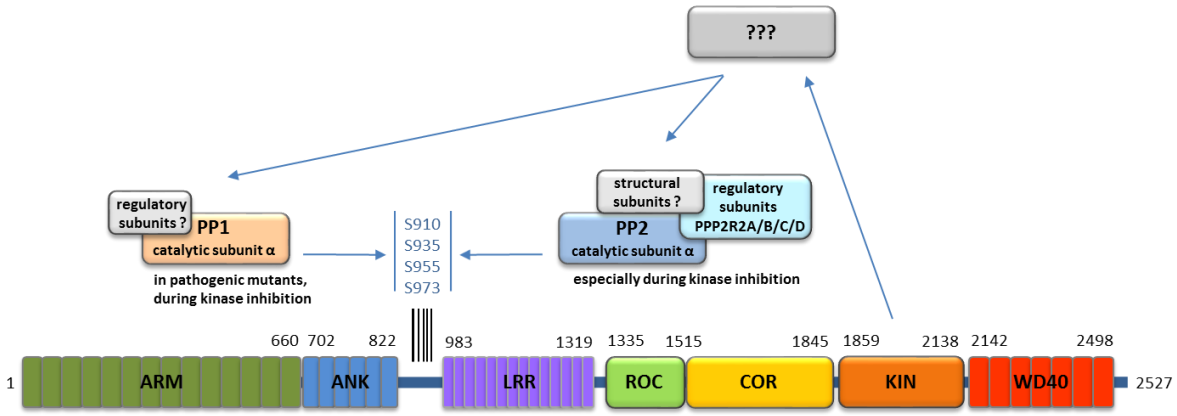


Figure 6.1. – Composition of phosphatase complexes proposed to act on heterologous LRRK2 phosphorylation sites.

A second consequence of LRRK2 kinase inhibition is a decrease in total LRRK2 protein levels. This event has long remained undetected, as LRRK2 kinase inhibition induces quick dephosphorylation of both autophosphorylation and heterologous phosphorylation sites[143,161,164,165,206]. Consequently, most studies with LRRK2 kinase inhibitors only applied short-term LRRK2 kinase inhibitor treatment. However, we and several other research groups have shown that sustained LRRK2 kinase inhibition can result in reduced LRRK2 protein levels[169,184,198,203–205]. In Chapter 4, we describe a gradual LRRK2 protein loss after LRRK2 kinase inhibition using six different LRRK2 kinase inhibitors[169]. This study was performed in SH-SY5Y cells overexpressing WT LRRK2 and is in line with the report from Zhao et al. (2015) in which HEK293T-Rex cells with overexpression of WT LRRK2 showed decreased total LRRK2 protein levels after treatment with the LRRK2 kinase inhibitor GNE1023[184]. Moreover, several studies have shown that LRRK2 kinase inhibition also induces LRRK2 destabilization *in vivo*. First of all, we show that four i.p. injections of the LRRK2 kinase inhibitor MLi-2 over two days induces LRRK2 protein loss in brain, lung and kidney of mice[169]. Furthermore, oral administration or in-diet dosing of LRRK2 kinase inhibitors for a period of 11 to 15 days was also reported to induce LRRK2 destabilization in mice. However, this was only observed in the kidney[204,205]. Longer term dosing induces also LRRK2 destabilization in mouse lung tissue (after administration of 15 weeks)[204], and in the brain of non-human primates (after 29 days)[205]. On the cellular level, LRRK2 kinase inhibition can induce morphological changes in the type II pneumocytes of the lung, which relate to lysosomal deficits. These changes were observed both in rodents[204] and non-human primates[205] that were treated with LRRK2 kinase inhibitors. Remarkably, analogous lysosomal abnormalities were observed in the lung, but also in the kidney of LRRK2 knockout animals[203,205,211,291,292,330]. This difference might be explained by our observation that a truncated LRRK2 variant is present in the kidney that cannot be destabilized by LRRK2 kinase inhibition and which is thought to be lacking in LRRK2 knockout animals. Potentially, this truncated LRRK2 variant may possess enough residual LRRK2-like (enzymatic) function during LRRK2 kinase inhibition to avoid the development of lysosomal defects.

Next, we have investigated the reversibility of LRRK2 destabilization after LRRK2 kinase inhibition. In Chapter 5, we show that both LRRK2 dephosphorylation at S935 and S1292 and LRRK2 protein loss are maintained during chronic LRRK2 kinase inhibition, but are reversible after removal of the inhibitor. This finding is in line with the normalization of the lung phenotype in non-human primates after removal of the LRRK2 kinase inhibitor[326]. Combined, these observations seem to indicate that LRRK2 kinase inhibition induces reversible LRRK2 destabilization and affects peripheral lysosomal functioning. Consequently, more insight in the exact mechanism of LRRK2 kinase inhibition will be pivotal with regard to its further development as a potential PD therapy.

In Chapter 4, we aimed to gain more insight in the mechanism by which LRRK2 is destabilized during LRRK2 kinase inhibition. In physiological conditions, LRRK2 is degraded via the proteasomal system[133,208,209,322] and we have now shown that LRRK2 kinase inhibition results in increased proteasomal degradation of LRRK2. This observation is in line with the report of Zhao et al. (2015) that LRRK2 kinase inhibition results in increased LRRK2 ubiquitination[184].

Next, we set out to identify the molecular mechanisms of LRRK2 kinase inhibitor-induced destabilization. First of all, in the kidney and in neutrophils, a truncated variant of LRRK2 can be detected, which is about 170 kDa in size[169,203]. We have observed that this truncated variant in the kidney becomes dephosphorylated, but not destabilized after treatment with LRRK2 kinase inhibitors[169]. We and others hypothesize that the N-terminus is lacking in this truncated variant, based on mass-spectrometry analysis and antibody detection. Consequently, we cloned artificially truncated LRRK2 variants lacking parts of the N-terminus, to further study them in cells. Interestingly, we found that truncated LRRK2 variants that were overexpressed in SH-SY5Y cells, show strongly reduced heterologous phosphorylation levels at S935 in basal conditions. Alternatively, the epitope for the P-S935 antibody might be inaccessible in these truncated variants, explaining the lack of phosphorylated S935 detection. Moreover, these truncated variants do not display protein loss after treatment with LRRK2 kinase inhibitors. Consequently, we propose the involvement of the N-terminus and of dephosphorylation in LRRK2 kinase inhibitor-induced destabilization. However, Fan and colleagues have reported a truncated variant of about the same size in neutrophils, which is quickly destabilized after LRRK2 kinase inhibitor treatment[207]. This observation highlights the importance of further investigation on the potential differential regulation of these truncated LRRK2 variants with relation to their specific sequence identity, distribution, expression and function.

More evidence for the importance of dephosphorylation of heterologous phosphorylation sites in LRRK2 kinase inhibitor-induced destabilization comes from the pathogenic variants of LRRK2. We examined whether LRRK2 kinase inhibitor-induced destabilization also applies for the pathogenic LRRK2 variants R1441C, R1441G, Y1699C, G2019S and I2020T. We observed LRRK2 protein loss after LRRK2 kinase inhibitor treatment in two out of five mutants tested; G2019S and R1441C. LRRK2 variants R1441G, Y1699C and I2020T did not show LRRK2 kinase inhibitor-induced destabilization, which is in line with the observation that these three mutants do not show increased ubiquitination upon LRRK2 kinase inhibition[184]. The two pathogenic LRRK2 mutants R1441C and G2019S are destabilized to the same extent as LRRK2 WT by LRRK2 kinase inhibition and show the same degree of heterologous phosphorylation at S935 (for G2019S) or only a modest reduction in S935 phosphorylation (for R1441C). On the other hand, LRRK2 variants R1441G, Y1699C and I2020T show a strong reduction in S935 phosphorylation in basal conditions[134,163,184]. These observations indicate the potential involvement of LRRK2 heterologous phosphorylation in LRRK2 kinase inhibitor-induced destabilization and should be taken into account in clinical studies as potential protective effects of LRRK2 kinase inhibitors could be mutation-specific. Moreover, we could only observe LRRK2 protein loss in WT, R1441C and G2019S LRRK2 starting after 8 h of LRRK2 kinase inhibitor treatment. This indicates that LRRK2 dephosphorylation precedes LRRK2 destabilization after LRRK2 kinase inhibition and further supports our above-mentioned hypothesis.

To further investigate which heterologous LRRK2 phosphorylation sites are crucial for the induction of LRRK2 kinase inhibitor-induced destabilization, we generated several phospho-dead LRRK2 variants. They were stably overexpressed in SH-SY5Y cells and consequently subjected to LRRK2 kinase inhibitor treatment. We observed LRRK2 protein loss after inhibitor treatment for both the S910A and the S935A variant, which indicates that the dephosphorylation of these two residues is not a crucial step in LRRK2 protein loss after LRRK2 kinase inhibition. Furthermore, our observations are in line with the observation of increased ubiquitination after LRRK2 kinase inhibition in the combined S910A/S935A mutant[184]. As the kinase CK1α was reported to control phosphorylation of six heterologous phosphorylated serine-residues (S908, S910, S935, S955, S973 and S976)[168], we next investigated the effect of CK1 inhibition on the stability of LRRK2. Interestingly, pharmacological CK1 inhibition did result in a stronger and faster reduction in WT LRRK2 protein levels than LRRK2 kinase inhibition. Moreover, we found a CK1 inhibition-induced decrease in LRRK2 protein levels in all LRRK2 variants tested which were insensitive to LRRK2 kinase inhibitor-induced destabilization. Indeed, LRRK2 protein loss after CK1 inhibition was observed in pathogenic or truncated LRRK2 variants that show strong dephosphorylation at S935 in basal conditions. Combined, these observations suggest that CK1 inhibition affects LRRK2 protein homeostasis more downstream than LRRK2 kinase inhibition and that heterologous phosphorylation under control of CK1 is important in the process. In Chapter 5 we show that CK1 inhibition induces a significant LRRK2 protein destabilization in the lung of mice, which we could not observe in the brain or kidney. As the CK1 inhibitor used in these experiments was not described to be specific for the α isoform of CK1, which was previously reported as a LRRK2 interactor[168], we cannot exclude the involvement of other CK1 isoforms in different tissues. In addition, low brain permeability of the compound cannot be excluded either, which might explain the lack of LRRK2 protein destabilization after CK1 inhibition in the brain. Moreover, we could not observe decreased protein levels of the truncated variant in the kidney after CK1 inhibition, while our artificial truncated LRRK2 variants were still sensitive to CK1 inhibition. Consequently, the potential dissimilarity of these artificial truncated LRRK2 variants compared to the truncated variant in the kidney of mice should be kept in mind. Altogether, these in vivo observations do correspond to the hypothesis that CK1 inhibition is involved in LRRK2 protein homeostasis, both in cell culture and *in vivo*.

We also generated a LRRK2 phospho-dead and phosphomimetic variant in which we mutated the six serine residues controlled by CK1α[168] and subjected these to LRRK2 kinase inhibition and CK1 inhibition. However, we still observed LRRK2 protein loss after LRRK2 kinase inhibition and after CK1 inhibition, indicating that LRRK2 kinase inhibitor-induced destabilization of LRRK2 is not crucially controlled by these six phosphorylation sites, but probably by a currently unknown phosphorylation site under control of CK1. Consequently, we propose CK1 as a new regulator of LRRK2 homeostasis**.** However, the CK1 inhibitors we used in this project, IC261 and PF-670462, are not specific for the α-isoform. Consequently, the involvement of other CK1 isoforms in the regulation of LRRK2 protein stability cannot be excluded and more research is necessary to exclude indirect or kinase-independent effects of CK1. **Altogether, we propose that LRRK2 kinase inhibition induces N-terminal changes that disrupt the LRRK2-CK1 interaction, leading to reduced heterologous phosphorylation and LRRK2 protein degradation. We hypothesize a currently unknown heterologous LRRK2 phosphorylation site as the primary inductor of LRRK2 kinase inhibitor- and CK1 inhibitor-induced LRRK2 protein destabilization (**Figure 6.2.)**.**

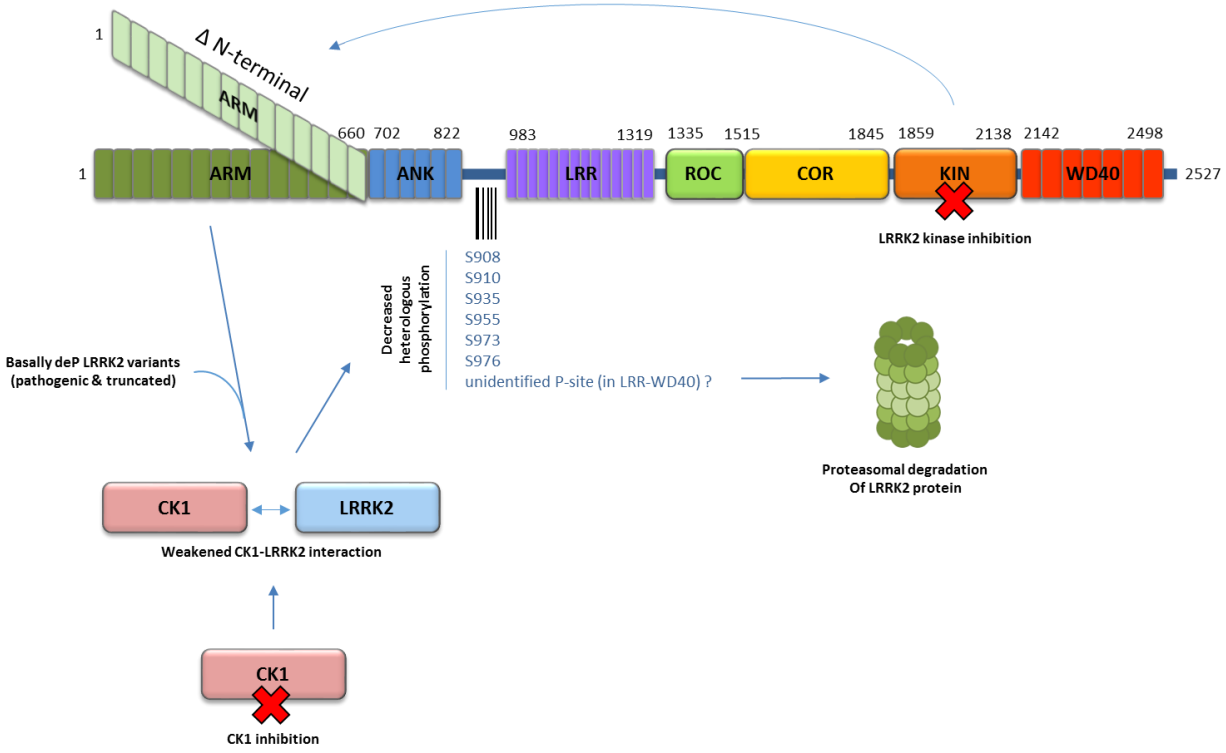


Figure 6.2. – Schematic representation of our hypothesis on LRRK2 kinase/CK1 inhibitor-induced destabilization of the LRRK2 protein, based on the findings discussed in Chapter 4 and 5.

Generally, the Goldilocks principle might apply when it comes to the relation between LRRK2 kinase activity, LRRK2 protein levels, LRRK2 kinase inhibition and toxicity. First, it was hypothesized that elevated LRRK2 kinase activity, which is characteristic to LRRK2 pathogenic variants, is detrimental. The link between pathogenic mutations and the induction of toxicity was repeatedly shown[74,142–145], which can be rescued by LRRK2 kinase inhibition[132,143–145,151,315]. However, Skibinski and colleagues hypothesized that the beneficial effects of LRRK2 kinase inhibition can be attributed to reduced LRRK2 protein levels, rather than to a reduction of its kinase activity *per se*[198].

On the other hand, a complete loss of LRRK2 protein is neither considered beneficial as LRRK2 knockout animals show kidney and lung pathology, which relate to lysosomal deficits[203,205,211,291,292,330]. Remarkably, LRRK2 kinase inhibition induces a reminiscent pathology in the lung, but not in the kidney[204,205]. We propose that this discrepancy originates from our observation that a truncated LRRK2 variant is present in the kidney, which is not destabilized after LRRK2 kinase inhibition, and not present in LRRK2 knockout animals. It might be possible that this truncated LRRK2 variant displays enough residual LRRK2 kinase activity in the kidney to prevent the development of kidney pathology after LRRK2 kinase inhibition, although binding and (partial) inhibition of a LRRK2 kinase inhibitor cannot be excluded either. However, it remains to be determined whether the kidney truncated LRRK2 variant displays kinase activity, which is comparable to full length WT LRRK2. This possibility is further supported by the observation that heterozygous LRRK2 knockout mice do not present morphological lung changes[203]. Combined, this suggests that a modest reduction of LRRK2 protein levels can by-pass the observed lung phenotype. Furthermore, the idea of therapeutic reduction of LRRK2 protein levels is recently supported by the finding that LRRK2 antisense oligonucleotides diminish the formation of α-synuclein inclusions, a typical hallmark of PD, in a PD mouse model[212]. Consequently, more insight in the regulation of LRRK2 protein homeostasis is crucial. In conclusion, this PhD project provides important new insights in the molecular regulation of LRRK2 protein homeostasis and stability, especially after LRRK2 kinase inhibition, and in the potential side effects of LRRK2 kinase inhibition, which are essential for the further development of disease-modifying therapies.

Chapter 7 – References

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Chapter 8 – Curriculum vitae

Tina DE WIT (°08/05/1991, Duffel, Belgium)

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Education

2015-present	**PhD in Biomedical Sciences**, KU Leuven, Leuven, Belgium
2014 (5 months)	**Pre-doctoral student in Biomedical Sciences**, KU Leuven, Leuven, Belgium Promotor: Prof. Dr. Veerle Baekelandt Laboratory for Neurobiology and Gene Therapy Subject: Insight in the molecular consequences of LRRK2 kinase inhibition
2012-2014	**Master in Biochemistry & Biotechnology**, KU Leuven, Leuven, Belgium Promotor: Prof. Dr. Veerle Baekelandt Laboratory for Neurobiology and Gene Therapy Subject: Characterization of phosphorylation and stability of leucine-rich repeat kinase 2 (LRRK2), a protein important in Parkinson's disease
2009-2012	**Bachelor in Biochemistry & Biotechnology**, KU Leuven, Leuven, Belgium

Professional positions

2013 (6 months):	External internship at Galapagos nv (Mechelen, Belgium) Supervisor: Luc Nelles Subject: Development and application of tests for evaluating antibiotic characteristics of chemical compounds

Trainings

Scientific Integrity (KU Leuven)
Safety, health and environment in the laboratory (KU Leuven)
Advanced molecular & cellular medicine: viral vector technology (KU Leuven)
Course for animal sciences (KU Leuven)
Radiation protection (KU Leuven)
Creating effective posters (KU Leuven)

Teaching experience

Laura Carrera Carballes: master thesis student biomedical sciences (academic year 2015-2016)
Senna Staessens: labrotation student biomedical sciences (april 2015)

Scientific awards & honors

Travel grant 'Broaden Your Horizon' (for the conference 'LRRK2: ten years along the road to therapeutic intervention' – Greenlands, UK (2016)
VLAIO doctoral fellowship (2015-2018)

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PP2A holoenzyme regulates LRRK2 phosphorylation and expression.
Article in preparation

De Wit, T., Baekelandt, V. and Lobbestael, E. (2018) Inhibition of LRRK2 or casein kinase 1 results in LRRK2 protein destabilization. *Molecular Neurobiology*, 2018 Dec 27, DOI: 10.1007/s12035-018-1449-2.

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Conference proceedings (attended *)

Lobbestael, E., **De Wit, T.**, Baekelandt, V. Inhibition of LRRK2 or casein kinase induces LRRK2 protein destabilization. 2-4 September 2018. Biennial International LRRK2 meeting. Padova, Italy.

Lobbestael, E., Civiero, L., **De Wit, T**., Taymans, J.-M., Greggio, E., Baekelandt, V.. Pharmacological LRRK2 kinase inhibition induces LRRK2 protein destabilization and proteasomal degradation. 14-17 June 2017. Mechanisms of neurodegeneration. Heidelberg, Germany.

De Wit, T.*, Lobbestael, E., Bolliger, M., Drouyer, M., Chartier-Harlin, M.-C., Nichols, J. Taymans, J.-M., Baekelandt, V.. Phosphatome-wide siRNA screen reveals phosphorylation regulators of the Parkinson's disease protein LRRK2. 22 May 2017. The 12th National Congress of the Belgian Society for Neuroscience (BSfN). Ghent, Belgium.

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Lobbestael, E., Civiero, L., **De Wit, T.**, Taymans, J.-M., Greggio, E., Baekelandt, V.. Pharmacological LRRK2 kinase inhibition induces LRRK2 protein destabilization and proteasomal degradation. 29 March – 2 April 2017. The 13th International Conference on Alzheimer's & Parkinson's Disease. Vienna, Austria.

Drouyer, M., Emanuele, M., Lobbestael, E., Bolliger, M., **De Wit, T.**, Baekelandt, V., Chartier-Harlin, M.-C., Nichols, J., Taymans, J.M.. Comparative analysis of PP1 and PP2A as regulators of LRRK2 phosphorylation. 29 March – 2 April 2017. The 13th International Conference on Alzheimer's & Parkinson's Disease. Vienna, Austria.

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De Wit, T.*, Lobbestael, E., Bolliger, M., Drouyer, M., Chartier-Harlin, M.-C., Baekelandt, V., Nichols, J., Taymans, J.-M.. Phosphatome-wide siRNA screen for LRRK2 phosphorylation regulators. Leucine-rich repeat kinase 2: Ten Years Along the Road to Therapeutic Intervention. 11-13 July 2016. Greenlands, United Kingdom.

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Lobbestael, E., Deman, S., **De Wit, T.**, Taymans, J.-M., Baekelandt, V.. LRRK2 kinase inhibition reduces endogenous LRRK2 protein levels *in vivo*. Society for Neuroscience. 15-19 November 2014. Washington, DC, United States of America (oral presentation).