Instituto de Parasitología y Biomedicina "López-Neyra"
CSIC
Universidad de Granada

# Estudio de la Fosforilación de parkina y sus implicaciones en la Enfermedad de Parkinson

Elena Rubio de la Torre Gil
Tesis Doctoral
2009

Instituto de Parasitología y Biomedicina "López-Neyra"
Consejo Superior de Investigaciones Científicas

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# *Estudio de la fosforilación de parkina y sus implicaciones en la enfermedad de Parkinson*

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Dª: Sabine Nicole Navarro Hilfiker, Investigadora científica del Departamento de Biología Molecular del Instituto de Parasitología y Biomedicina "López-Neyra" del CSIC en Granada:

CERTIFICA que D.ª Elena Rubio de la Torre Gil, Licenciada en Farmacia y en Bioquímica, ha realizado bajo su dirección y en el Departamento de Biología Molecular del Instituto de Parasitología y Biomedicina "López-Neyra" del CSIC en Granada, el trabajo titulado: Estudio de la fosforilación de parkina y sus implicaciones en la enfermedad de Parkinson, reuniendo el mismo las condiciones necesarias para optar al grado de Doctor Europeo por la Universidad de Granada.

En Granada , a 10 de Julio del 2009

Vº Bº Directora

La interesada

Sabine N. Navarro Hilfiker

Elena Rubio de la Torre Gil

Portada: Brainbow system. Livet et al 2007.
Mouse Dentate gyrus neurons expressing brainbow transgene.

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de lo que yo misma soy capaz de ver.

# ÍNDICE

# I. RESUMEN:

Parkinson's disease (PD) is a progressive and substantially disabling neurodegenerative disorder [1]. Its clinical symptoms primarily result from the progressive and selective death of dopaminergic neurons of the substantia nigra pars compacta. Besides cell death, a pathological hallmark of PD in surviving neurons comprises Lewy bodies, ubiquitylated intraneuronal inclusions rich in α-synuclein [2]. Even though largely a sporadic disorder, there are several genes associated with inherited forms of PD. One commonly implicated is PARK2, the gene encoding for parkin [3]. Mutations in the parkin gene are responsible for a large percentage of autosomal recessive juvenile parkinsonism [4, 5].

Parkin functions as an E3 ubiquitin ligase [6], and inactivation of its catalytic activity may lead to dopaminergic cell death due to accumulation of its toxic substrates. Studies suggest that changes in parkin solubility comprise the major mechanism of parkin inactivation, both in familial and sporadic PD [7-9]. In addition, some post-translational modifications lead to dramatic changes in parkin solubility, highlighting a mechanism for parkin inactivativation [7, 8].

This thesis work demostrates that parkin is subject to compound phosphorylation by Casein Kinase 1 and Cdk5 *in vitro* and in cultured cells. Such compound phosphorylation enhances its insolubility, leading to aggregation and concomitant inactivation. Although phosphorylation does not change parkin's E3 ligase activity, increased aggregation effectively decreases the amount of soluble parkin protein able to exert a neuroprotective role. An increase in parkin phosphorylation was observed in brain samples from PD brains as compared to controls patients in caudate, with no

changes in cortex and no detection of phosphorylated parkin in cerebellum. The neuroanatomical differences in parkin phosphorylation between control and PD correlate with the relative extent to which these distinct brain areas are affected by disease pathology.

Further, an increase in p25, the activator of Cdk5, was observed in the caudate of PD when compared to control samples, indicating that such increase may lead to parkin phosphorylation.

Importantly, compound inhibition of kinase activity was found to display beneficial effects in decreasing the aggregative properties of pathogenic parkin mutants.

In conclusion, the results presented in this thesis indicate that regulating the phosphorylation status of parkin has beneficial effects in reducing parkin aggregation and concomitant inactivation. These findings may help in the design of novel therapeutic strategies against PD.

# II. ABREVIATURAS:

Ab: Antibody (Anticuerpo)

AD: Alzheimer's disease (Enfermedad de Alzheimer)

ARS: Aminoacyl tRNA synthetase (Aminoacil tRNA sintetasa)

ATP: Adenosine triphosphate (Adenosín trifosfato)

BAG: Bcl-2 associated gene (Gen asociado a Bcl-2)

Bcl-2: B cells lymphoma-2 (Linfoma de células B tipo 2)

Cdk5: Cyclin dependent Kinase 5 (Quinasa dependiente de ciclina 5)

CK1: Casein Kinase I (Caseína Quinasa I)

CL: Cuerpo de Lewy

COR: C-Terminal of ROC (Extremo C-terminal de ROC)

CRL: Cullin RING Ligase (Ligasa RING Culina)

DA: Dopamine (Dopamina)

DAT: Dopamine transporter (Transportador de Dopamina)

ER: Endoplasmic reticulum

LB: Lewy Body

LBD: Lewy body dementia (demencia con cuerpos de Lewy)

EGFR: EGF Receptor (Receptor de EGF)

EP: Enfermedad de Parkinson

FBP-1: Far upstream Binding Protein 1

GSH: Glutathione (Glutatión)

GTP: Guanosine triphosphate (Guanosín trifosfato)

HECT: Homologous to E6-associated protein C-terminus

IBR: In between RING

IKKγ: Inhibitor of Nuclear Factor-κB γ

JNK: c-Jun N-terminal Kinase (Quinasa asociada al extremo N-terminal de c-Jun)

K: Lysine (Lisina)

KO: Knock-out

LN: Lewy neurites (Neuritas de Lewy)

LRRK2: Leucine rich repeat Kinase 2 (Quinasa con repeticiones ricas en leucina 2)

MAPK: Mitogen Activated Protein Kinase (Proteína quinasa activada por mitógenos)

MPP+: 1-metil-4-fenilpiridinium (1-methil-4-phenil-piridinium)

MPTP: 1-metil-4-fenil-1,2,3,6-tetrahidropiridina (1-methil-4-phenil-1,2,3,6-tetrahidropyridine)

NF-κB: Nuclear factor κB (Factor nuclear κB)

NO: Nitric Oxide (Óxido nítrico)

6-OHDA: 6-Hidroxy- Dopamine (6-Hidroxi-Dopamina)

Pael-R: Putative endothelin like Receptor

PD: Parkinson's Disease

PINK-1: PTEN-induced kinase 1

PI3K: Phosphoinositide-3-Kinase (Fosfoinosítido 3-Quinasa)

PKA: Protein Kinase A (Proteína quinasa A)

PKC: Protein Kinase C (Proteína Quinasa C)

PolyUb: Polyubiquitylation (Poli-Ubiquitinación)

PDZ: PSD-95 Disc large Zone ocludens

PSD-95: Post-synaptic density 95

PTEN: Phosphatase and tensin homologue

RE: Retículo endoplásmico

RIR: RING In between RING

ROC: Ras in complex protein

ROS: Reactive oxygen species (Especies reactivas del oxígeno)

α-SN: α-Synuclein (α-Sinucleína)

α-Sp22: Glycosylated form of α-Synuclein (Forma glicosilada de α-Sinucleína)

TNF: Tumor Necrosis Factor (Factor de necrosis tumoral)

TRAF2: TNF Receptor-associated factor 2 (Factor asociado al receptor de TNF 2)

Ub: Ubiquitin (Ubiquitina)

UBL: Ubiquitin like domain (Dominio con similitud a ubiquitina)

UCH: Ubiquitin carboxy terminal hydrolase (Hidrolasa de ubiquitina en el extremo C-terminal)

WB: Western Blotting

# INTRODUCCIÓN

# III. INTRODUCCIÓN:

## 1- ENFERMEDAD DE PARKINSON:

### 1.A- Síntomas y etiología:

La enfermedad de Parkinson (EP) fue descrita por primera vez por James Parkinson en 1817, en su "Tratado sobre la parálisis agitante". En la actualidad, es el trastorno del movimiento más frecuente, afectando a un 1-2% de individuos cuya edad ronda los 65 años [10]. La edad media de comienzo es de 55 años y la incidencia aumenta significativamente con la edad. En España, unas 65.000 personas están diagnosticadas, aunque se estima que el número total de afectados podría superar los 100.000 (datos obtenidos de la Federación Española de Parkinson).

Los principales síntomas clínicos son temblor en reposo, bradiquinesia, rigidez o aumento del tono muscular, y trastornos posturales [1].

Los síntomas clínicos aparecen como consecuencia de una pérdida progresiva y selectiva de las neuronas dopaminérgicas en el sistema nervioso central. La pérdida neuronal parece tener lugar por apoptosis, aunque también se han descrito procesos inflamatorios [11]. Se cree que el proceso patológico se produce de forma ascendente e implica al sistema límbico, al visceromotor y al somatomotor. La progresión de la enfermedad se puede clasificar en seis fases según el estudio patológico de Braak y colaboradores [12].

En la primera fase de la enfermedad, se ve afectado el núcleo dorsal motor del nervio vago (cerebro medio, medula oblongata), así como algunas zonas del sistema nervioso entérico [13]. Los síntomas motores comienzan en las fases 3 y 4, cuando las neuronas dopaminérgicas de la sustancia negra pars compacta comienzan a morir (cerca de un 60%). Se produce una depleción de dopamina y sus metabolitos (hasta un 80% cuando los síntomas comienzan) en el estriado, donde estas neuronas proyectan. Las fases 5 y 6 son las más severas, los pacientes presentan el registro completo de síntomas clínicos asociados con la enfermedad (Figura 1) [12].

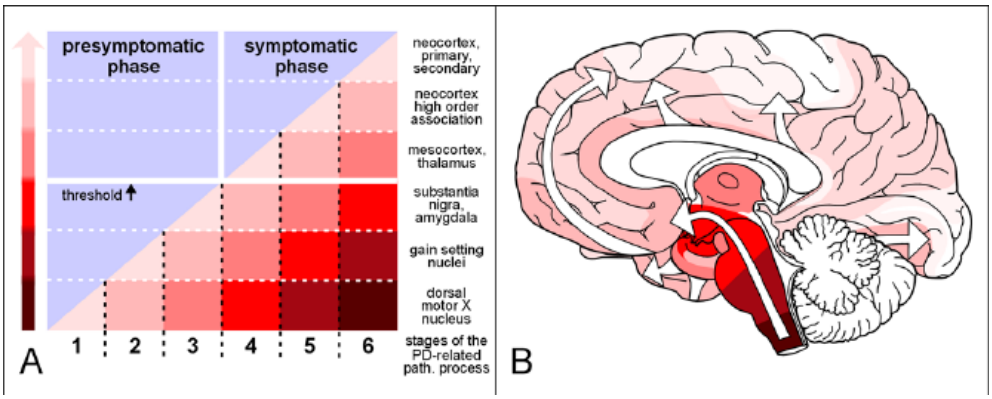


**Figure 1:** Adaptado de [12]. PD presymptomatic and symptomatic phases. **A.** The presymptomatic phase is marked by the appearance of Lewy bodies or Lewy neurites. In the symptomatic phase, the individual neuropathological threshold is exceeded (black arrow). The increasing slope and intensity of the colored areas below the diagonal indicate the growing severity of the pathology in vulnerable brain regions (right). The severity of the pathology is indicated by darker degrees of shading in the colored arrow left. **B.** Diagram showing the ascending pathological progress (white arrows). The shading intensity of the colored areas correspond to that in A.

La forma progresiva en la que las diferentes áreas del cerebro se ven afectadas, concuerda generalmente con la aparición de los síntomas en los pacientes, tanto los tempranos como tardíos.

La hipótesis de Braak y colaboradores, aunque ampliamente extendida, está sujeta a controversia. Diferentes estudios patológicos muestran presencia de inclusiones de α-SN en zonas del cerebro no descritas por Braak [14-16]. Además, también se pueden encontrar inclusiones fibrilares de α-SN en cerebros de pacientes de edad avanzada sin síntomas de EP, con una prevalencia cercana al 20% [14-17]. Estos datos sugieren que la aparición de inclusiones de α-SN no sólo representaría una fase preclínica de la EP, si no también un fenómeno no maligno asociado con el envejecimiento.

## 1.B- Cuerpos de Lewy:

El principal marcador histopatológico de la EP son los cuerpos de Lewy (CL), inclusiones citoplasmáticas eosinofílicas, compuestas de un núcleo denso, rodeado por un halo, que aparecen en las neuronas supervivientes de los cerebros de pacientes con EP (Figura 2) [18].

La microscopía electrónica revela que el centro de los CL contiene un material granular denso mientras que el halo periférico presenta una disposición ordenada de filamentos en forma de radio [19]. α-SN es su principal componente, aunque también contienen ubiquitina (Ub) y son ricos en neurofilamentos [20]. Otros componentes son elementos del sistema ubiquitín proteosoma y proteínas de choque térmico [19]. Los CL clásicos presentan α-SN en el halo que rodea al núcleo, el cual es rico en Ub. Están presentes en diferentes tipos de células neuronales y suelen tener forma ovalada. Además de estos cuerpos, se pueden observar diversos tipos de inclusiones ricas en α-SN, las cuales se piensa que representan diferentes fases en la evolución de los CL [21-23] Las estructuras con una tinción tipo nube representarían una fase temprana, éstas evolucionarían a unas estructuras más compactas, positivas para ubiquitina, llamadas cuerpos pálidos, los cuales se compactarían aún más para formar el CL clásico [18].

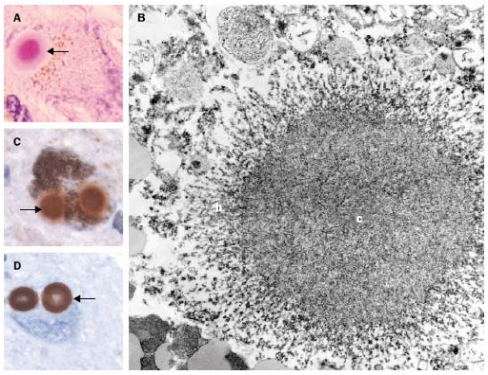


**Figure 2**: From [19].
Demonstration of Lewy bodies in substantia nigra dopaminergic neurons in sporadic PD. **A**. Conventional haematoxylin (blue) and eosin (pink) histological staining reveals a spherical Lewy body (arrow) with a distinct central core and a peripheral halo. **B**. Electron micrograph of a Lewy body reveals that the core contains granular material and the outer halo (h) is composed of radiating filaments. **C, D**. Standard immunohistochemical protocol shows two Lewy bodies (arrow) with the ubiquitin concentrated in the core (**C**) and two Lewy bodies (arrow) with α-synuclein concentrated in the halo (**D**).

Las neuritas de Lewy (LN) (Figura 3), son terminales distróficas que también se tiñen para α-SN y que están presentes en los cerebros de pacientes con EP. En algunos casos, los estudios muestran LN y no CL, por lo que son empleadas como un marcador más sensible en el estudio histopatológico de la enfermedad [20].

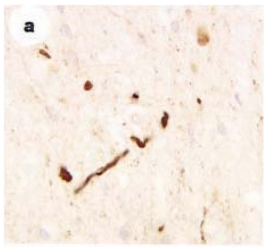


**Figure 3:** From [20]. α-synuclein-positive Lewy neurites in the substantia nigra from patients with DLB.

Como en otros trastornos degenerativos donde aparecen cuerpos de inclusión, el papel de los CL en la viabilidad neuronal se encuentra sometido a un intenso debate. La principal característica de la EP es la pérdida selectiva de neuronas dopaminérgicas y la presencia de CL en las neuronas supervivientes. Dicha asociación lleva a la teoría de que la aparición de inclusiones de α-SN es tóxica para las neuronas. *In vitro*, la oligomerización y la formación de protofibrillas de α-SN es tóxica para las células (para revisión ver [24], más detallado en el apartado 1.C.2.A), pero no se ha demostrado *in vivo*. La presencia de inclusiones de gran tamaño, podría además alterar la arquitectura celular, modificando así diversas funciones esenciales.

Sin embargo, no existe una asociación clara entre la muerte neuronal y la aparición de inclusiones de α-SN [25]. La presencia de CL en pacientes sin EP [14], sugiere que dichas inclusiones pueden no ser tóxicas. Además, se ha observado que las neuronas que presentan CL, son más grandes y parecen mucho más sanas que las que no los contienen [26, 27]. Por lo que la formación de los CL podría representar un mecanismo de defensa mediante el cual la célula secuestraría moléculas potencialmente tóxicas.

La imposibilidad de determinar qué es causa y qué es efecto mediante estudios histopatológicos y la falta de un modelo tanto celular como animal que

recapitule todas las características morfológicas básicas de los CL, no ha permitido aclarar el papel de estas inclusiones en la viabilidad celular.

## 1.C- Formas de la enfermedad:

Clínicamente, cualquier enfermedad que curse con una disminución de DA en el estriado, o con daño directo en esta zona, provoca el llamado síndrome parkinsoniano. La EP es la causa más frecuente de este síndrome, representando cerca del 80% de los casos [1]. La enfermedad tiene dos formas básicas, la idiopática y la hereditaria:

### 1.C.1 Parkinson idiopático:

El 90-95% de los casos de EP son considerados como esporádicos o idiopáticos [28] ya que no tienen una relación genética aparente. Se cree que la disfunción mitocondrial y el estrés oxidativo juegan un papel fundamental en su patogénesis [29-31]. Existen dos evidencias principales que implican la disfunción mitocondrial y la EP. La primera es la disminución de la actividad del complejo I de la cadena de transporte de electrones en las neuronas dopaminérgicas de la sustancia negra de pacientes con EP respecto a controles [32, 33]. La segunda es, que el DNA mitocondrial de las neuronas dopaminérgicas de pacientes con EP presenta una tasa de deleción mayor, lo que podría producir un déficit de las proteínas implicadas en la cadena de transporte. El análisis de la sustancia negra de pacientes con EP muestra un incremento del estrés oxidativo, reflejado en una elevación de los niveles de hierro [34], de peroxidación lipídica [35] y de oxidación de DNA y proteínas [36, 37], así como una disminución en los niveles de GSH [38, 39]. Además, las neuronas de la sustancia negra están sometidas a un estrés oxidativo crónico [40], debido a factores como la elevada actividad aeróbica en el cerebro, la autooxidación de la dopamina [41] y los niveles de hierro incrementados [29].

Se ha visto que ciertos factores ambientales incrementan el riesgo de padecer EP. De esta observación ha surgido la "hipótesis ambiental", en la cual agentes químicos presentes en el ambiente, podrían dañar de forma selectiva las neuronas dopaminérgicas, contribuyendo así al desarrollo de la enfermedad [42]. Existe una

fuerte asociación entre la exposición prolongada a pesticidas y el desarrollo de la EP [43-45]. La Rotenona, un insecticida lipofílico, produce inhibición del complejo I de la cadena de transporte de electrones [46] y despolimerización de microtúbulos [47]. Otra molécula que tiene el mismo mecanismo de acción y que está ampliamente demostrado que produce EP, es MPP+ (metabolito activo de MPTP) [48, 49]. Dieldrina y Maneb son otros dos pesticidas que inhiben de forma selectiva el complejo III de la cadena de transporte de electrones [50]. Se ha visto que la concentración de Dieldrina en el cerebro de pacientes con EP es mayor que en el de controles [51] y que la exposición crónica a Maneb provoca síndrome parkinsoniano crónico [52]. La exposición crónica a Paraquat, un herbicida, también incrementa el riesgo de padecer EP [52], ejerciendo toxicidad mediante la producción de ROS [53, 54].

### 1.C.2 - Parkinson hereditario:

Entre un 5-10% de los casos con los síntomas clínicos de EP tienen un historial familiar positivo y compatible con herencia mendeliana (autosómico dominante o autosómico recesivo). Hasta la fecha, se han descrito seis formas monogénicas de la enfermedad [55] (Tabla 1).

| LOCUS | CHROMOSOME | GENE | INHERITANCE | PROBABLE FUNCTION |
|---|---|---|---|---|
| PARK1/4 | 4q21.3 | α-synuclein | AD | Presynaptic protein/chaperone |
| PARK2 | 6q25.2-27 | Parkin | AR | Ubiquitin E3 ligase |
| PARK5 | 4p14 | UCH-L1 | AD | Ubiquitin C-terminal hydrolase |
| PARK6 | 1p35-36 | PINK-1 | AR | Kinase |
| PARK7 | 1p36 | DJ-1 | AR | Chaperone |
| PARK8 | 12p11.2-q13.1 | LRRK2 | AD | Kinase |

**Table 1**: Loci involved in genetic PD, chromosomic location, gene, inheritance and possible function.

1.C.2.1- α-sinucleína:

Es el primero de los genes asociados con la EP que fue descrito, y su modo de herencia es autosómico dominante. Hasta la fecha se han descrito tres mutaciones puntuales asociadas con la enfermedad (A53T, A30P y E46K) [56-58], multiplicaciones de la secuencia original [59], y variaciones en la secuencia del gen y del promotor, asociadas con un mayor riesgo de padecer la enfermedad [60, 61].

Parece ser que su papel fisiológico está relacionado con la dinámica de las vesículas sinápticas [62-65], la liberación de DA [66] y con el aprendizaje y la plasticidad sináptica [67].

Aunque las mutaciones y multiplicaciones en este gen son extremadamente raras, la importancia radica en que α-SN es el mayor componente de los CL. Por lo que se piensa que la EP idiopática y la genética comparten mecanismos patológicos comunes.

α-SN es una proteína que no posee una estructura definida en condiciones fisiológicas [68]. En solución aparece totalmente desplegada como monómeros [69], y en las células es mayoritariamente citosólica soluble, aunque una parte se encuentra unida a membranas [70, 71] (Figura 4). Puede encontrarse en forma de dímeros y oligómeros [72, 73]. Los oligómeros forman protofibrillas [74]. Una vez que éstas alcanzan un determinado umbral, se forman las llamadas fibrillas, las cuales darían lugar a la formación de agregados irregulares por un mecanismo aún no conocido [74-78].

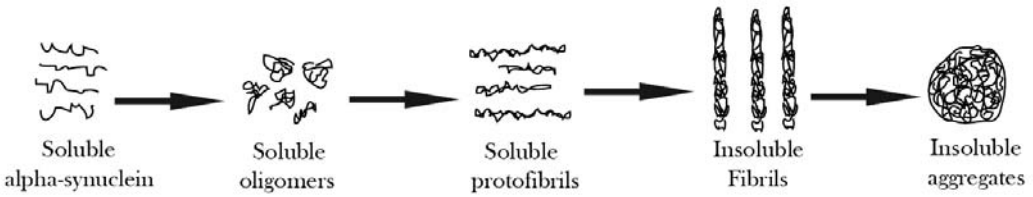


**Figure 4**: Aggregatory mechanism of α-SN. In normal cells, α-SN is in an unfolded and soluble monomeric state. Through disease-associated mechanisms, a transition to various insoluble forms is possible.

Los agregados fibrilares de α-SN son el principal componente de los CL [20]. Además, en pacientes con EP, está incrementada la fracción de especies oligomerizadas de α-SN asociada a membranas [79] y es mayor la proporción de α-SN insoluble, comparada con controles [70]. Se ha visto que los oligómeros y protofibrillas podrían ejercer toxicidad mediante su unión a membranas, formando poros que permeabilizarían las vesículas [80], también alterando el aparato de Golgi y los lisosomas [81], o mediante inhibición del proteosoma [82, 83], reduciendo así la viabilidad celular.

La agregación de α-SN se ve inducida por un incremento en su concentración, que aumenta la tasa de fibrilación *in vitro* [84] y por alteraciones [85] y mutaciones patológicas en la secuencia [86, 87]. Además, la expresión de α-SN en animales de experimentación induce pérdida de neuronas dopaminérgicas y la aparición de inclusiones que contienen α-SN [88, 89].

También se ve inducida por tóxicos ambientales como Paraquat y Rotenona [90], los cuales promueven la formación de agregados de α-SN cuando son administrados a animales de experimentación [91] [92, 93]. *In vitro* se ha demostrado que la agregación de α-SN se ve incrementada por iones metálicos [94], disolventes orgánicos [95], policationes [96] y por oxidación y nitrosilación [97, 98].

La fosforilación de la proteína también parece desempeñar un papel en la agregación. α-SN es fosforilada en la serina 129 por Caseína quinasa II [99]. Dicha fosforilación promueve la formación de fibrillas *in vitro* [100]. Bajo condiciones normales, α-SN se encuentra en su mayoría en la forma no fosforilada, pero sí lo está en los agregados procedentes de pacientes con patología por CL [100, 101], así como en las inclusiones de α-SN presentes en moscas y ratones que sobreexpresan la proteína [102, 103]. Sin embargo, la fosforilación no tiene por qué suponer toxicidad celular. Los últimos resultados obtenidos en ratas que expresan la forma pseudofosforilada de la proteína muestran menor pérdida neuronal que con la forma no fosforilable [104, 105]. La forma no fosforilable induce mayor toxicidad [104, 105] mediante la formación de formas filamentosas insolubles de α-SN [104].

1.C.2.2- LRRK2 (Leucine Rich repeat Kinase II):

Otro gen asociado con la EP, con herencia autosómica dominante es LRRK2 [106-108]. Las mutaciones en este gen suponen un 5-6% de los casos de parkinson hereditario y cerca de un 2% de los esporádicos [109]. El comienzo de la enfermedad es tardío, con síntomas no diferenciables de los de la EP idiopática. La patología que presenta es pleomórfica, variando incluso entre individuos de una misma familia que poseen la misma mutación [110].

La proteína tiene una estructura en multi-dominios y pertenece a la familia de proteínas ROCO (Figura 5). Desde el extremo N- al C-terminal posee un dominio LRR (Leucine Rich Repeat), un dominio Roc (Ras on complex), seguido de uno COR (C-terminal of Roc), un dominio MAPKKK (Microtubule Associated Protein Kinase Kinase Kinase) y un dominio WD40 (Figura 5). El dominio Roc pertenece a la superfamilia de GTP-asas pequeñas. El dominio MAPKKK pertenece a la familia de quinasas con similitud a tirosin-quinasas (tyrosine kinase-like, TKL), su secuencia tiene homología tanto con serín-treonín quinasas como con tirosín-quinasas, pero sólo fosforilan residuos de serina o treonina [111]. La presencia del dominio quinasa y el GTP-asa sugiere que la proteína tiene actividad enzimática, mientras que la existencia de los dominios LRR y WD40 implican un posible papel como proteína de andamiaje [112, 113].

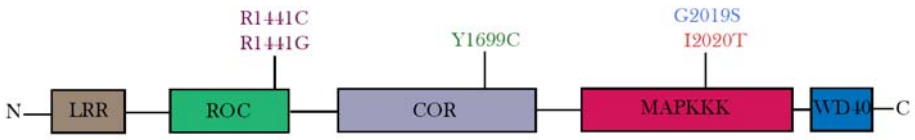


**Figure 5**: LRRK2 domain structure and pathogenic mutations. LRRK2 encodes a leucine rich repeat domain (LRR), a GTP-ase domain (Roc) with a COR (C-terminal of Roc) domain, a Kinase domain and a WD40 domain. Pathogenic mutations are depicted above the domain structure.

LRRK2 se expresa en diversas regiones neuronales del cerebro [114]. A nivel subcelular su localización es citoplasmática y está presente en las terminales, donde se asocia con vesículas y estructuras membranosas [115]. Este patrón de

localización también se ha descrito en células en cultivo que sobrexpresan la proteína, cultivos de neuronas primarias y cerebro de roedores [116, 117]. Se encuentra enriquecida en la fracción microsomal y en la asociada a vesículas sinápticas y mitocondria [115], lo que sugiere un papel en la dinámica de dichas vesículas y en funciones inherentes al Golgi, lisosomas y mitocondria.

LRRK2 es una quinasa capaz de llevar a cabo autofosforilación [116] y fosforilación de sustratos modelo [118]. Mientras que se ha demostrado que la mutación patogénica G2019S incrementa la actividad quinasa, [119, 120], las otras mutaciones, tanto en el dominio quinasa, como en el Roc y COR, están sujetas a controversia, ya que parecen incrementar o no alterar dicha actividad [113]. LRRK2 también tiene actividad GTP-asa, hidrolizando GTP [121, 122]. Las mutaciones patogénicas dentro de los dominios Roc y COR resultan en un descenso de dicha actividad [121, 122]. Mientras que la actividad GTP-asa es independiente de la actividad quinasa, la proteína requiere que dicha actividad esté intacta para poder fosforilar [121].

Su expresión en células provoca toxicidad, que induce muerte celular por apoptosis [117, 119]. Aunque también provoca reducción de la longitud y de la ramificación de neuritas mediante activación de autofagia [120, 123].

1.C.2.3-PINK-1 (PTEN-induced Kinase 1):

Mutaciones homocigóticas y heterocigóticas compuestas en el gen de PINK-1 causan parkinsonismo juvenil autosómico recesivo [124, 125].

El gen de PINK-1 codifica para una proteína que se expresa de forma ubicua en cerebro humano [126]. Su estructura en dominios muestra un motivo de señalización mitocondrial, el cual dirige la proteína a la mitocondria, aunque también se puede encontrar de forma citosólica [127, 128], y un dominio serín-treonín-quinasa [129].

PINK-1 tiene un papel neuroprotector dependiente de su actividad quinasa frente a diferentes estímulos provenientes de la mitocondria, como estrés oxidativo [130], señales proapoptóticas [131] o el cambio del potencial de membrana mitocondrial [132]. Por lo que debe ser fundamental en el mantenimiento de la

funcionalidad y la integridad estructural de la mitocondria y en la protección frente a su disfunción [133].

1.C.2.4. DJ-1:

Mutaciones en su gen producen parkinsonismo juvenil autosómico recesivo [134, 135]. La frecuencia de aparición es baja, ya que sólo supone un 1-2% de los casos de parkinsonismo juvenil [136]. La proteína se expresa de forma ubicua tanto en el cerebro como en tejidos periféricos [137] y forma homodímeros [138]. Su localización es mayoritariamente citoplasmática, aunque también está presente en mitocondria y núcleo [139] . DJ-1 tiene capacidad protectora y está involucrada en diferentes procesos celulares. Funciona como antioxidante, mediante auto-oxidación de sus cisteínas [140], protegiendo frente a la muerte celular [141], siendo la células que no expresan DJ-1 más sensibles al estrés oxidativo inducido por diversos tóxicos [142].

1.C.2.5. UCH-L1 (Ubiquitín carboxi terminal hidrolasa):

Es otro de los genes descritos con herencia autosómica recesiva. Su mutación (I93M) fue descrita en una única familia con síntomas similares a los de la EPI [143]. También se ha descrito un polimorfismo que da lugar al cambio de un aminoácido (S18Y), el cual tiene un posible efecto protector frente a la enfermedad [144]. La proteína es una de las más abundantes en el cerebro y se localiza exclusivamente en neuronas [145]. En un principio fue identificada como una ubiquitina carboxi-terminal hidrolasa [143]. Posteriormente se han descrito dos actividades más, como E3 ubiquitín ligasa (cuando se encuentra en forma de dímero) [146] y otra como estabilizador de mono-ubiquitina, asociándose a ella y evitando su degradación [147]. Las tres actividades de UCH-L1 implican a la proteína en la vía de degradación de proteínas por el proteosoma y en señalización celular mediante mono-ubiquitinación.

En resumen; los genes asociados con las formas hereditarias de la EP descritos hasta la fecha están involucrados en la supervivencia celular mediante

procesos de fosforilación, control del plegamiento de proteínas, agregación y mantenimiento de la integridad mitocondrial.

# 2- PARKINA:

## 2.A- Modo de herencia y mutaciones descritas:

Descrita por primera vez en 1998, se ha demostrado que las mutaciones en el gen que codifica para parkina (PARK2) en ambos alelos, co-segregan con la enfermedad, que tiene un modo de herencia autosómico recesivo, asociado a un comienzo temprano del trastorno [3]. Las mutaciones en este gen son la causa más frecuente del parkinsonismo juvenil en pacientes con una historia familiar positiva, variando el porcentaje entre un 5% y un 53% dependiendo del estudio [5],[148],[149], [150]. En los casos de parkinson esporádico de comienzo temprano, el porcentaje de mutaciones en parkina oscila entre un 9% y un 33% [148], [151], [152], [153]. Sin embargo, también han sido descritas variaciones en la secuencia de este gen en pacientes con un comienzo de la enfermedad tardío con [154],[150] y sin antecedentes familiares [155].

También aparecen mutaciones heterocigóticas (en un único alelo) en pacientes con la enfermedad [156-158]. Aunque si estas mutaciones heterocigóticas son la causa o suponen un incremento del riesgo de padecer el trastorno no está claro.

PARK2 se compone de 12 exones y de largas regiones intrónicas, su tamaño es de 1380Kb, siendo el segundo gen más grande del genoma humano después del de la distrofina [159]. Se han descrito cerca de 100 mutaciones diferentes en el gen, algunas de las cuales son recurrentes entre diferentes grupos étnicos. Entre éstas se incluyen mutaciones puntuales, microinserciones y microdeleciones, duplicaciones-triplicaciones y deleciones de uno o más exones [160]. También se han detallado mutaciones que afectan a sitios de *splicing* [156], [153], [161], así como diferentes polimorfismos.

## 2.B- Síntomas y patología:

El parkinsonismo juvenil autosómico recesivo (AR-JP) se caracteriza por un comienzo temprano de la enfermedad (antes de los 40-45 años), con una edad media de iniciación de 24 [162]. Las autopsias de pacientes con mutaciones en parkina revelan una muerte selectiva de las neuronas pigmentadas de la sustancia negra y del locus ceruleus, así como gliosis y depósitos de neuromelanina intra y extraneuronales [163], [164]. Al contrario que en la EP idiopática, el estudio patológico muestra ausencia de CL [163],[164], [165], [166], [167], aunque su presencia si ha sido descrita en algunos pacientes [168]. Las evidencias sugieren que parkina sería necesaria para la formación de los CL, ya que si no está presente, no aparecen dichas inclusiones. En los pacientes que sí se observó su presencia, pudo detectarse la proteína truncada, la cual podría ser parcialmente activa, contribuyendo así a la formación de dichos cuerpos [168], [169], [28].

## 2.C- Ubiquitinación y la vía ubiquitín proteosoma:

Existe una asociación entre la EP y la vía ubiquitín-proteosoma (VUP). Una de las evidencias directas es que la mutaciones que provocan una pérdida de actividad en el gen que codifica para parkina producen parkinsonismo juvenil autosómico recesivo, ya que parkina es una E3 ligasa de ubiquitina [6]. Además, junto con α-SN, la Ub es uno de los componentes mayoritarios de los CL [20].

La regulación de los niveles basales de proteínas que hay en la célula dependen de su tasa de síntesis y degradación. Tal degradación puede tener lugar en el citosol y en los lisosomas [163], [164]. Para que una proteína sea degradada en el citosol debe mostrar algún tipo de señal, como secuencias específicas de reconocimiento, exposición de residuos hidrofóbicos debido a la pérdida de plegamiento o alguna modificación post-traduccional como fosforilación u oxidación [170].

Las proteínas marcadas con Ub (una proteína de 76 aminoácidos) generalmente son degradadas por el proteosoma [171-173]. En el primer paso de la ruta, una enzima E1 activa la Ub con gasto de ATP, formando un enlace tiol-ester E1-ubiquitina. Éste es reconocido por una enzima E2 o enzima conjugadora de Ub,

la cual transporta la Ub activada a una enzima E3 o ligasa de Ub, encargada de transferir la Ub al sustrato, confiriendo especificidad al proceso de degradación. La Ub se une covalentemente a la proteína objeto de degradación mediante un enlace isopeptídico entre la glicina del extremo carboxi-terminal de la Ub y el grupo ε-amino de una lisina en la proteína a degradar (Figura 6) [170]. Para que la proteína sea degradada por el complejo proteosoma 26s, es necesaria una cadena de al menos 4 residuos de Ub. Para conseguirla, se vuelve a formar un enlace isopeptídico entre la glicina terminal de la Ub y el grupo ε-amino de un residuo de lisina de la molécula de Ub previamente unida.

El complejo proteosoma 26s se compone de dos subunidades principales, la tapa 19s y el núcleo 20s [174]. La tapa 19s elimina la cadena de Ub y despliega el péptido para que pueda entrar por el poro al núcleo 20s, donde reside la actividad proteasa. Los polipéptidos son procesados en pequeños péptidos que pueden salir por el poro. La Ub es reutilizada mediante la acción de las enzimas ubiquitín-carboxi-terminal hidrolasas (UCH) (Figura 6) [170].

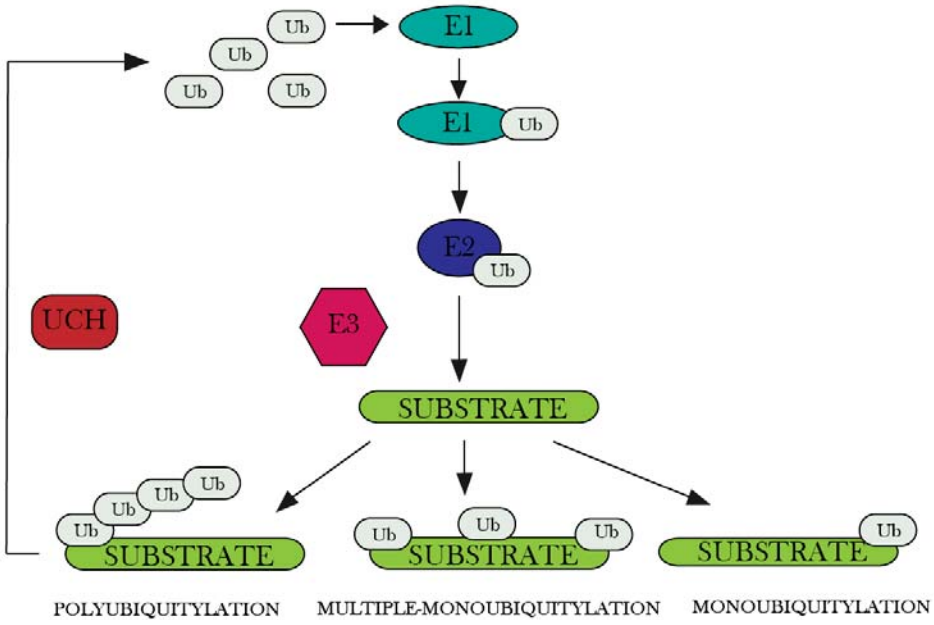


**Figure 6**: Ubiquitylation process. A three-step cascade mechanism where an E1 enzyme activates Ub. One of the several E2 enzymes transfers the activated Ub from E1 to an E3 ligase, that transfers the Ub to a substrate protein. In some cases, proteins are modified by a single Ub moiety (monoubiquitylation) or multiple Ubs in different lysine residues within the same protein (multiple-monoubiquitylation). In other cases, the first Ub moiety is bound to new Ub moieties, leading to chain elongation (polyubiquitylation).

La Ub tiene 7 residuos de lisina (lisinas 6, 11, 27, 29, 33, 48 y 63), lo que posibilita la formación de diferentes tipos de cadenas, resultando en una amplia gama de señales celulares. La poliubiquitinación clásica es la que usa la lisina 48 (K48) de la Ub para la extensión de la cadena, y que está ligada a la degradación de proteínas mediante el proteosoma [175]. Sin embargo, se han descrito diversos tipos de cadenas [176], llamadas atípicas (Figura 7).

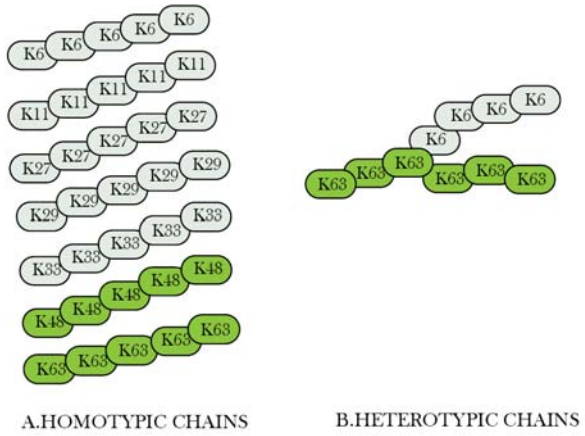


**Figure 7**: A schematic model of possible Ub chain formations on a target protein. **A.** Homotypic atypical (blue) and typical chains (green). **B.** Mixed-linkage atypical chains are formed by the use of different lysine residues for sequential Ub conjugation, leading to the formation of bifurcated chains.

Las cadenas formadas mediante el empleo del mismo residuo de lisina en la Ub son las llamadas "homotípicas", mientras que las formadas mediante el ensamblaje de diferentes residuos dentro de la molécula de Ub, son llamadas "de unión mixta" [177](Figura 7).

Aunque *in vivo* se ha visto que todas las lisinas de la molécula de Ub pueden ser usadas bajo diferentes condiciones, aún no se conoce su significado biológico. Aparte de K48, la cadena típica más estudiada ha sido la formada mediante la lisina 63 (K63), la cual está involucrada en la traducción de señales mediante la via NF-κB, endocitosis de receptores y reparación de daño en el DNA [178, 179] (Figura 8). La formación de cadenas mediante lisina 29 y 33 (K29 y K33) está implicada en la regulación de la familia de quinasas AMPK, mediante bloqueo de la fosforilación del segmento de activación por ubiquitinación [180], aunque K29 también se ha visto implicada en la degradación lisosomal de proteínas [181].

La monoubiquitinación y la multiplemonoubiquitinación están involucradas fundamentalmente en la internalización y tráfico de receptores de membrana [182].

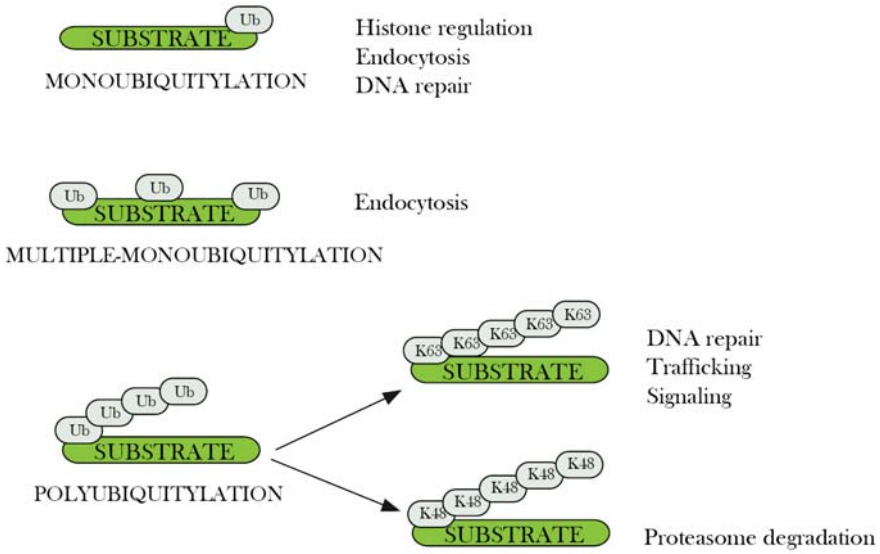


**Figure 8**: Physiological implications of protein ubiquitylation. Depending on the number of Ub moieties attached (mono-versus polyubiquitylation) and the mode of Ub linkage, ubiquitylation serves various physiological functions.

Existen más E2s que E1s y más E3s que E2s, por lo que en cada paso, el número de proteínas involucradas aumenta, confiriendo especificidad al proceso de ubiquitinación. La enzima E1 es producto de un solo gen, que da lugar a dos isoformas [183]. Las enzimas E2 son miembros de una familia que comparten un dominio central conservado, el cual confiere la capacidad de formar enlaces tiol-ester con Ub [184]. Las E2 más simples sólo poseen este dominio y pertenecen a la clase I. Otras poseen extensiones tanto en el extremo amino (clase II) como en el carboxilo (clase III) o en ambos (clase IV). Dichas extensiones confieren especificidad a la hora de interaccionar con diferentes E3 ligasas [185, 186]. Aunque la mayoría de las E2 no tienen selectividad por un residuo de lisina aceptor en concreto [186], algunas si pueden estimular la formación de ciertos tipos de cadenas. Un ejemplo es el complejo MMS2-UBC13 [187]. En este modelo, UBC13 es catalíticamente activa, cuando se ubiquitina, se une a MMS2, una pseudo-E2 con capacidad de unir una Ub aceptora. En consecuencia, la Ub aceptora es posicionada de tal manera que sólo la K63 es accesible para ser transferida desde la enzima UBC13 al sustrato, formando exclusivamente cadenas de Ub K63 [187].

## 2.D- Estructura y función de parkina como E3 ligasa:

Parkina es una proteína de 465 aminoácidos y 52 KD con una estructura modular conservada a lo largo de la evolución, tanto en vertebrados como en invertebrados. Los 76 primeros aminoácidos de la proteína comenzando por el extremo amino terminal comparten un 62% de homología con Ub y forman el dominio UBL (Ubiquitin Like Domain) (Figura 9) [188]. Posee un dominio central UPD (Unique Parkin Domain) no presente en otras proteínas, dentro del cual se ha identificado recientemente un nuevo dominio RING (Real Interesting New Gene), llamado RING0 [189]. El extremo carboxi-terminal contiene dos dominios RING finger, que flanquean un dominio central rico en cisteína llamado IBR (In Between RING) [3]. Tanto los dominios RING como el IBR son capaces de unir zinc, lo cual parece ser importante para la estabilidad de la proteína [189, 190]. Los tres últimos aminoácidos de parkina (FDV) en el extremo carboxi-terminal pertenecen a la clase II de motivos de unión a dominios PDZ (PSD-95 (Post synaptic density 95)/disc large/ZO (zone occludens)-1).

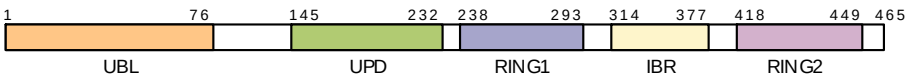


**Figure 9**: Schematic representation of parkin domain structure, with domain boundaries shown by aminoacid residue numbers above. UBL (Ubiquitin Like Domain), UPD (Unique Parkin Domain), RING (Real Interesting New Gene), IBR (In Between Ring).

*In vitro*, parkina tiene actividad E3 ubiquitín-ligasa en colaboración con las enzimas E2 conjugadoras de Ub UbcH7 [6], UbcH8 [191], Ubc4 [192], Ubc6 y Ubc7 [193] y UbcH13/Uev1a [194], [195]. La interacción con las enzimas E2 es llevada a cabo con el dominio RING [191, 196], siendo la cisteína 418 del dominio RING2 imprescindible para la actividad catalítica [192]. La proteína tiene actividad aunque el dominio UBL no esté presente [197].

Las E3 ligasas se clasifican en 3 tipos según su estructura: Las que tienen dominios HECT (Homologuos to E6-associated proteín C-Terminus), las que contienen dominios RING y las que contienen dominios U-box [198, 199] (Figura

10). Dentro de las RING E3 ligasas existen dos subclases, las RIR (RING In between RING-RING) y las que forman parte de complejos multiproteicos CRL (Cullin-RING Ligase) [198]. Su estructura determina su mecanismo de actuación.

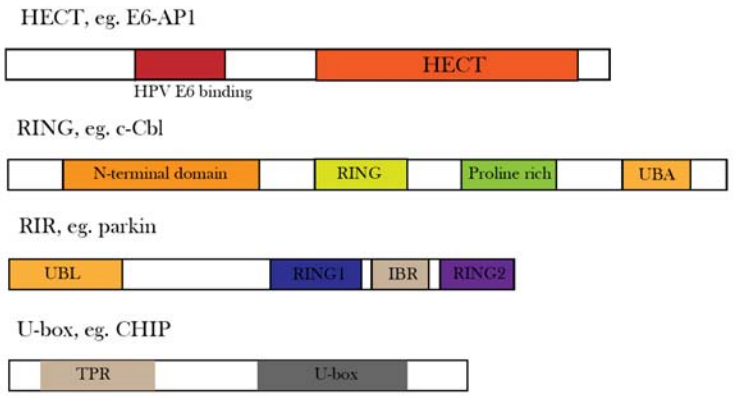


**Figure 10**: Domain structure of E3s. Structures of the main E3 ligases, HECT, RING, RIR and U-box, an example of each type is shown. RING domains of the RIR E3s are of the RING-HC type. Most E3 also contain other protein interaction domains. These vary widely between members of the same E3 type. HPV (Human PapillomaVirus), UBL (Ubiquitin Like Domain), TPR (TetratricoPeptide Repeats).

Las E3 ligasas que tienen dominios HECT poseen un residuo de cisteína en el centro del dominio que actúa como aceptor de Ub, formándose un enlace entre ambos antes de ser transferida al sustrato (Figura 11). En el extremo C-terminal es donde tiene lugar la actividad catalítica, mientras que el amino-terminal es el encargado de la unión al sustrato [200, 201]. Las E3 que contienen dominios RING son las más abundantes y complejas y parece que no tienen un papel catalítico directo en la ubiquitinación del sustrato, sino que actúan como proteínas de andamiaje facilitando la interacción entre la enzima E2 y la proteína aceptora [185] (Figura 11). El dominio RING es rico en cisteína e histidina. Este tipo de dominios se ha visto que promueve las interacciones proteína-proteína y proteína-DNA. La presencia de una histidina o una cisteína en la posición 5 del dominio da lugar a una subdivisión, RING-H2 o RING-HC respectivamente. Parece ser que este residuo tiene importancia estructural en el reconocimiento de la enzima E2 [198]. Las E3 ligasas RIR (dentro de las cuales se incluye parkina) parecen pertenecer al subgrupo

HC e interaccionan con las $E_2$ UbcH7 y UbcH8 para su actividad [198]. El RING1 es esencial para la unión de la enzima E2 y la actividad ligasa, la función del IBR aún no está clara, pero podría promover la interacción proteína-proteína, aunque también podría actuar como un espaciador flexible que facilitaría la interacción con las enzimas E2 [190]. El RING2 es generalmente más pequeño, y es necesario para el reconocimiento y la unión del sustrato.

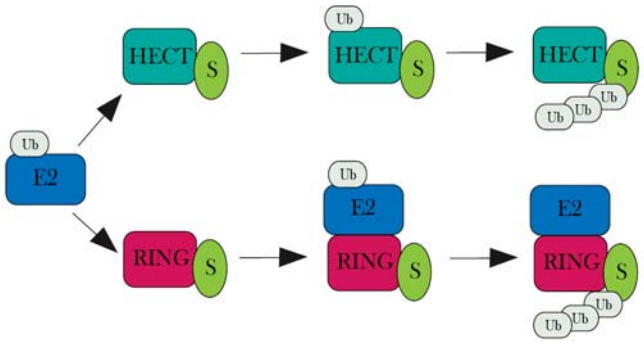


**Figure 11**: Ub is transferred from an E1 to an E2. E3 facilitates the ubiquitination of the substrate protein. HECT E3 transfer Ub directly to the substrate. RING E3 do not directly catalyze the transfer of Ub to the target protein, but require the presence of an E2, and often, additional components.

CRLs (Cullin-RING-Ligase) son complejos con múltiples componentes en los que se incluyen un gran número de ligasas. En ellos, se encuentra como mínimo una cullina y una RING ligasa, siendo la cullina el componente principal que actúa como andamiaje [202]. Cambiando uno solo de los componentes del complejo, se forman nuevas E3 ligasas, con especificidad por un sustrato diferente, como ocurre por ejemplo en los complejos SCF (Skp1, Cullina y F-box) [202].

El dominio U-box de las U-box E3 ligasas es estructuralmente muy similar al RING. Inicialmente se pensó que eran proteínas auxiliares que complementaban la actividad de otras E2-E3, sin embargo, se ha visto que pueden tener actividad E3 ligasa de forma independiente [203]. El miembro más representativo de esta familia es CHIP que actúa conjuntamente con chaperonas en el control del re-plegamiento o la degradación de proteínas [198, 199].

Las E3 ligasas reconocen sus sustratos mediante diferentes mecanismos. Uno de ellos es el reconocimiento de la secuencia primaria de la proteína [204]. Las modificaciones post-traduccionales como la adición de polisacáridos [205], oxidación [206] o fosforilación [207, 208] también influyen en el reconocimiento. Ciertas chaperonas como Hsp70 participan de forma conjunta con E3 ligasas, las cuales ubiquitinan los sustratos de éstas que no han sido replegados [209], o tiene proteínas adaptadoras, las cuales confieren la selectividad hacia el sustrato [210].

Parkina es una E3 ligasa que promueve la formación de cadenas de poliubiquitina tanto vía K48, en colaboración con UbcH7 [6] y UbcH8 [191] y K63, en colaboración con UbcH13/Uev1 [195, 211] y Ubc7. Además, su actividad autocatalítica parece ser llevada a cabo mediante múltiple monoubiquitinación [212], [213].

## 2.E- Localización:

Parkina se expresa prácticamente en todos los tejidos del cuerpo humano (próstata, testículos, ovario, intestino delgado, colon, corazón, hígado, músculo esquelético, riñón, estómago, tiroides, médula espinal, glándulas adrenales y médula ósea) [3]. En el sistema nervioso central su expresión es generalizada (cerebelo, corteza, medula, lóbulos frontal, temporal y occipital, putamen, amígdala, núcleo caudado, cuerpo calloso, hipocampo, sustancia negra, núcleo subtalámico y tálamo) [3]. La expresión en el locus ceruleus y en núcleo basal de Meynert es baja [3]. Parkina se expresa fundamentalmente en sustancia gris, sugiriendo que la proteína es predominantemente neuronal, aunque también se ha visto su expresión en astrositos y glia [214].

Mediante el uso de anticuerpos específicos se ha confirmado su localización en cerebro humano. Parkina se localiza en neuronas y en las terminales de éstas, en células gliares, endoteliales [215] y vasculares [216]. Se encuentra ampliamente distribuída en todas las áreas del cerebro, tanto en neuronas catecolaminérgicas como en las que no lo son, así como en las áreas donde las neuronas dopaminérgicas proyectan [215].

A nivel subcelular, la proteína es principalmente citoplasmática [217], aunque también es recluida de forma específica en mitocondrias dañadas [218]. Cuando es sobrexpresada en células puede localizarse de forma punteada a lo largo de microtúbulos [219], asociada a filamentos de actina [220], en la región perinuclear [221],[222], dentro del núcleo [223], asociada a la membrana de vesículas sinápticas y secretoras [224] y en la membrana mitocondrial externa [225].

Parkina está presente en los CL de pacientes con EP idiopática, hereditaria y demencia con CL, así como en los que aparecen fuera del sistema nervioso central durante el desarrollo de la enfermedad [215, 217] [226]. Se encuentra en el centro del CL, donde co-localiza con Ub [217].

## 2.F- Agregación de parkina y agregosomas:

Cuando las proteínas intracelulares no deseadas se acumulan (debido a un incremento en su producción o a una disminución en su eliminación), pueden agregar si exceden la capacidad local del proteosoma de ser eliminadas [227]. Tales proteínas son transportadas de forma activa a través de los microtúbulos hasta el centrosoma [228]. De forma simultanea, componentes del proteosoma, proteínas de choque térmico y mitocondrias son también reclutadas, en un intento por facilitar su eliminación [228]. Cuando los niveles de proteínas acumuladas en el centrosoma son elevados, se organizan en estructuras electrodensas en el centro, rodeadas de una red de elementos del citoesqueleto formada por filamentos intermedios, principalmente vimentina, formando una estructura de mayor tamaño en el centrosoma llamada agregosoma (Figura 12) [228]. Se cree que mediante la formación de agregosomas en células en cultivo, las proteínas mal plegadas que son tóxicas son secuestradas, para facilitar su eliminación por la vía lisosomal autofágica [227].

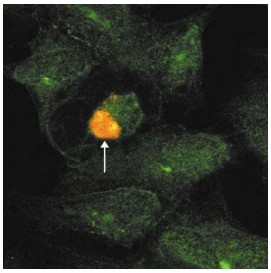

**Figure 12**: From [19]. Aggresomes in HEK 293 cells in culture. Double immunostaining to reveal the presence of 20s proteasomes (green) and ubiquitin (orange)

La similitud en cuanto a estructura, composición y localización entre los CL y los agregosomas convierten a éstos en un buen modelo celular para el estudio de los mecanismos implicados en la agregación de proteínas que tienen lugar en diversas enfermedades neurodegenerativas [19].

Se ha visto que la inhibición del proteosoma provoca la acumulación de parkina endógena en agregosomas en células neuronales [229], [230]. La sobre-expresión de parkina en células en cultivo también provoca la formación de inclusiones en un pequeño porcentaje de células (dependiendo del tipo celular) [9, 229-234]. Sin embargo, la sobreexpresión junto con la inhibición del proteosoma produce un incremento elevado en el número de células que contienen dichas inclusiones, las cuales son agregosomas, y que permanecen en la célula aún cuando la inhibición del proteosoma ha cesado [234], [231]. Otros insultos también promueven la insolubilidad y posterior agregación de parkina. El estrés oxidativo provocado por sustancias como DA (Dopamina) [8, 9, 230], 6OH-DA (6-Hidroxi-dopamina), Rotenona, Paraquat, MPP+(1-metil-4-fenil-piridinium), NO (óxido nítrico) y hierro [9], $H_2O_2$ (Peróxido de Hidrógeno) [235], así como el estrés procedente del RE [236], provoca la agregación en especies de alto peso molecular y la formación de inclusiones intracitoplasmáticas de parkina, fundamentalmente perinucleares.

## 2.G- Sustratos y proteínas de interacción:

Actualmente, se han descrito numerosos sustratos y proteínas de interacción de parkina, con diversas localizaciones y funciones celulares. Una proteína que sea

sustrato real, debería acumularse en el cerebro de los ratones Knock-out (KO) para parkina así como en el cerebro de pacientes con EP juvenil autosómica recesiva debido a mutaciones en parkina (Tabla 2).

### 2.G.1- Componentes del proteosoma:

Se ha descrito que parkina interacciona, pero no ubiquitina, tres proteínas componentes del proteosoma 26s [188, 237, 238]. El dominio UBL interacciona con las subunidades reguladoras de la tapa 19s Rpn10 y Rpt6 [188, 238, 239], participando Hsp70 en la interacción con Rpt6. También interacciona con su dominio IBR-RING2 con α4, una subunidad reguladora del núcleo 20s [237]. Parece ser que parkina modula la actividad del proteosoma mediante su interacción con α4, ya que su sobreexpresión aumenta ligeramente su actividad proteasa [237].

### 2.G.2- Otras proteínas implicadas en la EP:

#### 2.G.2.1- αSp-22 (forma glicosilada de α-sinucleína):

Parkina interacciona con la forma glicosilada de α-SN (αSp-22) [196]. αSp-22 es poliubiquitinada por parkina en conjunción con UbcH7. Dicha forma se acumula en el cerebro de pacientes con EP juvenil autosómica recesiva [196]. Sin embargo, la presencia de esta forma de α-SN es extremadamente baja en cerebro [196]. Su acumulación en los KO no ha sido descrita.

#### 2.G.2.2- Sinfilina-1:

Descrita por primera vez debido a su interacción con α-SN, sinfilina-1 también interacciona con el dominio RING2 de parkina [240]. Esta proteína se encuentra a nivel celular en las terminales sinápticas asociada a vesículas, dicha asociación está modulada por la presencia de α-SN [241], sin embargo, su función en la célula aún no ha sido establecida. Cuando la proteína es sobreexpresada tiene tendencia a agregar formando inclusiones [242]. La cotransfección de sinfilina-1 y α-SN da lugar a inclusiones citoplasmáticas similares a agregosomas [243], lo que se usa como modelo celular para el estudio de la agregación. Además, se ha descrito

que Sinfilina-1 está presente en los CL [244]. Sin embargo, no se ha referido su acumulación en cerebro de ratones KO para parkina o en el cerebro de pacientes con mutaciones en su gen.

2.G.2.3- LRRK2:

Se ha descrito que LRRK2 interacciona con parkina (dominio COR de la quinasa y dominio RING2 de la ligasa) [119]. LRRK2 no es ubiquitinada por parkina, sin embargo, la presencia de LRRK2 parece potenciar la actividad autocatalítica de ésta. No obstante, otro estudio no ha podido reproducir la interacción [245], permaneciendo el tema sujeto a cuestión.

2.G.2.4- PINK-1:

La interacción entre PINK-1 y parkina fue demostrada en principio de forma indirecta [246, 247], usando *Drosophila melanogaster* como modelo animal. Aunque también se ha visto en células humanas en cultivo [132, 248]. Se ha observado que la supresión de PINK-1 produce como fenotipo esterilidad y degeneración muscular y de las neuronas dopaminérgicas, acompañado de irregularidades en la morfología y función de la mitocondria [246, 247, 249]. La degeneración se produce por apoptosis y se ha observado que la expresión del homólogo en *Drosophila* de Bcl-2 (moléculas protectoras de la integridad y funcionalidad mitocondrial) la disminuye [247]. El fenotipo que presentan las moscas deficientes en PINK-1 es prácticamente igual al obtenido con las moscas nulas para parkina [246]. La expresión de parkina en ausencia de PINK-1, rescata el fenotipo presentado por los animales, pero no de forma inversa [247]. Lo que implica que parkina se encuentra abajo en la cascada de señalización de PINK-1 que promueve protección frente a la muerte celular inducida por daño mitocondrial [247], [246], [249], modificando los procesos de fusión y fisión mitocondrial [250].

Mediante interacción directa, parkina parece estabilizar a PINK-1, evitando su degradación por el proteosoma [248]. Mientras que la actividad quinasa de PINK-1 promueve la relocalización de parkina hacia la mitocondria [251].

Parkina no solo interacciona con proteínas relacionadas con la EP, hasta la fecha se han descrito numerosas proteínas de interacción y sustratos, con diversas funciones fisiológicas y diferentes localizaciones celulares. Sin embargo, la relevancia de dichas interacciones todavía debe ser determinada.

| INTERACTOR | POSSIBLE FUNCTION | SUBSTRATE | COLOCALIZATION | AR-JP BRAIN ACCUMULATION | KO MOUSE ACCUMULATION | REFERENCE |
|---|---|---|---|---|---|---|
| CDC-Rel1 | Septin/Exocytosis | Yes | No | Yes | ND | Zhang et al 2000, Choi et al 2003 |
| CASK | Synaptic transmission | No | Yes | No | ND | Fallon et al 2001 |
| Pael-R | Endotelin like receptor | Yes | ND | Yes | ND | Imai et al 2001 |
| Synphilin-1 | Presynaptic protein | Yes | Yes | ND | ND | Chung et al 2001 |
| α-Sp22 | Glycosilated α-Synuclein | Yes | ND | Yes | ND | Shimura et al 2001 |
| CDC-Rel2 | Septin/Exocytosis | Yes | ND | Yes | ND | Choi et al 2003 |
| hSel-10 | F-box protein, E3 ligase complex | No | Yes | ND | ND | Staropoli et al 2003 |
| γ-tubulin | Microtubuls | ND | Yes | ND | ND | Zhao et al 2003 |
| α/β-Tubulins | Microtubulus | Yes | Yes | ND | ND | Ren et al 2003 |
| Synaptotagmin XI | Synaptic transmission | Yes | Yes | ND | ND | Huynh et al 2003 |
| p38 | t-RNA syntethase subunit | Yes | Yes | Yes | Yes/ND | Corti et al 2003, Ko et al 2005 |
| BAG5 | Cochaperone | No | Yes | ND | ND | Suneil et al 2004 |
| DAT | Transporter | Yes | Yes | ND | ND | Jiang et al 2004 |
| Nrdp-1 | E3 ligase | No | Yes | ND | ND | Zhong et al 2005 |
| LRRK2 | Kinase | No | Yes | ND | ND | Smith et al 2005 |
| 14-3-3η | Chaperone | No | ND | ND | ND | Sato et al 2006 |
| SUMO-1 | Post-translational modifier | No | Yes | ND | ND | Um et al 2006 |
| RanBP2 | SUMO E3 ligase | Yes | Yes | ND | ND | Um et al 2006 |
| Eps-15 | Adaptor protein, Endocytosis | Yes | ND | ND | ND | Fallon et al 2006 |
| FBP-1 | DNA binding protein | Yes | ND | Yes | ND | Ko et al 2006 |
| Ataxin-2 | Unknown | Yes | Yes | ND | ND | Huynh et al 2007 |
| LIM Kinase1 | Actin polimerization regulador | Yes | Yes | ND | ND | Lim et al 2007 |
| PICK1 | PKC modulator | Yes | Yes | ND | ND | Joch et al 2007 |
| DJ-1 | Chaperone | Yes | Yes | ND | ND | Olzmann et al 2007, Xiong et al 2009 |
| Phospholipase C-γ1 | Signal transduction | Yes | Yes | ND | Yes/ND | Dehvari et al 2008 |
| Hsp70 | Chaperone | Yes | ND | ND | ND | Moore et al 2008 |
| PINK-1 | Kinase | No | No | ND | ND | Shiba et al 2009 |

**Table 2**: Parkin substrates and interacting proteins. Abbreviations: AR-JP (Autosomal recessive juvenile Parkinson disease), BAG5 (bcl-2 associated athanogene 5), (CDC-Rel ½ (Cell division cycle related protein 1/2), DAT (Dopamine transporter), FBP-1 (Far upstream binding protein 1), KO (Knock-out), ND (Not determined), PICK-1 (Protein interacting with C-kinase 1), SUMO (Small ubiquitin modifier).

## 2.H- Modelos animales de parkina:

Los modelos animales de las formas hereditarias de la EP que conllevan una pérdida de función, como es el caso de parkina, se pueden llevar a cabo fácilmente mediante la anulación del gen (Knock-out).


**Figure 13**: Schematic representation of parkin exons. Aminoacid residue numbers are shown above relevant domains (shaded green). Numbers in boxes depict the exon coding for each region.

La anulación del gen de parkina ha sido llevada a cabo mediante la deleción del exón 2, el 3 y el 7 (Figura 13) [252-256]. Los tres tipos de ratones son viables, pero ninguno muestra degeneración selectiva de las neuronas dopaminérgicas de la sustancia negra. La deleción en el exón 2 no es un modelo robusto de EP, quizá porque sólo provoca la eliminación del dominio UBL y podría dar lugar a una proteína truncada parcialmente activa [255].

La deleción del exón 3 mostró una afectación sutil de la vía dopaminérgica (niveles elevados de DA extracelular, disminución de la liberación de DA, niveles reducidos de DAT y reducción en la excitabilidad sináptica), afectación de la función mitocondrial, menor capacidad antioxidante, mayor daño por estrés oxidativo y déficits locomotores y cognitivos [253], [252], [257]. Se ha visto que con la edad estos ratones pierden los mecanismos compensatorios que mantienen la función normal de las neuronas dopaminérgicas (control de estrés oxidativo y proteínas mal plegadas), incluso que acumulan la proteína tau [258]. Estudios proteómicos han revelado alteraciones del metabolismo energético, en el control de

calidad de proteínas y en la función sináptica [259], así como disfunción mitocondrial y daño oxidativo [254].

La deleción del exón 7 parece tener un fenotipo diferente, ya que muestra pérdida de neuronas dopaminérgicas del locus ceruleus, una de las primeras áreas del cerebro afectadas por la EP [256]. La pérdida del dominio RING1 (Figura 13) provocaría la pérdida de la actividad catalítica de la proteína, siendo quizá éste el modelo más robusto para el estudio de la enfermedad.

Estos modelos animales son útiles para estudiar la posible acumulación de los sustratos de parkina. p38 ha sido el único sustrato del que se ha descrito acumulación [260] (Tabla 2), pero sólo en uno de los ratones KO generados, por lo que la acumulación podría deberse a mecanismos indirectos.

Otro modelo animal utilizado en el estudio de la función de parkina ha sido *Drosophila melanogaster* [261-263]. Las moscas que no expresan parkina presentan esterilidad en los machos y degeneración muscular, provocada por disfunción mitocondrial [261, 262]. El estudio de este modelo reveló una inducción de genes implicados en la respuesta al daño por estrés oxidativo e inflamación [261]. La expresión de mutantes patológicos de parkina en moscas, provoca la muerte selectiva, progresiva y dependiente de edad de las neuronas dopaminérgicas [263], [262]. Drosophila al no ser vertebrado y presentar grandes diferencias tanto anatómicas como fisiológicas con el ser humano, no es un buen modelo animal para el estudio de la EP, sin embargo, si presenta homólogo de parkina y la generación de los KO ha sido de gran utilidad para conocer la función de esta proteína.

## 2.I- Efecto neuroprotector de parkina:

### 2.I.1- Frente proteínas con tendencia a agregar:

Se ha visto que la sobreexpresión de α-SN y sus mutantes, tanto en células en cultivo como en animales de experimentación promueve su agregación [103] y conduce a la muerte neuronal [264]. La sobreexpresión de parkina disminuye la muerte de neuronas dopaminérgicas causada por α-SN, tanto en cultivos

mesencefálicos [83], [265] como en ratones [266], ratas [264] y moscas [267] (Figura 14).

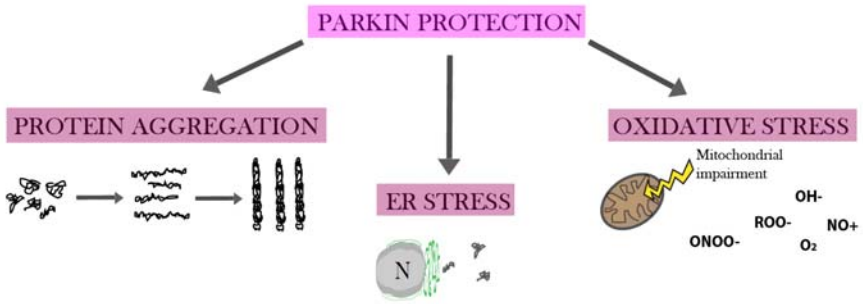


**Figura 14**: Parkin protection: Parkin has a protective role against different insults like protein aggregation, endoplasmic reticulum stress and oxidative stress and mitochondrial impairment.

### 2.I.2- Frente a estrés en el retículo endoplásmico:

La acumulación de proteínas desplegadas en el RE provoca estrés y muerte celular [268]. Frente a este tipo de estrés, la célula reacciona transcribiendo genes que facilitan el plegamiento de proteínas o su eliminación [268]. Cuando se induce estrés en el RE mediante tunicamicina [269] o manganeso [236], se produce un aumento de la transcripción y de los niveles de parkina. La sobreexpresión de parkina disminuye la muerte celular inducida por este tipo de estrés, especialmente en células dopaminérgicas [236] (Figura 14), pero sólo tiene lugar cuando la actividad E3 ligasa de parkina se encuentra intacta [269].

Cuando el estrés en el RE es inducido por sobreexpresión de proteínas de membrana, parkina también protege frente a la muerte celular, promoviendo la formación de inclusiones citosólicas que contienen dichas proteínas [270, 271].

### 2.I.3- Frente a estrés oxidativo y daño mitocondrial:

Parece que parkina tiene un papel protector frente al estrés oxidativo (Figura 14), ya que su expresión en células produce una disminución de radicales libres [272]. Además, su ausencia provoca daño mitocondrial en Drosophila [261], y la patología inducida por dicha supresión puede ser rescatada si se sobreexpresa el gen

de la glutatión-S-transferasa, un factor implicado en la respuesta al estrés oxidativo [263].

Parkina ejerce protección cuando las células son tratadas con múltiples agentes que generan estrés oxidativo [9] [225] [273] [274]. Cuando se tratan las células con Rotenona [9], parkina protege de la despolimerización de microtúbulos inducida por este agente [47, 275]. También ejerce neuroprotección frente al tratamiento con ceramida [225], evitando el daño mitocondrial, la liberación de citocromo c y la activación de caspasa 3, para lo cual es necesario que la actividad ligasa esté intacta [225].

El tratamiento con DA provoca un aumento en la expresión de parkina [273], la cual protege frente a este tóxico. Su derivado 6-OHDA, es un tóxico ampliamente utilizado, implicado en estrés oxidativo y daño mitocondrial [276, 277]. Parkina ejerce protección frente a este tóxico reduciendo los niveles de radicales libres y proteínas carboniladas, así como la activación de la apoptosis por la vía JNK [274]. La protección frente a 6-OHDA también ha sido demostrada *in vivo*, mediante inyección de vectores lentivirales que expresan parkina en ratas tratadas con 6-OHDA [278]. Sin embargo, la protección no es generalizada, ya que no protege frente a agentes oxidantes como $H_2O_2$ [225], [274]. Los resultados son contradictorios para algunos tóxicos, lo que puede deberse a la inactivación de parkina por una toxicidad excesiva [279], [280] (ver más adelante en modificaciones post-traduccionales).

La excitotoxicidad es un proceso patológico inducido por la sobreexcitación de los receptores NMDA (N-metil-D-aspartato) y AMPA (α-amino-3-hidroxi-5-metil-ácido isoxazolepropionico) por el neurotransmisor excitatorio glutamato o sustancias tóxicas como kainato. Dicha sobreexcitación provoca un incremento de calcio intracelular, el cual promueve la activación de calpaínas y otras enzimas que dañan estructuras celulares, induciendo muerte neuronal [2, 281]. La excitotoxicidad inducida por kainato puede ser aliviada por parkina [280], evitando la apoptosis mediante degradación de Ciclina E [210]. Se ha visto que parkina también regula la función y la estabilidad de las sinapsis glutaminérgicas excitatorias, disminuyendo la

transmisión sináptica excitatoria y la proliferación de dichas sinapsis, reduciendo la vulnerabilidad de las neuronas a la excitotoxicidad [282].

### 2.I.4- Posibles vías a través de las cuales ejerce neuroprotección:

Existen diferentes formas a través de las cuales parkina ejerce un efecto neuroprotector. Además de regular el daño producido por la agregación de proteínas y las diferentes rutas de estrés, también ha sido implicada en diversas cascadas de transducción de señales. Una de ellas es la vía antiapoptótica de señalización celular del Factor Nuclear-κB (NF-κB) [280]. La expresión de parkina aumenta la activación de este factor y su capacidad de unión al ADN, promoviendo la transcripción de genes implicados en la supervivencia celular [280]. El bloqueo de esta cascada de señalización, suprime el efecto protector que parkina ejerce frente al estrés por Kainato y Rotenona [280]. Mediante ubiquitinación vía K63 no degradativa de TRAF2 e IKKγ, parece ser que parkina activa la vía de señalización, protegiendo las células de la muerte por apoptosis [280].

Otra vía antiapoptótica en la que parkina ha sido implicada es la PI[123]K-Akt [283]. Parkina se une a la proteína Eps15, ubiquitinándola, lo que impide su unión al receptor del factor de crecimiento epidérmico (EGFR), previniendo su internalización y degradación. La señalización a través de la vía de supervivencia PI3K-Akt se ve entonces prolongada [283].

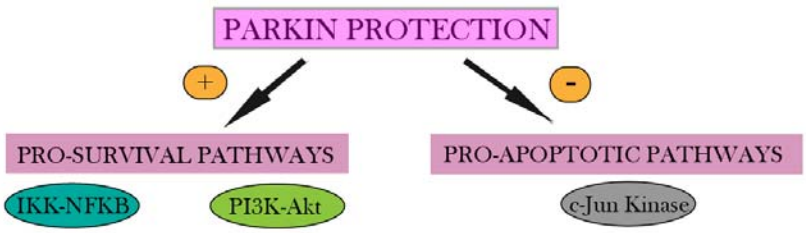


**Figure 15**: Parkin protection pathways: Parkin has a protective role through different cellular pathways, promoting cell survival (IKK-NFκB or PI3K-Akt pathways) or inhibintg cell death (c-Jun Kinase pathway).

La vía JNK (c-Jun $NH_2$-terminal Kinase) es una ruta proapoptótica que se activa en respuesta a estrés celular [284]. Cuando las células son estresadas sobreexpresando tirosinasa (la cual genera ROS a través de una producción excesiva de DA), la vía JNK se activa y se produce muerte celular [285]. La sobre-expresión de parkina protege frente a este tipo de estrés reduciendo la activación de esta vía, lo que conlleva una menor muerte por apoptosis [284]. La regulación negativa que parkina ejerce sobre la vía JNK también ha sido observada en *Drosophila* [286] y en células en cultivo estresadas con DA [274]. Mediante monoubiquitinación de Hsp70, parkina inhibe la fosforilación de c-jun por JNK, inactivando la ruta proapotótica [287].

Parkina también está implicada en la ruta de las MAPK (microtubule associated protein kinase), inhibiendo la activación de Erk, p38 y JNK [275], reprimiendo así la activación de rutas proapoptóticas [284].

El knock-down de parkina en células dopaminérgicas en cultivo produce una disminución en la viabilidad celular y un aumento en la apoptosis, lo cual ha sido observado por un incremento en el procesamiento de las caspasas 9, 6 y 3 [288]. El efecto es específico de células dopaminérgicas, y es más evidente cuando éstas son diferenciadas [288].

Parkina también está implicada en la regulación de la funcionalidad y morfología de la mitocondria, como se ha visto en varios estudios en *Drosophila melanogaster* [249, 250, 289]. Parkina es reclutada de forma selectiva en las mitocondrias dañadas con bajo potencial de membrana [218], promoviendo su eliminación mediante autofagia [218], por lo que podría proteger a las neuronas del daño inducido por acumulación de mitocondrias no funcionales.

## 2.J- Mecanismo patológico de las mutaciones puntuales de parkina:

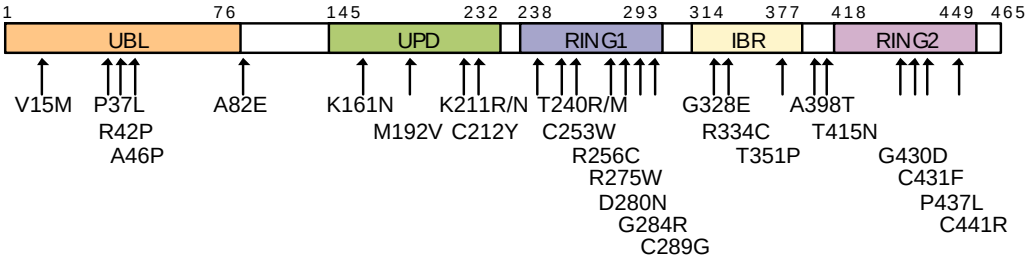


**Figure 16**: Schematic representation of parkin domain structure, with domain boundaries shown by aminoacid residue numbers above and pathogenic point mutations depicted below.

Se han llevado a cabo estudios estructurales con los dominios UBL [239] e IBR [189, 190] de parkina, los cuales han aportado algo de conocimiento al mecanismo mediante el cual las mutaciones patogénicas ejercen su acción. Sin embargo, todavía no se ha llevado a cabo ningún estudio estructural con la proteína completa, lo que aportaría gran información sobre los mecanismos implicados en la pérdida de actividad de la proteína, ya que los datos actuales vienen fundamentalmente de estudios *in vitro* usando proteínas recombinantes o de células en cultivo.

Las mutaciones presentes en el dominio UBL, en principio deberían afectar a la estructura de parkina o a su unión con diversas proteínas y no a su actividad catalítica. Por ejemplo, la mutación R42P (la más estudiada) perjudica la interacción de parkina con α-Sp22 [196], p38 [260], 14-3-3η [290], Eps15 [283] y la subunidad Rpn10 del proteosoma [188]. Parece que este mutante conserva la actividad catalítica [260], [213], [291], pero pierde capacidad protectora frente a diversos insultos [270], [132], [280], [284]. Algunos trabajos sugieren que esta mutación induce un cambio conformacional [292, 293], lo que provocaría su agregación, y en consecuencia su inactivación [9], [213, 239].

Las mutaciones en el dominio UPD (K161N, M192L, K211N y C212Y) parecen no tener consecuencias en la interacción con sustratos, solubilidad y actividad. La excepción es la mutación C212Y, la cual tiene mayor tendencia a

agregar [233], [9], [212], [213], ya que afecta a un residuo de cisteína, que parece tener importancia estructural en parkina [225], [270], [274].

T240R, R256C, C268Stop, R275W, D280N, C289G, G311Stop y E409Stop son las mutaciones más estudiadas del dominio RING1. La característica general de todas ellas excepto T240R y G311Stop es que parkina conserva la actividad E3 ligasa, al menos hacia ella misma [233], [212], [213]. La pérdida de actividad producida por T240R y G311Stop parece ser consecuencia de la disminución en la interacción con UbcH7 [269]. El mecanismo patológico de todos ellos parece ser consecuencia de su gran tendencia a agregar, formando inclusiones citoplasmáticas [294], [233], [9, 295], [213].

Sólo se han estudiado tres mutaciones del dominio IBR (T415N, R334C y C418R). El mecanismo por el cual producen EP implica agregación en el caso de R334C [9], [233] y por pérdida de actividad catalítica (debido al fallo en la interacción con UbcH8) en el caso de C418R [191].

Por último, las mutaciones en el dominio RING2 (G430D, C431F, P437L, C441R y W453Stop) se ha visto que producen un incremento en la insolubilidad, que conlleva su agregación [9], [213], [293], principalmente en el mutante W453Stop, el cual adopta una conformación desplegada tras ser sintetizado [235], [293]. La agregación provocaría una pérdida de la actividad E3 ligasa de la proteína [212].

## 2.K- Modificaciones post-traduccionales:

### 2.K.1- S-Nitrosilación:

Parkina es una E3 ligasa con dominios RING que contiene un elevado número de cisteínas, algunas de las cuales pueden ser modificadas por S-nitrosilación [7, 296]. La nitrosilación es uno de los procesos a través de los cuales el óxido nítrico (NO) puede regular la función de múltiples proteínas [297]. La modificación en parkina (dominios RING1-IBR-RING2) se ha visto tanto *in vitro*, *in situ* como *in vivo* [296], con muestras de pacientes con EP esporádico, demencia

con CL y con modelos animales de EP [7]. La nitrosilación provoca un aumento en la actividad E3 ligasa de parkina, que posteriormente se ve inhibida [7, 296]. La relevancia de esta modificación en la capacidad protectora de parkina se encuentra sujeta a controversia. El incremento inicial en la actividad promovería la autoubiquitinación de parkina, lo que podría provocar disfunción proteosomal y agregación de proteínas [296]. Sin embargo, también podría contribuir a la patogénesis de la EP mediante inhibición crónica de la actividad ligasa, perdiendo así parkina su capacidad protectora [7].

### 2.K.2- Procesamiento por caspasas:

La muerte celular por apoptosis conlleva la activación de caspasas, proteasas que reconocen residuos de aspártico en las proteínas a procesar [298]. La inducción de apoptosis por ácido okaídico, estaurosporina o camptotecina promueve el procesamiento de parkina por caspasas [299]. El corte se produce en el residuo aspártico 126, lo que provoca la pérdida del dominio UBL de la proteína [299]. Se ha descrito que Caspasa 1 y 8 (las cuales se activan en los procesos iniciales de apoptosis y que están implicadas en la vía extrínseca e intrínseca respectivamente) son las encargadas del corte [300].

### 2.K.3- Dopamina y estrés oxidativo:

El metabolismo celular de la DA genera dopamina-quinona, un metabolito reactivo con tendencia a unirse a residuos de cisteína de proteínas [301]. Parkina es covalentemente modificada por este metabolito, tanto *in vitro* como *in situ* e *in vivo* [8]. La modificación por dopamina-quinona produce una disminución de la actividad catalítica y un aumento de su insolubilidad, el cual puede ser detectado en pacientes con EP comparado con pacientes control [8]. Parkina se encuentra modificada sólo en las regiones del cerebro que contienen neuronas dopaminérgicas [8]. Parece ser que la DA modifica selectivamente las cisteínas 268 y 323, únicas en parkina. Dicha especificidad explicaría la susceptibilidad de parkina y no de otras E3 ligasas presentes en neuronas dopaminérgicas [302].

### 2.K.4- Fosforilación de parkina:

Otra modificación post-traduccional que ha sido descrita es la fosforilación. Las quinasas implicadas son CKI (Caseína Quinasa I), PKC (Proteína quinasa C) y PKA (Proteína quinasa A). Los sitios de fosforilación de PKC no han sido determinados, pero si se ha descrito que CKI fosforila en las serinas 101 y 378 y que PKA fosforila las serinas 101, 131 y 136 *in vitro* [303], disminuyendo la actividad catalítica [303]. Se observó que la fosforilación de la proteína disminuía cuando se inducía estrés por proteínas desplegadas, pero no por estrés oxidativo [303].

Parkina también es fosforilada por la Quinasa dependiente de ciclina 5 (Cdk5) en la serina 131 [232] y parece ser que también disminuye su actividad catalítica [232]. En este mismo trabajo se observó como el mutante no fosforilable (S131A) tenía una mayor actividad catalítica hacia sus sustratos y mayor tendencia a agregar, aumentando también el número de inclusiones formadas al cotransfectar parkina, sinfilina-1 y α-SN. Los autores proponen que la disminución en la actividad catalítica de parkina mediante fosforilación por Cdk5 disminuiría la formación de agregados celulares [232].

Se ha descubierto recientemente que parkina es fosforilada por PINK-1 en la treonina 175 [251] y que dicha fosforilación promueve la traslocación de parkina a la mitocondria [251].

No obstante, a pesar de la publicación de estos artículos, no se ha llevado a cabo ningún estudio exhaustivo de la fosforilación de parkina, de sus posibles efectos tanto en su actividad como en su solubilidad, ni sobre la presencia de parkina fosforilada en muestras de cerebro, tanto de pacientes con EP como de controles.

# SPECIFIC AIMS

# IV. SPECIFIC AIMS

1- Study parkin phosphorylation *in vitro* using recombinant proteins.

2- Determination of the phosphorylation sites.

3- Generation of phospho-state specific antibodies.

4- Study the effect of parkin phosphorylation on parkin function *in vitro* (E3 ligase activity).

5- Study the effect of parkin phosphorylation on its aggregative properties.

6- Study the parkin phosphorylation status in control patients compared to Parkinson's disease patients.

7- Establish whether inhibiting parkin phosphorylation by kinase inhibition has beneficial effects on solubility

# RESULTS

# 1- COMBINED KINASE INHIBITION MODULATES PARKIN INACTIVATION

# V. RESULTS

## 1- Combined Kinase Inhibition Modulates Parkin inactivation:

### 1.A- Resumen:

Las mutaciones en el gen que codifica para la proteína parkina causan Parkinsonismo juvenil autosómico recesivo. Además, parkina parece desempeñar un papel importante en la patogénesis del Parkinson esporádico. Se sabe que parkina tiene un papel neuroprotector, sin embargo, su función específica aún no ha sido establecida. Existen numerosos estudios que muestran como las modificaciones post-traduccionales de parkina (como la S-nitrosilación o la modificación por dopamina), conllevan la inactivación de la proteína, y que dichas modificaciones están asociadas con la enfermedad.

Otro tipo de modificación post-traduccional es la fosforilación de proteínas, la cual ha sido recientemente ligada a la patogénesis de la EP. A pesar de ello, aún no se ha descrito con exactitud qué papel desempeña dicha modificación en parkina. En el siguiente estudio mostramos cómo la fosforilación simultanea de parkina por Caseína Quinasa I (CKI) y por la Quinasa dependiente de Ciclina 5 (CdK5), disminuye la solubilidad de la proteína, provocando su agregación e inactivación. La inhibición combinada de ambas quinasas revierte parcialmente la agregación en cultivo de ciertos mutantes patogénicos de parkina. Además, se ha visto que existe un incremento en la fosforilación de parkina en diferentes zonas del cerebro de individuos con Parkinson esporádico, el cual correlaciona con un incremento en los niveles de p25, el activador de CdK5. Estos hallazgos indican que tanto CKI como CdK5 podrían representar nuevas dianas terapéuticas combinatorias para el tratamiento de la EP.

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# Combined kinase inhibition modulates parkin inactivation

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**Mutations in the parkin gene cause autosomal-recessive, juvenile-onset parkinsonism, and parkin dysfunction may also play a role in the pathogenesis of sporadic Parkinson disease (PD). Although its precise function remains largely unknown, parkin seems to play a neuroprotective role. Several studies indicate that changes in parkin solubility induced by post-translational modifications, such as S-nitrosylation or dopamine modification, comprise one mechanism of parkin inactivation associated with disease. Protein phosphorylation events have recently been linked to the molecular mechanism(s) underlying PD, but the role of this post-translational modification for parkin function has remained unclear. Here we report that compound phosphorylation of parkin by both casein kinase I and cyclin-dependent kinase 5 (cdk5) decreases parkin solubility, leading to its aggregation and inactivation. Combined kinase inhibition partially reverses the aggregative properties of several pathogenic point mutants in cultured cells. Enhanced parkin phosphorylation is detected in distinct brain areas of individuals with sporadic PD and correlates with increases in the levels of p25, the activator of cdk5. These findings indicate that casein kinase I and cdk5 may represent novel combinatorial therapeutic targets for treating PD.**

## INTRODUCTION

Parkinson disease (PD) is a progressive and substantially disabling neurodegenerative disorder (1–3). Its clinical symptoms primarily result from the progressive and rather selective degeneration of dopaminergic neurons of the substantia nigra pars compacta. Besides cell death, a pathological hallmark of PD in surviving neurons comprises Lewy bodies, ubiquitylated intraneuronal inclusions rich in α-synuclein (4).

Even though largely a sporadic disorder, there are several genes associated with inherited forms of PD. One commonly implicated is PARK2, the gene encoding for parkin (5). Indeed, mutations in the parkin gene are responsible for a large percentage of autosomal-recessive, juvenile-onset parkinsonism (6,7). A variety of homozygous and compound heterozygous mutations have been reported, and although mutations that reduce but do not abolish parkin function are accompanied by dopaminergic cell loss in the presence of Lewy bodies (8,9), homozygous loss-of-function parkin mutations seem to be associated with a lack of Lewy bodies (10), raising the possibility that parkin may be involved in Lewy body biogenesis. Furthermore, parkin may also play a role in sporadic PD, given that it is present in Lewy bodies from sporadic PD patients (11,12).

Parkin functions as an E3 ubiquitin ligase (13), and inactivation of its catalytic activity may lead to dopaminergic cell death due to the accumulation of toxic substrate protein(s). Recent studies suggest that changes in parkin solubility comprise a major mechanism of parkin inactivation both in familial and sporadic PD. For example, a wide range of pathogenic parkin point mutations result in decreased parkin solubility and promote its aggregation (14–16). In addition, an array of oxidative stressors (17), as well as direct post-translational parkin modifications, including dopamine modification (18) or

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S-nitrosylation (19,20), lead to dramatic changes in the solubility of parkin, thereby highlighting a mechanism for parkin dysfunction in the pathogenesis of idiopathic PD.

Protein phosphorylation is another post-translational modification which has recently been linked to mechanism(s) underlying PD (e.g. 21). As protein kinases are tractable drug targets, these findings may help in the design of novel therapeutic strategies. Parkin has been previously described to be subject to phosphorylation by casein kinase I or by cyclin-dependent kinase 5 (cdk5), with modest changes in its enzymatic E3 ubiquitin ligase activity in either case (22,23). Given that compound or hyperphosphorylation of proteins can have profound effects on their aggregative properties, we sought to determine whether compound phosphorylation of parkin may modulate its aggregative properties. We hypothesized that such phosphorylation-induced changes could contribute directly to the inactivation of parkin and concomitantly reduced survival of dopaminergic neurons in PD.

## RESULTS

### Compound phosphorylation of parkin *in vitro* and in cells

We first carried out *in vitro* phosphorylation experiments using a set of purified protein kinases and recombinant full-length human parkin protein, or select domains thereof (Fig. 1A and B). Purified full-length parkin displayed both mono- and polyubiquitylation activity *in vitro* (Fig. 1C) and was phosphorylated by casein kinase I (Fig. 1D–F), as previously reported (22). Site-directed mutagenesis using full-length parkin as well as parkin fragments confirmed that S101 and S378 were phosphorylation sites for casein kinase I (22), and an additional site was identified as S127 (Fig. 1D and E). Indeed, mutations of these three serine residues to alanines almost completely abrogated phosphorylation, indicating that these sites are the major phosphorylation sites for casein kinase I *in vitro* (Fig. 1D and F). Parkin was not an *in vitro* substrate for a series of other protein kinases analyzed here (Fig. 1G), indicating that only certain signal transduction cascades may impact upon parkin function *in vivo*.

To assess phosphorylation of parkin in cells, we generated phospho-state-specific antibodies against phospho-S101 and phospho-S378. Their phosphorylation-state-specificity was confirmed using recombinant, phosphorylated parkin protein *in vitro*. The antibodies only detected the protein when previously phosphorylated by casein kinase I, but not its non-phosphorylated form (Fig. 2A). Constitutive phosphorylation of parkin on both S101 and S378 was detected when HEK293T cells were transiently transfected with human parkin (Fig. 2B). Upon treatment of transfected cells with IC261, a selective inhibitor of casein kinase I (24,25), a significant decrease in the phosphorylation state of both S101 and S378 of parkin was detected (Fig. 2C). Similarly, treatment of transfected cells with 35 μM D4476 (26), a distinct, structurally non-related and potent inhibitor of casein kinase I led to a significant decrease in parkin phosphorylation (by $44 \pm 10\%$, mean $\pm$ S.E.M., $n = 3$) in the absence of measurable cytotoxicity. Together, these data indicate that S101 and S378 of parkin are phosphorylated by casein kinase I in intact cells as well.

Apart from casein kinase I, parkin is also subject to phosphorylation by cdk5 on S131 (23). A phospho-mimetic mutant on this site displayed enhanced casein kinase I-mediated phosphorylation when compared with wild-type *in vitro* ($307 \pm 12\%$; mean $\pm$ S.E.M., $n = 5$) (Fig. 3A). Conversely, a phospho-mimetic mutant on all three casein kinase I sites served as a better substrate for cdk5 when compared with wild-type parkin *in vitro* ($540 \pm 240\%$; mean $\pm$ S.E.M., $n = 3$) (Fig. 3B). Similar findings were observed in parkin-transfected HEK293T cells using phospho-state-specific antibodies. On the one hand, an S131E parkin mutant, which mimicks constitutive phosphorylation by cdk5, displayed enhanced phosphorylation by casein kinase I when compared with wild-type parkin (Fig. 3C). On the other hand, blocking the activity of cdk5 by treating transfected HEK293T cells with roscovitine, a highly specific inhibitor for cdk5 (27), decreased phosphorylation on the casein kinase I sites of parkin (to $47 \pm 10\%$; mean $\pm$ S.E.M., $n = 4$) (Fig. 3D). Together, these data indicate that phosphorylation of parkin by cdk5 enhances its propensity to serve as a substrate for casein kinase I and *vice versa* (Fig. 3E).

### Effects of parkin phosphorylation on activity and inclusion formation

Phosphorylation of parkin may regulate its E3 ubiquitin ligase activity or modulate its insolubility, with downstream effects on dopaminergic cell survival in either case. To determine whether phosphorylation of parkin would affect its catalytic activity, we performed autoubiquitylation assays *in vitro* using various phospho-mimetic mutants (Fig. 4). A parkin phospho-mimetic mutant for cdk5 phosphorylation (131E) displayed ubiquitylation activity similar to wild-type, whereas its non-phosphorylatable counterpart (131A) was slightly more active, as previously described (23) (Fig. 4A). Parkin phospho-mimetic mutants for the individual casein kinase I sites (101E or 378E) displayed ubiquitylation activity similar to wild-type (Fig. 4B–D). Similarly, a phospho-mimetic mutant for all three casein kinase I sites (101E/127E/378E) did not display significant changes in ubiquitylation activity (Fig. 4E–G), indicating that neither mimicking individual nor combined phosphorylation of the casein kinase I sites affect parkin's ubiquitylation activity. A phospho-mimetic mutant for the adjacent casein kinase I and cdk5 sites (127E/131E) did not display changes in activity (Fig. 4C and D), whereas a phospho-mimetic mutant for compound phosphorylation by both casein kinase I and cdk5 (101E/127E/131E/378E) displayed slightly enhanced autoubiquitylation activity (Fig. 4E–G). However, this modest enhancement was also observed for the respective non-phosphorylatable mutant counterpart (101A/127A/131A/378A), and therefore does not seem to reflect specific effects related to parkin phosphorylation (Fig. 4E–G).

We next addressed whether parkin phosphorylation may modulate its tendency to form inclusions in cell culture and concomitantly alter its solubility in detergent. For this purpose, HEK293T cells were transfected with parkin or various phospho-mimetic or non-phosphorylatable mutants, treated with the proteasome inhibitor MG-132, and the amount of parkin inclusion bodies determined by immunocytochemistry

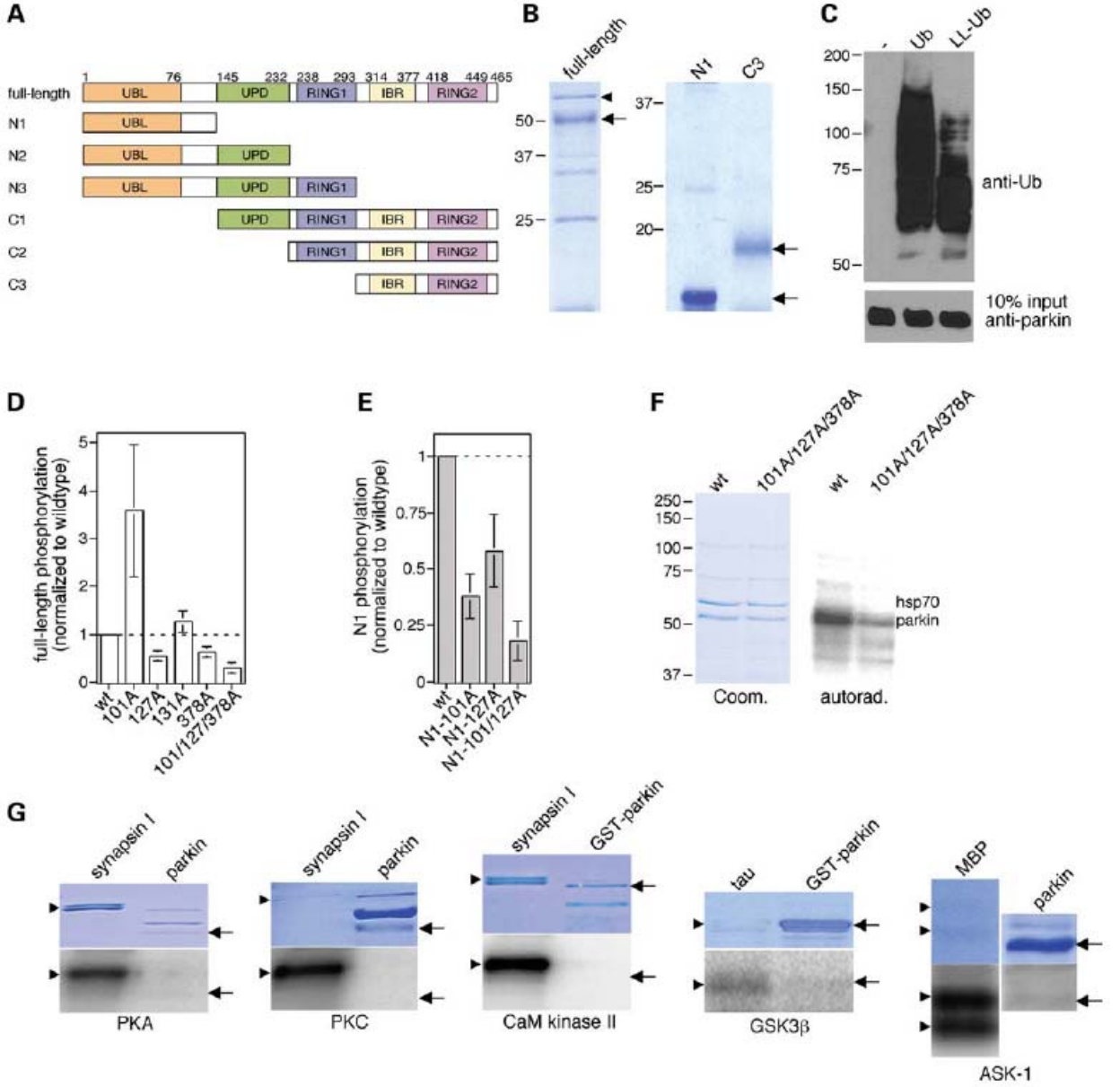


**Figure 1.** Parkin phosphorylation by casein kinase I *in vitro*. (**A**) Schematic representation of parkin domain structure, with domain boundaries shown by amino acid residue numbers above, and of recombinant parkin domain combinations (full-length, N1–N3 and C1–C3) analyzed by *in vitro* phosphorylation assays. (**B**) The different recombinant parkin domains were purified as described in Materials and Methods, and analyzed for purity by SDS–PAGE and Coomassie staining. Full-length recombinant parkin as well as N1 and C3 truncated forms are indicated by arrows. Bacterial hsp70 (arrowhead), as determined by mass spectroscopy, co-purified with full-length parkin. (**C**) Full-length recombinant parkin was catalytically active, as assessed by *in vitro* autoubiquitylation in the presence of wild-type ubiquitin (Ub) or a lysine-less derivative (LL-Ub) lacking the conjugation sites necessary for polyubiquitylation. When the reaction was performed with LL-Ub, lower levels of ubiquitylation were detected, indicating that recombinant parkin displays both multiple monoubiquitylation as well as polyubiquitylation activity towards itself. (**D**) Full-length recombinant parkin (wt), or equal amounts of the indicated point-mutated versions (S101A, $n = 4$; S127A, $n = 4$; S131A, $n = 4$; S378A, $n = 4$; S101A/S127A/S378A, $n = 3$), were subjected to *in vitro* phosphorylation reactions using casein kinase I, and phosphate incorporation quantified by using a PhosphorImager (mean ± S.E.M.). Note that the S101A mutant displayed increased phosphate incorporation in the context of the full-length protein, possibly reflecting conformational effects. (**E**) The N1 fragment of recombinant parkin (wt) or equal amounts of the indicated point-mutated versions (S101A, $n = 6$; S127A, $n = 4$; S101A/S127A, $n = 2$) were subjected to *in vitro* phosphorylation reactions using casein kinase I, and phosphate incorporation quantified by using a PhosphorImager (mean ± S.E.M.). (**F**) Example of a phosphorylation experiment using full-length recombinant parkin (wt) or the triple mutant S101A/S127A/S378A. (**G**) Parkin phosphorylation by additional protein kinases *in vitro*. Full-length recombinant parkin or glutathione S-transferase-parkin (arrows) was subjected to *in vitro* phosphorylation reactions using cAMP-dependent protein kinase (PKA), PKC, $Ca^{2+}$/calmodulin-dependent protein kinase II (CaM kinase II), GSK3β or ASK-1, as indicated, using various proteins as positive controls (synapsin, tau or MBP, respectively) (arrowheads). None of these kinases significantly phosphorylated parkin *in vitro*.

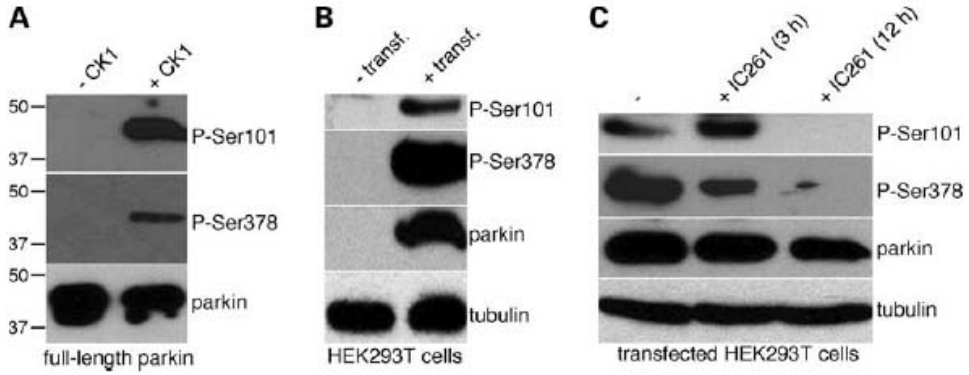


**Figure 2.** Parkin phosphorylation by casein kinase I in cells assessed using phospho-state-specific antibodies. (**A**) Phospho-state-specific antibodies against phospho-S101 (P-Ser101) or against phospho-S378 (P-Ser378) recognize recombinant full-length parkin only upon its *in vitro* phosphorylation by casein kinase I (+CK1), but not if the protein is not previously subjected to phosphorylation (−CK1). (**B**) Exogenous human parkin, upon transfection in HEK293T cells (+transf.), is constitutively phosphorylated on both S101 and S378 residues. Cells were treated for 1 h with 500 nM okadaic acid before cell lysis to stabilize the phosphorylation state of parkin. (**C**) Exogenous human parkin phosphorylation on S101 and S378 decreases upon treatment of HEK293T cells with IC261 (50 μM), a specific inhibitor of casein kinase I.

(Fig. 5A). Wild-type and all mutant parkin proteins were overexpressed to similar degrees (Fig. 5B), and the parkin mutant mimicking phosphorylation by only casein kinase I (101E/127E/378E) or by only cdk5 (131E) did not show a significant increase in the number of parkin inclusion bodies when compared with wild-type (Fig. 5C). These results indicate that individually mimicking phosphorylation by either casein kinase I or by cdk5 does not lead to changes in parkin aggregation. In contrast, a pronounced, $6.2 \pm 2$-fold increase (mean $\pm$ S.E.M., $n = 4$) in cells with parkin inclusions was found with the parkin mutant mimicking compound phosphorylation by both protein kinases (101E/127E/131E/378E) (Fig. 5C). Such enhanced aggregation was not observed with a non-phosphorylatable mutant counterpart (101A/127A/131A/378A) (Fig. 5C) and was only observed when mimicking phosphorylation on all four serine residues (Fig. 5D), indicating that it was specific for mimicking compound phosphorylation of parkin by both casein kinase I and cdk5.

An increased propensity of parkin to be confined to intracellular aggregates should result in changes in its detergent extractability, as previously described for several pathogenic parkin mutants and stress-induced parkin alterations (14–18). Indeed, although a significant amount of wild-type parkin was found in the Triton-soluble fraction in the presence of MG-132, the quadruple mutant mimicking compound phosphorylation displayed decreased solubility (Fig. 5E and F). In contrast, no change in detergent extractability was observed for the non-phosphorylatable mutant counterpart, which was present in the soluble fraction to a degree similar to wild-type parkin (Fig. 5E and F). Together, these data suggest that simultaneous phosphorylation of parkin on both casein kinase I and cdk5 sites profoundly facilitates its aggregation into inclusion bodies.

### Increased phospho-parkin in Parkinson brain

To determine whether parkin undergoes phosphorylation in human brain, and in sporadic PD, we chose to analyze parkin and phospho-parkin levels from distinct brain areas reported to be differentially affected by disease pathology (28). Samples from three distinct brain areas were available from each individual patient, and control and PD cases matched for postmortem interval, tissue-handling and storage conditions as important variables in these comparisons (Fig. 6A). The levels of total parkin were similar in caudate, cortex and cerebellum (Fig. 6B), and there were no differences in the levels of total parkin between control and PD cases in any of the three brain regions analyzed (Fig. 6C and D), as previously described for cortical samples (29). However, we found statistically significant increases in phospho-Ser101 parkin levels in PD versus control cases (Fig. 6C and D). These increases were detected in the caudate, known to be particularly affected by the presence of Lewy bodies and Lewy neurites during relatively early stages of disease development (28). No significant changes in phospho-parkin levels in PD versus control cases were observed in the cortex, and no phospho-parkin could be detected in the cerebellum (Fig. 6C and D), a brain area largely devoid of Lewy bodies and Lewy neurites (27). Together, these data demonstrate a neuroanatomical and disease-specific alteration in the phosphorylation status of parkin.

As a first step towards identifying the possible underlying mechanism(s) for the differences in phospho-parkin levels in PD when compared with control brains, we analyzed the total levels of casein kinase I α, δ and ε, as well as of cdk5 and its activator p25/p35. Similar levels of the α and ε isoforms of casein kinase I were detected in all three brain areas, whereas the levels of the δ isoform were lower in the cerebellum when compared with caudate and cortex, as previously described (30) (Fig. 6B). Similar levels of cdk5 were detected in all three brain areas, but the levels of p35 were lower in the cerebellum when compared with caudate and cortex, as previously described in rodent brain (31), and no p25 could be detected in the cerebellum (Fig. 6B). No differences were observed in the levels of the three casein kinase isoforms or of cdk5 in PD versus control in any of the three brain areas analyzed (Fig. 6E and F). However, the total levels of p25 were significantly higher in PD when compared

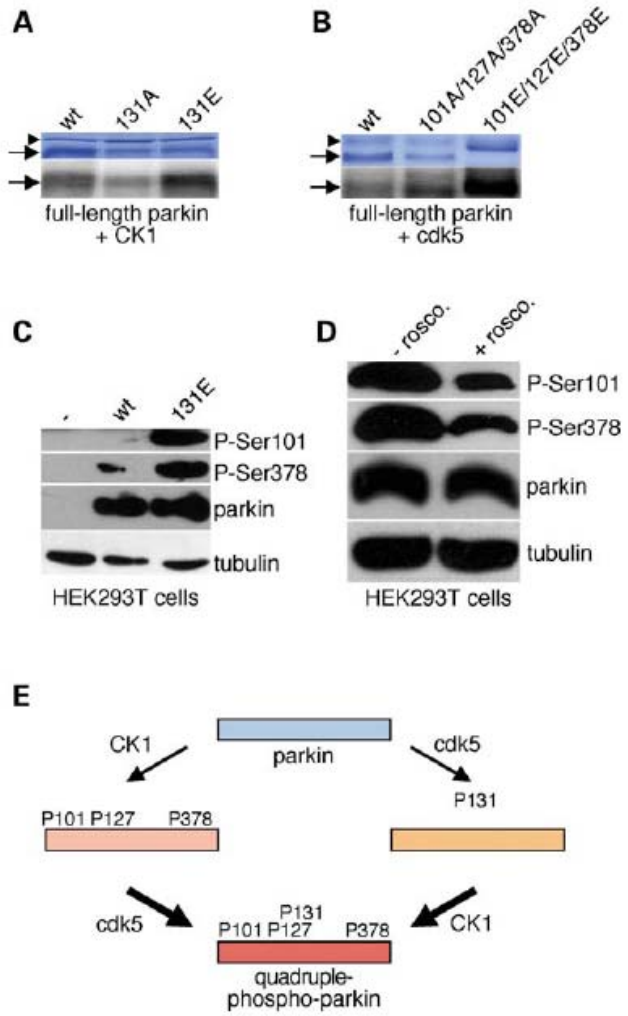


**Figure 3.** Compound phosphorylation of parkin *in vitro* and in cells. (A) Example of an *in vitro* phosphorylation experiment using full-length recombinant parkin (wt), or the indicated point-mutated versions (S131A, S131E), and casein kinase I (CK1). (B) Example of an *in vitro* phosphorylation experiment using full-length recombinant parkin (wt), or the indicated point-mutated versions (S101A/S127A/S378A, S101E/S127E/S378E), and cdk5. Arrows, full-length parkin and mutant versions thereof; arrowhead, bacterial hsp70. (C) HEK293T cells were transfected with either wild-type (wt) or S131E-mutant parkin, and equal amounts of cell extracts analyzed for parkin phosphorylation using phospho-state-specific antibodies. (D) Parkin-transfected HEK293T cells were treated with or without roscovitine (1 μM) for 12 h, and equal amounts of cell extracts analyzed for parkin phosphorylation using phospho-state-specific antibodies. (E) Schematic model depicting how parkin phosphorylation by one kinase may increase its propensity to serve as a substrate for the other kinase, thereby leading to a multiple-phosphorylated state.

with control samples in the caudate, with no changes in the cortex (Fig. 6C and D). Thus, the observed increase in phospho-parkin levels in PD when compared with control may, at least in part, be due to changes in the total levels of p25 in the distinct brain areas analyzed, with concomitant changes in cdk5 activity followed by compound parkin phosphorylation and aggregation.

### Modulation of mutant parkin aggregation by a combination of kinase inhibitors

As increased parkin phosphorylation seems to correlate with sporadic PD *in vivo* and enhances the aggregative properties of parkin in cultured cells, inhibiting casein kinase I and cdk5 activities may have beneficial effects in preventing such protein aggregation. To address this possibility, we quantified the number of aggregates in cells transfected with three pathogenic parkin mutants (R256C, R275W, C289G) previously described to display enhanced aggregative properties when compared with wild-type parkin (14–16). Indeed, all three mutants showed an enhanced propensity to form intracellular aggregates in the absence of proteasome inhibition, with C289G>R275W>R256C (Fig. 7A). These pathogenic mutants did not display significant differences in their auto-ubiquitylation activity (Fig. 7B) and were subject to phosphorylation by casein kinase I and cdk5 *in vitro* (Fig. 7C), indicating that their major pathogenic mechanism of action seems to involve enhanced aggregation. Concomitant with their reduced detergent extractability, we observed that they were present in the Triton-insoluble fraction in a heavily phosphorylated manner (Fig. 7D). Therefore, we next studied whether inhibiting casein kinase I and cdk5 activities may modulate the aggregative properties of these mutant proteins.

Cells were transfected with the aggregative R256C mutant and analyzed 72 h after transfection. Around 20% of transfected cells displayed large perinuclear inclusions, and treatment of cells during the last 12 h using two structurally dissimilar cdk5-specific inhibitors (roscovitine and GW8510 (32)), or two distinct casein kinase I-specific inhibitors (CKI-7 (24,25)) and D4476 (25,26)) profoundly decreased the number of cells with R256C-mutant parkin aggregates (Fig. 8A). Statistically significant additive effects in decreasing the number of cells with aggregates were observed when using combinations of a cdk5-specific and a casein kinase I-specific inhibitor (Fig. 8A), further indicating that the effects were specific for inhibiting the activity of these two protein kinases. The extent of the additive effect was relatively small, in agreement with the observed crosstalk between casein kinase I and cdk5 phosphorylation of parkin observed *in vitro* (Fig. 3). Compound inhibition of cdk5 and casein kinase I activities resulted in a roughly 70% decrease in the number of cells displaying aggregates for the R256C mutant, when compared with a 25% decrease for the R275W mutant, with no effects observed for the most aggregative C289G mutant (Fig. 8B). Addition of a casein kinase I-specific and a cdk5-specific inhibitor decreased the phosphorylation status of the three pathogenic parkin mutants analyzed to a similar degree (Fig. 8C and D), indicating that the relative efficiency by which compound kinase inhibition affects aggregation of the respective mutants is likely due to the observed distinct inherent aggregative properties of the mutants (Fig. 7A).

As the C289G-parkin mutant accumulated in perinuclear aggregates in virtually all transfected cells (Fig. 8B), we reasoned that the effects of inhibiting parkin phosphorylation may be best studied by analyzing the size and number of aggregates for this potent aggregative parkin mutant. Indeed, although treatment of transfected cells during the last 12 h

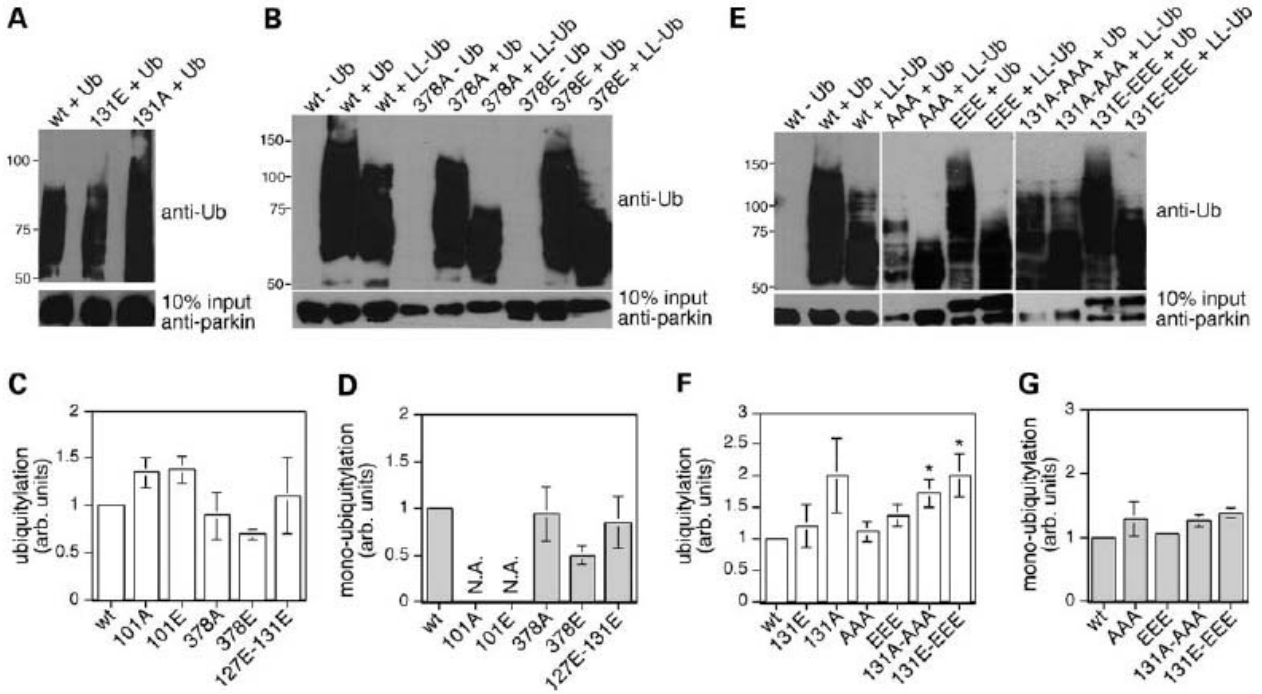


**Figure 4.** Phosphomimetic parkin mutants do not display drastic changes in E3 ligase activity. (A) Example of an autoubiquitylation experiment using wild-type, 131A-mutant or 131E-mutant parkin forms, displaying enhanced activity of the 131A-mutant. (B) Example of an autoubiquitylation experiment using wild-type, 378A-mutant or 378E-mutant parkin forms and either using ubiquitin (Ub) or a lysine-less derivative (LL-Ub) to assess polyubiquitylation versus monoubiquitylation activity, respectively. (C) Quantification of experiments of the type depicted in (B), indicating that the individual mutants do not lead to significant changes in the ubiquitylation activity of parkin. The ubiquitin-positive ladder obtained with various parkin-mutant proteins and wild-type ubiquitin was quantified, values were corrected for differences in protein input as analyzed separately using an anti-parkin antibody (844) and normalized to the activity of wild-type parkin. Bars depict mean ± S.E.M. (D) Quantification of experiments of the type depicted in (B), indicating that the individual mutants do not lead to significant changes in the monoubiquitylation activity of parkin. The ubiquitin-positive ladder obtained with various parkin-mutant proteins and lysine-less ubiquitin, reflecting multiple mono-ubiquitylation activity, was quantified, corrected for differences in protein input and normalized to the activity of wild-type parkin. Bars depict mean ± S.E.M. N.A., not analyzed. (E) Example of an autoubiquitylation experiment using wild-type or the indicated parkin mutants (AAA, 101A/127A/378A; EEE, 101E/127E/378E; 131A-AAA, 101A/127A/131A/378A; 131E-EEE, 101E/127E/131E/378E), and either wild-type ubiquitin (Ub) of a lysine-less derivative (LL-Ub) to assess monoubiquitylation activity. (F) The ubiquitin-positive ladder obtained with various parkin-mutant proteins (131E, $n = 3$; 131A, $n = 2$; AAA, $n = 3$; EEE, $n = 3$; 131A-AAA, $n = 4$; 131E-EEE, $n = 4$) and wild-type ubiquitin was quantified. Values were corrected for differences in protein input as analyzed separately using an anti-parkin antibody (844), and normalized to the activity of wild-type parkin. Bars depict mean ± S.E.M. *$P < 0.05$. (G) The ubiquitin-positive ladder obtained with various parkin mutant proteins (AAA, $n = 3$; EEE, $n = 3$; 131A-AAA, $n = 4$; 131E-EEE, $n = 4$) and lysine-less ubiquitin, reflecting multiple mono-ubiquitylation activity, was quantified, corrected for differences in protein input and normalized to the activity of wild-type parkin. Bars depict mean ± S.E.M. Error bars are only depicted when larger than column lines. The 131A-AAA and 131E-EEE mutants displayed a significant increase in autoubiquitylation activity only as assessed using ubiquitin, but not lysine-less ubiquitin.

using distinct cdk5 inhibitors, casein kinase I inhibitors or a combination thereof did not decrease the number of cells with mutant parkin inclusions, we observed a decrease in the number of cells displaying a large perinuclear aggregate (Fig. 8E). The effects of individual inhibitor treatment were significant on its own, and statistically significant additive effects were observed when using a combination of both inhibitors, with a nearly 50% reduction in the number of cells with large perinuclear aggregates (Fig. 8E). In addition, prolonged and repetitive incubation of cells with a mixture of a cdk5-specific and a casein kinase I-specific inhibitor resulted in additive beneficial effects in decreasing the number of cells with large C289G-mutant parkin aggregates with time (Fig. 8F). Together, these data show that inhibiting compound phosphorylation of parkin can regulate its aggregative properties in culture, indicating that such treatment is useful in maintaining parkin's protective function in cultured cells.

## DISCUSSION

Here, we report that parkin, a protein essential for the sustained survival of dopaminergic neurons, is subject to compound phosphorylation *in vitro* and in cultured cells. Such compound phosphorylation by casein kinase I and cdk5 enhances its insolubility, leading to aggregation and concomitant inactivation. Although phosphorylation does not change parkin's E3 ubiquitin ligase activity, increased aggregation effectively decreases the amount of soluble parkin protein able to exert a neuroprotective role (33). Notably, only simultaneous, but not individual phosphorylation by either casein kinase I or cdk5 seems to alter parkin solubility. Moreover, phosphorylation of parkin by one kinase seems to enhance its susceptibility to serve as a substrate for the other kinase. In this manner, simultaneous activation of both kinases may cooperatively contribute towards parkin inactivation.

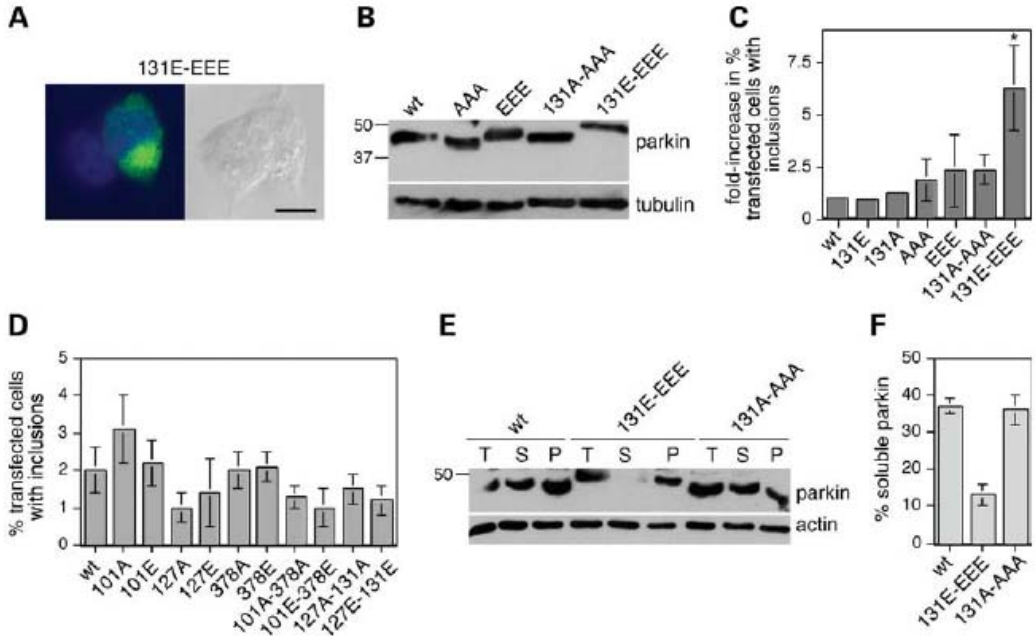


**Figure 5.** Phospho-mimetic parkin mutants display profound changes in aggregative properties. (A) HEK293T cells were transfected with 101E/127E/131E/378E-mutant parkin, and treated with 5 μM MG-132 for 12 h prior to immunocytochemistry using a polyclonal anti-parkin antibody (844) (green). Nuclei were stained with DAPI. Scale bar, 10 μm. (B) To assure equal levels of overexpression of wild-type and mutant parkin forms, cells were transfected with the various forms as indicated and 50 μg of cell extracts were subjected to western blot analysis 72 h later using an anti-parkin antibody (Abcam) or an anti-tubulin antibody to correct for differences in protein loading. Wild-type and mutant parkin constructs were overexpressed to similar degrees. (C) HEK293T cells were transfected with wild-type parkin or various mutant forms, treated with 5 μM MG-132 for 12 h followed by immunocytochemistry as described above. The percent of transfected cells displaying large perinuclear inclusions was determined by an investigator unaware of treatment groups, and the data plotted as an overall fold-increase in the number of transfected cells displaying large perinuclear inclusions when compared with wild-type parkin-expressing cells (131E; $n = 3$; 131A; $n = 3$; AAA, $n = 3$; EEE, $n = 4$; 131A-AAA, $n = 4$; 131E-EEE; $n = 4$). Bars depict mean ± S.E.M. $^*P < 0.05$. Error bars are only depicted when larger than column lines. (D) HEK293T cells were transfected with wild-type parkin or various mutant forms, treated with 5 μM MG-132 for 12 h followed by immunocytochemistry. None of the combinations analyzed led to significant changes in the number of transfected cells with inclusions, indicating that only the quadruple mutant parkin-variant mimicking compound phosphorylation by casein kinase I and cdk5 displays an increased tendency to aggregate. Bars depict mean ± S.E.M. ($n = 3$). (E) Analysis of the distribution of overexpressed wild-type (wt) or mutant parkin variants (treated with 5 μM MG-132 for 12 h before analysis) (total, T) in the Triton X-100-soluble (S) and insoluble pellet (P) fractions of HEK293T extracts upon blotting with an anti-parkin antibody (Abcam) or an anti-actin antibody. (F) Quantification of experiments of the type depicted in (E), where the amount of protein in the supernatant, when compared with that in the pellet, is plotted as % soluble parkin. Bars depict mean ± S.E.M. ($n = 3$).

To assess whether parkin phosphorylation may occur in humans, we analyzed samples from caudate nucleus, cortex and cerebellum of control and PD brains. We observed enhanced parkin phosphorylation in PD when compared with control in the caudate, with no changes in the cortex. Of note, parkin phosphorylation could not be detected in the cerebellum in either control or PD samples. The neuroanatomical differences in parkin phosphorylation between control and sporadic PD samples correlate with the relative extent to which these distinct brain areas are affected by disease pathology (28), indicating that abnormal parkin phosphorylation and concomitant inactivation may contribute to neuronal degeneration in sporadic PD.

We further examined whether the increase in parkin phosphorylation in PD brains may correlate with increases in the levels of cdk5, its activator p25/p35 or casein kinase I. Although we observed no changes in the levels of cdk5 or the levels of distinct casein kinase isoforms, we did find an increase in the levels of p25 in the caudate in PD when compared with control samples, with no changes in the cortex and the absence of detectable p25 in the cerebellum. In this context, it is interesting to note that p25 is known to lead to prolonged activation of cdk5 (34) and that cdk5 and p35 have been reported to accumulate in Lewy bodies in postmortem PD brains (35,36). Furthermore, an increase in cdk5 levels and activity has been observed in an 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine mouse model of PD (37), and various neurotoxic insults induce generation of p25 from p35 (38), indicating that the activity of cdk5 may play a key role in the pathogenesis of sporadic PD (39).

Little is known regarding de-regulation of casein kinase I activities during neurodegeneration (40), but most casein kinase I isoforms seem to be basally active (41). Our present data suggest that increased cdk5-mediated parkin phosphorylation would lead to a concomitant increase in casein kinase I-mediated phosphorylation events, as detected here using phospho-state-specific antibodies, thereby generating a compound phosphorylated protein with decreased solubility.

Finally, the generally high basal casein kinase I and cdk5 activities suggest a very active role for protein phosphatases in controlling the steady-state levels of parkin phosphorylation. Indeed, a variety of studies indicate that inhibition of

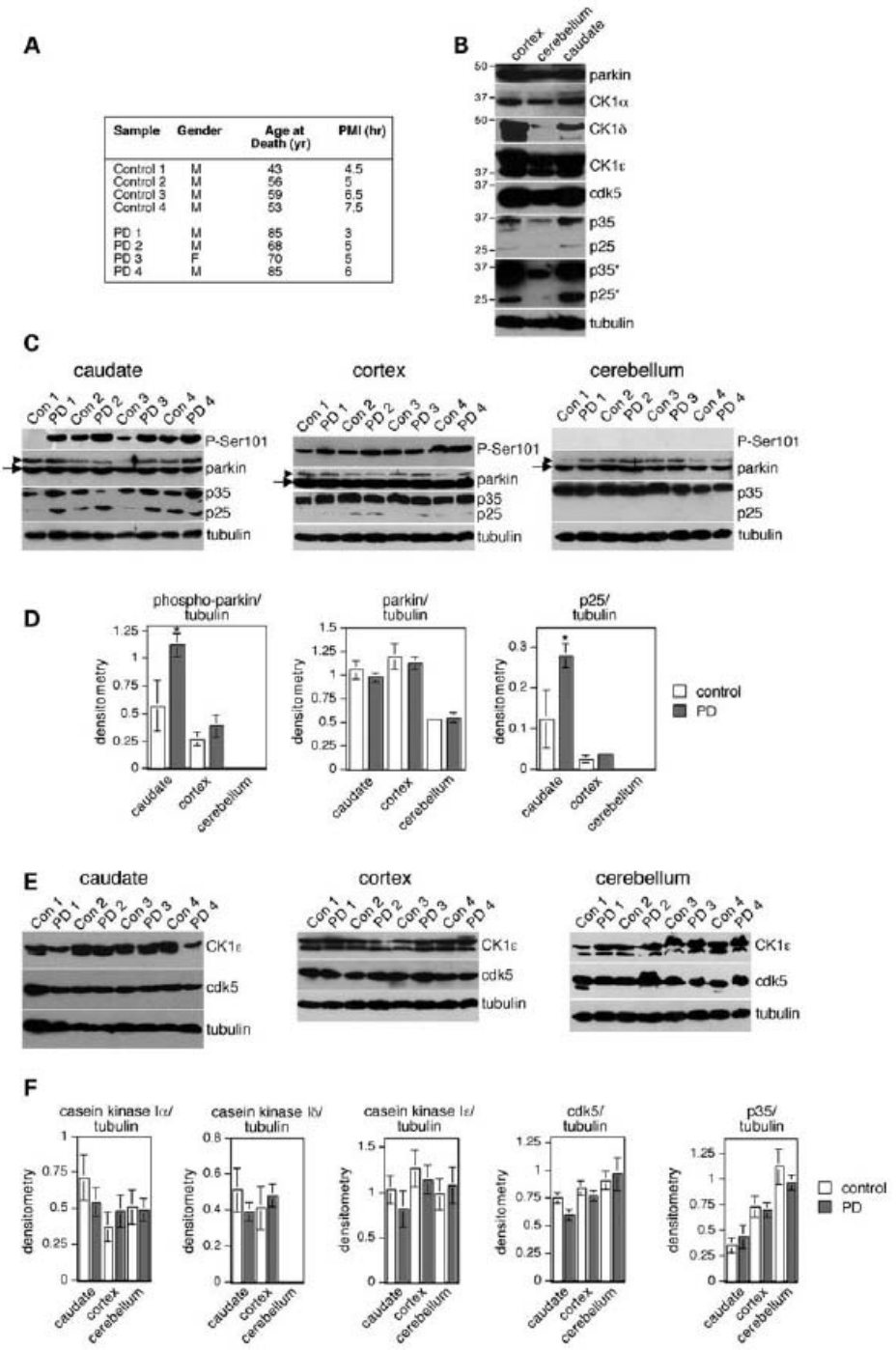


Figure 6. Selective increase in phospho-parkin and p25 in distinct areas of idiopathic PD brains. (A) Gender, age at death (years) and postmortem interval (PMI, h) for the eight human brains analyzed here. (B) Relative levels of total parkin, casein kinase I isoforms α, δ and ε, cdk5, p35 and p25 in three distinct brain areas from a control patient, and of tubulin as a protein loading control. * Overexposed blot shows the absence of detectable p25 levels in the cerebellum. (C) Levels of phospho-parkin (P-Ser101), parkin, p35/p25 and tubulin as a protein loading control were analyzed in caudate, cortex and cerebellum from four control and four PD patients which were matched for postmortem interval tissue collection. Arrows indicate parkin band, arrowheads indicate cross-reacting band detected with the anti-parkin antibody (Abcam). (D) Levels of phospho-parkin, parkin and p25 were normalized to tubulin levels and plotted on a histogram ($n = 4$; mean ± S.E.M., $^*P < 0.05$). Error bars are only depicted when larger than column lines. (E) Levels of casein kinase Iε (CK1ε), cdk5 and tubulin as a protein loading control were analyzed in caudate, cortex and cerebellum from four controls and four PD patients matched for postmortem interval tissue collection. (F) Levels of CK1α, δ and ε, cdk5 and p35 were normalized to tubulin levels and plotted on a histogram ($n = 4$; mean ± S.E.M.). Error bars are only depicted when larger than column lines.

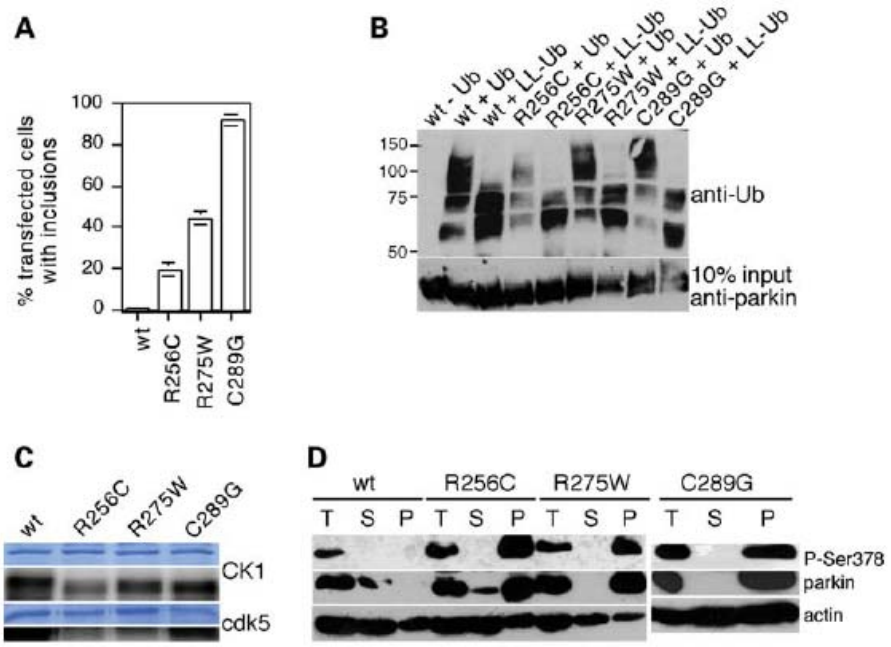


**Figure 7.** Analysis of pathogenic parkin mutants. (A) The percentage of transfected cells displaying parkin aggregates using wild-type or various pathogenic parkin point mutants (wt, $n = 5$; R256C, $n = 6$; R275W, $n = 11$; C289G, $n = 10$). Bars depict mean ± S.E.M. (B) Example of an autoubiquitylation experiment using wild-type or the indicated parkin mutants, and either wild-type ubiquitin (Ub) or a lysine-less derivative (LL-Ub). (C) Example of *in vitro* phosphorylation experiments using full-length recombinant parkin (wt), or the indicated point-mutated versions, and either casein kinase I (CK1, top) or cdk5 (bottom). (D) Analysis of the distribution of overexpressed wild-type (wt) or the various mutant parkin proteins (total, T) in the Triton X-100-soluble (S) and insoluble pellet (P) fractions of HEK293T extracts upon blotting with a phospho-state-specific parkin antibody (P-Ser378), an anti-parkin antibody (Abcam) or an anti-actin antibody. Note that the phosphorylation status of wild-type parkin in the soluble and insoluble fractions could only be detected in the presence of okadaic acid or upon prolonged exposure of the blot (not shown).

phosphatase activities, with concomitant hyperphosphorylation of proteins, can lead to PD-related neurodegeneration (42,43). Thus, a complex balance between kinase and phosphatase activities, possibly in a cell-type and region-specific manner, may be subject to acute and/or chronic regulation.

To determine whether inhibition of casein kinase I and cdk5 activities would display beneficial effects in decreasing the aggregative properties of pathogenic parkin-mutant protein, we chose to analyze three distinct point mutants whose pathogenic mechanism of action seems to involve enhanced aggregation (14–16). Importantly, using a combination of inhibitors against casein kinase I and cdk5, we could decrease the number of cells displaying parkin aggregates for two out of three mutants tested (R256C and R275W). For the third point mutant (C289G), which displayed the most dramatic aggregative phenotype, combined kinase inhibition led to a decrease in the number of cells displaying large perinuclear aggregates, indicating beneficial effects of such inhibitor treatment in modulating the profound aggregation even of this pathogenic mutant.

In conclusion, our experiments indicate that regulating the phosphorylation status of parkin has beneficial effects in reducing parkin aggregation and concomitant inactivation. Although future studies will be necessary to determine the neuroprotective or beneficial effects of combined kinase inhibition for PD subjects, the present results may have additional implications for other approaches in treating this neurodegenerative disorder, including gene replacement (44,45) or transplantation therapies (46).

## MATERIALS AND METHODS

### Plasmid construction

Human full-length parkin was amplified by PCR (primer sequences: 5′-TTA TGA ATT CAT ATA GTG TTT GTC AGG TTC AAC-3′ and 5′-TTT AAA GCT TTT ACA CGT CGA ACC AGT GGT CCC-3′) using a full-length piTrex human parkin cDNA construct and cloned into the *Eco*RI/*Hin*dIII restriction sites of the pGEX-KG vector (47). Constructs encoding sequences for the different human parkin domains (N1, N2, N3, C1, C2, C3) were generated by PCR using the piTrex parkin cDNA construct as a template and subcloned into the pGEX-KG vector as described above. For expression of parkin in mammalian tissue culture cells, human full-length parkin was amplified by PCR and subcloned into the *Eco*RI/*Xba*I restriction sites of the pCMV5-SV40-hGH vector (48). Point-mutated constructs were generated using the QuickChange Site-Directed Mutagenesis Kit (Stratagene) according to the manufacturer's instructions. The entire coding sequences of all constructs used in this study were verified by DNA sequencing. The sequences of all primers used in the present study are available upon author's request.

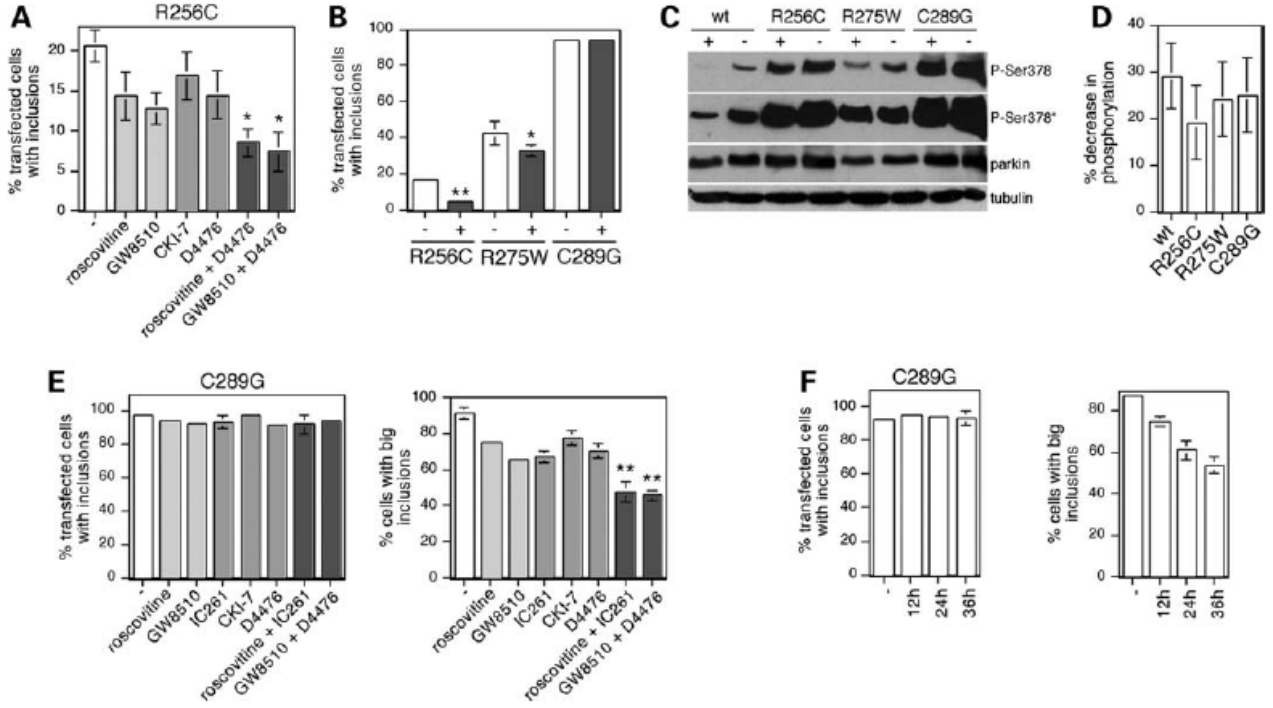


Figure 8. Inhibition of parkin phosphorylation decreases mutant parkin aggregation. (A) The percentage of transfected cells displaying R256C-mutant parkin aggregates were quantified in the absence (−) or presence of various kinase inhibitors, or combinations thereof, present for 12 h before fixation. Inhibitors used included roscovitine (0.5 μM), GW8510 (0.25 μM), CKI-7 (150 μM) and D4476 (17.5 μM). Bars depict mean ± S.E.M. ($n = 3$). Statistically significant additive effects were observed when using a combination of kinase inhibitors, when compared with individual inhibitor treatments. *$P < 0.1$. (B) Quantification of the percentage of transfected cells displaying R256C-mutant, R275W-mutant or C289G-mutant parkin aggregates in the absence (−) or presence (+) of 0.25 μM GW8510 and 17.5 μM D4476 (R256C, $n = 5$; R275W, $n = 3$; C289G, $n = 4$). Bars depict mean ± S.E.M. *$P < 0.1$; **$P < 0.01$. Error bars are only depicted when larger than column lines. (C) Analysis of the phosphorylation status of parkin in HEK293T cells transfected with wild-type (wt) or mutant parkin variants, treated without (−) or with (+) 0.25 μM GW8510 and 17.5 μM D4476 for 12 h before analysis upon blotting with the phospho-Ser378 antibody (*overexposed blot to show phosphorylation of wild-type parkin), an anti-parkin antibody (Abcam) or an anti-tubulin antibody. (D) Quantification of experiments of the type depicted in (C), where the amount of phospho-parkin in the presence of inhibitors, when compared with that in the absence of inhibitors, is plotted as % decrease in phosphorylation. Bars depict mean ± S.E.M. ($n = 3$). (E) The percentage of transfected cells displaying C289G-mutant parkin aggregates (left) and the percentage of those cells displaying (at least) one large perinuclear aggregate (right) were quantified in the absence (−) or presence of various kinase inhibitors, or combinations thereof, present for 12 h before fixation. Inhibitors used included roscovitine (0.5 μM), GW8510 (0.25 μM), IC261 (25 μM), CKI-7 (150 μM) and D4476 (17.5 μM). Bars depict mean ± S.E.M. ($n = 3$). Statistically significant additive effects were observed when using a combination of kinase inhibitors, when compared with individual inhibitor treatments. **$P < 0.05$. (F) The percentage of transfected cells displaying C289G-mutant parkin aggregates (left) and the percentage of those cells displaying (at least) one large perinuclear aggregate (right) were quantified in the absence (−) or presence of 0.5 μM roscovitine and 150 μM CKI-7. Inhibitors were added 12 h before fixation (12 h), 24 and 12 h before fixation (24 h) or 36, 24 and 12 h before fixation (36 h).

## Protein purification

All recombinant proteins were expressed as N-terminally tagged glutathione S-transferase fusion proteins in *Escherichia coli* BL21 DE3 cells and purified essentially as described (49) with minor modifications as outlined below. A 5 ml overnight culture of cells was diluted 200-fold and grown at 37°C to an $OD_{600}$ of around 0.6. Cells were incubated at 16°C for 15 min, followed by induction with 0.1 mM IPTG for 16–20 h at 16°C. Bacteria were pelleted at 10 000*g* for 10 min at 4°C, and the cell pellet resuspended in 12 ml (per liter culture) of resuspension buffer (PBS containing 1% TX-100, 1 mM PMSF, 1 mM DTT, 50 μg/ml RNAse, 50 μg/ml DNAse and 100 μg/ml lysozyme). The cell resuspension was incubated for 30 min at 4°C on a rotary shaker, followed by three sonication pulses (30 s each, separated by 30 s intervals) on ice. Upon centrifugation at 16 000*g* for 20 min at 4°C, the soluble fraction was filtered through a 0.22 μm filter and diluted with 6 ml of PBS containing 1% TX-100, 1 mM PMSF and 1 mM DTT (per liter of culture). Proteins were bound to glutathione Sepharose beads (Pharmacia) (750 μl of packed beads per liter of culture) for 2 h at 4°C. Beads were washed two times in PBS/1% TX-100, two times in PBS and two times in thrombin elution buffer (50 mM Tris–HCl, pH 8, 150 mM NaCl, 2.5 mM $CaCl_2$, 0.1% (v/v) β-mercaptoethanol). Pelleted beads were resuspended to a 50% slurry with thrombin elution buffer, and protein eluted with 50 units of thrombin (from bovine plasma; Sigma) per liter of culture for 1 h at 4°C with shaking. Purified proteins were dialyzed into PBS containing 1 mM DTT for 1 h at 4°C and small aliquots of recombinant parkin proteins were frozen at −20°C and only thawed up once. Protein concentration was determined by the BCA assay (Pierce) according to the manufacturer's instructions. Protein purity of all protein preparations was determined by Coomassie Blue staining, and in most cases was around 80–90%. In most cases, enzymatic activity of recombinant

full-length parkin was assayed by autoubiquitylation reactions (see below) before the protein was used in other assays.

### *In vitro* phosphorylation assays

Various recombinant wild-type and point-mutated full-length and truncated parkin proteins (1 μg) were subjected to *in vitro* phosphorylation reactions using buffer and enzyme conditions as outlined below. Phosphorylation by casein kinase I (0.5 units recombinant rat casein kinase Iδ; New England Biolabs) was performed in 50 mM Tris–HCl, 5 mM DTT, 10 mM $MgCl_2$, pH 7.5, using 20 ng casein as a positive control. Phosphorylation by cyclin-dependent protein kinase 5 (cdk5) (20 ng of human full-length cdk5/p25; Upstate) was performed in 8 mM MOPS/NaOH, 0.2 μM EDTA, 10 mM Mg-acetate, pH 7.0, using 500 ng of histone H1 as a positive control. Phosphorylation by casein kinase II (1 μg recombinant human casein kinase II; New England Biolabs) was performed in 20 mM Tris–HCl, 50 mM KCl, 10 mM $MgCl_2$, pH 7.5, using 20 ng of casein as a positive control. Phosphorylation by cAMP-dependent protein kinase A (PKA) (2.5 units of recombinant murine PKA; New England Biolabs) was performed in 50 mM Tris–HCl, 10 mM $MgCl_2$, pH 7.5, using 1 μg of purified bovine synapsin I as a positive control. Phosphorylation by glycogen synthase kinase 3β (GSK3β) (1 μg of recombinant human his-tagged GSK3β; Calbiochem) was performed in 25 mM Tris–HCl, pH 7.5, 5 mM β-glycerolphosphate, 12 mM $MgCl_2$, 2 mM DTT, 100 μM $Na_3VO_4$ using 100 ng of recombinant human tau protein (Calbiochem) as a positive control. Phosphorylation by 1 μg of protein kinase C (PKC) was performed in 50 mM HEPES, pH 7.4, 10 mM Mg-acetate, 1 mM EGTA, 1 mM EDTA, 1.5 mM $CaCl_2$, 1 mM DTT, 50 μg/ml phosphatidylserine and 4 μg/ml diacylglycerol using 1 μg of bovine synapsin I as a positive control. For phosphorylation by $Ca^{2+}$/calmodulin-dependent protein kinase II (CaMKII), 2.5 units of recombinant rat CaMKII (New England Biolabs) were activated for 10 min at 30°C in a buffer containing 50 mM Tris–HCl, pH 7.5, 10 mM $MgCl_2$, 2 mM DTT, 0.1 mM EDTA, 100 μM ATP, 1.2 μM calmodulin and 2 mM $CaCl_2$. One microgram of recombinant parkin was diluted in the same buffer and supplemented with 200 μM ATP, and 1 μg of bovine synapsin I served as a positive control. Phosphorylation by apoptosis signal-regulating kinase 1 (ASK1) (150 ng or recombinant human ASK1; Upstate) was performed in 8 mM MOPS/NaOH, 0.2 μM EDTA, 10 mM Mg-acetate, pH 7.0, using 1 μg of mouse myelin basic protein (MBP) (Sigma) as a positive control.

Unless otherwise stated, all reactions were carried out in a final volume of 40 μl and were initiated by the addition of ATP (final concentration of 100 μM) with trace amounts of [$\gamma-{}^{32}$P]ATP (GEHealthcare, specific activity 150 mCi/ml). After 30 min at 30°C with shaking (450 rpm), reactions were terminated by the addition of 0.2 volumes of 5× sample buffer and boiling for 5 min at 95°C. Proteins were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) on 10% polyacrylamide gels (or 12.5% gels for assays containing N1-parkin or C3-parkin domains), followed by staining with Coomassie blue dye. Incorporation of $^{32}$P was quantified using a PhosphorImager (Molecular Dynamics) and corrected for background values. Differences in protein amounts were quantified on Coomassie-stained gels using QuantityOne (Bio-Rad) and corrected for background values, and radioactivity values were corrected for differences in protein loading.

### Autoubiquitylation reactions

Recombinant full-length parkin (1 μg) was incubated with 80–150 ng of E1 enzyme (His-E1, Biomol), 2 μg of E2 enzyme (His-UbcH7, Biomol) and 5 μg of ubiquitin (Sigma), or lysine-less or methylated ubiquitin (Boston-Biochem), in a buffer containing 50 mM Tris–HCl, pH 7.4, 2 mM ATP, 1 mM DTT and 5 mM $MgCl_2$. The reactions were conducted at 30°C for 90 min with shaking (450 rpm) and terminated by adding 0.2 volumes of 5× sample buffer and heating for 5 min at 95°C. Samples were resolved in 7.5% SDS–PAGE gels, followed by immunoblotting using a monoclonal anti-ubiquitin antibody (clone 6C1, Sigma) at 1:1000 dilution overnight at 4°C, and equal amount of protein loading was assured by immunoblotting samples independently with an anti-parkin antibody (844) at 1:1000 dilution overnight at 4°C.

### Generation of anti-parkin and anti-phospho-parkin antibodies

Antibodies against parkin and phospho-parkin were generated in collaboration with PhosphoSolutions, Aurora, CO, USA. Briefly, antibodies were generated in rabbits against human parkin (around amino acids 400) (844) and against chemically phosphorylated S101 (EREPQS(phospho)LTRVDL) and S378 (GEGECS(phospho)AVFEAS) peptides. Phospho-state-specific antibodies were affinity-purified via sequential chromatography on phospho- and dephospho-peptide affinity columns. The p-Ser101 antibody was generally used to detect phospho-parkin in human brain extracts, whereas the p-Ser378 antibody was preferentially used to detect phospho-parkin in transfected HEK293 cell extracts.

### Cell culture, transfection and cell lysis

HEK293T cells were cultured in 100-mm dishes and grown at 37°C in 5% $CO_2$ in growth medium (Dulbecco's modified Eagle's medium) with 4 mM glutamine and non-essential amino acids (Sigma), 10% heat-inactivated fetal bovine serum (Invitrogen), penicillin (100 units/ml) and streptomycin (100 units/ml). Confluent cells were harvested using 0.05% trypsin, 0.02 mM EDTA in PBS and subcultured at a ratio of 1:4 to 1:6. Cells at 50–60% confluency were transfected using 8 μg of plasmid of interest and 80 μl of PolyFect transfection reagent (Qiagen) per 100 mm dish according to the manufacturer's specifications for 4 h in growth medium, followed by addition of a fresh medium.

For preparation of extracts, cells were collected 48 h after transfection. Prior to collection, transfected cells were treated with 1 μM roscovitine (Calbiochem) for 12 h to inhibit endogenous cdk5 activity, with 50 μM IC261 (3-[(2,4,6-trimethoxyphenyl)methylidenyl]indolin-2-one) (Calbiochem) for either 3 or 12 h to inhibit endogenous casein

kinase I activity, or with 35 μM D4476 for 12 h to inhibit endogenous casein kinase I activity. Cell viability was assessed by visual inspection as well as by Trypan blue exclusion assays. Although treatment of cells with 50 μM IC261 for 12 h caused a slight decrease in cell viability (by 3.6 ± 1.6%, mean ± S.E.M., $n = 3$), treatment of cells with IC261 for 3 h, or treatment of cells with any other kinase inhibitor analyzed in the present study did not display any effect upon cell viability. Where indicated, cells were further treated with 0.5 μM okadaic acid (Alomone Labs) for 1 h prior to processing to inhibit endogenous phosphatase activity. Cells were rinsed once in ice-cold PBS followed by resuspension in 1–1.5 ml of lysis buffer per 100 mm dish (1% SDS in PBS containing 1 mM PMSF, 1 mM $Na_3VO_4$ and 5 mM NaF). Resuspended cells were incubated for 15 min at 4°C in a rotary shaker, sonicated twice (two pulses for 1 s each) and centrifuged at 13 500 rpm for 10 min at 4°C. Protein concentrations of supernatants were estimated using the BCA assay (Pierce), followed by the addition of 0.2 volumes of 5× sample buffer and boiling for 5 min at 95°C. Samples were directly analyzed on western blots, as freeze–thaws were found to abolish detection of phospho-parkin levels. Unless otherwise stated, 80–100 μg of total protein extracts were loaded per lane to ensure sensitive detection of phospho-parkin levels.

For sequential fractionation studies, cells were collected 72 h after transfection. Cells were rinsed once in ice-cold PBS, followed by resuspension in 4 ml ice-cold PBS and the cell suspension equally split into two tubes. After centrifugation for 5 min (1500 rpm, 4°C), pellets were resuspended in 400 μl of buffer (50 mM Tris–HCl, pH 8.0, 300 mM NaCl, 1.5 mM $MgCl_2$, 2 μg/ml chymostatin, 100 units/ml aprotinin, 1 mM PMSF, 1 mM $Na_3VO_4$ and 5 mM NaF) either containing 1% SDS (total) or containing 1% Triton X-100. The latter tube was incubated for 10 min at 4°C on a rotary shaker, followed by centrifugation at 82 000*g* for 20 min at 4°C. The supernatant (Triton-soluble) was removed, and the pellet resuspended in 200 μl of buffer containing 1% SDS, followed by repetitive sonication to obtain complete solubilization (Triton-insoluble). Protein concentration was estimated by the BCA assay (Pierce), and 40 μg of the total and the Triton-soluble fractions and 20 μg of the Triton-insoluble fractions were resolved by SDS–PAGE and analyzed by means of Western blotting. Extracts were independently analyzed using a rabbit anti-actin antibody (Sigma, 1:100) to account for differences in protein loading.

### Immunocytochemistry

HEK293T or HEK293T/17 cells were plated onto 6-well plates at 40% confluency and transfected the following day (60% confluency) using 2 μg of DNA and 20 μl of PolyFect transfection reagent according to the manufacturer's instructions. Transfected cells were re-plated the next day at a 1:2 or 1:3 ratio onto coverslips and processed for immunocytochemistry 2 days later. Where indicated, cells were treated with 5 μM MG-132 (carbobenzoxy-L-leucyl-L-leucinal) (Calbiochem) for 12 h to inhibit proteasome activity, which enhances aggresome formation. Cells were then fixed for 30 min at 37°C with 4% (w/v) paraformaldehyde in PBS. Cells were permeabilized in 0.5% Triton X-100 in PBS (3×5 min washes) and preincubated in blocking buffer (10% goat serum; Vector Laboratories) in 0.5% Triton X-100 in PBS for 1 h at room temperature, followed by exposure to primary antibody [1:1000, polyclonal rabbit anti-parkin (844)] diluted in blocking buffer for 1 h at room temperature. Cells were washed in 0.5% Triton X-100 in PBS and incubated with goat anti-rabbit AlexaFluor-488-conjugated secondary antibody (1:1000, Invitrogen), diluted in 0.5% Triton X-100 in PBS, for 1 h at room temperature, followed by washes in 0.5% Triton X-100 in PBS, PBS, $H_2O$ and a rinse in 70% ethanol. Fixed cells were mounted using mounting medium containing DAPI (Vector Laboratories) and visualized on a Zeiss microscope using a 40× or 100× oil-immersion objective. Using this technique, transfection levels were usually found to be around 60%.

To determine the percentage of transfected cells containing parkin inclusions, cells overexpressing parkin were scored to display either diffuse cytosolic or aggregate staining. In addition, cells displaying aggregates were scored to either display at least one large perinuclear parkin aggregate or only multiple small aggregates. These values were used to determine the percentage of cells with (at least one) large inclusion. Perinuclear aggregates were independently verified to be aggresomes by double-staining with a monoclonal anti-vimentin antibody (1:200, clone V9, Sigma) and a goat anti-mouse AlexaFluor-594-conjugated secondary antibody (1:1000, Invitrogen). For all determinations, between 250 and 1000 transfected cells were analyzed, and in all cases, additional analysis was performed by an observer blind to conditions. Cells were also examined using a Leica TCS-SP5 confocal microscope and Leica Applied Systems (LAS-AF) image acquisition software. Images were collected using single excitation for each wavelength separately and channels subsequently merged using Adobe Photoshop.

### Human cases

All PD cases had suffered from classical parkinsonism and none of them had apparent cognitive impairment or dementia. The postmortem delay between death and tissue processing was between 3 and 7.5 h. The pH of the brain was between 6.6 and 6.9 in all cases. One-half of the brain was immediately cut on coronal sections, 1 cm thick, frozen in dry ice and stored at −80°C until use.

All sporadic PD cases were tested for the G2019S LRRK2 mutation (50) and were also tested for the c.255delA mutation in parkin (51) if their age at onset was before 55 years. None of the samples analyzed here were positive for either mutation.

Variable phenotypes and mixed pathologies can be observed in a significant fraction of postmortem cases, such as the presence of Alzheimer's disease-associated lesions combined with α-synuclein pathology in diagnosed PD cases, implying possible molecular crosstalks in protein aggregation mechanisms (52). In addition, Lewy body pathology has been observed in around 10% of brains from normal elderly subjects (53). To assure that our control subjects do not display Lewy body pathology and that our PD subjects display α-synuclein pathology in the absence of Alzheimer disease (AD)-related lesions, all samples were subjected to an extensive neuropathological study. Analysis was carried

out on de-waxed 4-μm-thick paraffin sections of the frontal (area 8), primary motor, primary sensory, parietal, temporal superior, temporal inferior, anterior gyrus cinguli, anterior insular, and primary and associative visual cortices; entorhinal cortex and hippocampus; caudate, putamen and globus pallidus; medial and posterior thalamus; subthalamus; Meynert nucleus; amygdala; midbrain (two levels), pons and bulb; and cerebellar cortex and dentate nucleus. The sections were stained with hematoxylin and eosin, Kluever Barrera and, for immunohistochemistry, to glial fibrillary acidic protein (Dako, 1:250), CD68 (Dako, 1:100), βA-amyloid (Boehringer Mannhein, 1:50), tau AT8 (Innogenetics, 1:500), tau 4R and tau 3R (Upstate, 1:200 and 1:2000, respectively), phosphorylation-specific tau Thr181, Ser202, Ser214, Ser262, Ser396 and Ser422 (all Calbiochem, 1:100, except Thr181, 1:250), αB-crystallin (Abcam, 1:100), ubiquitin (Dako, 1:200), α-synuclein (Chemicon, 1:3000), phosphorylation-specific α-synuclein Ser129 (WAKO, 1:2000) and nitrated/oxidized α-synuclein (Zymed, 1:400). Following incubation with the primary antibody, the sections were incubated with EnVision+system peroxidase for 15 min at room temperature. The peroxidase reaction was visualized with diaminobenzidine and $H_2O_2$. In all cases, control of the immunostaining included omission of the primary antibody.

Neuropathological characterization of PD was according to established criteria (54). All PD cases corresponded to stage 4 of Braak and Braak. This implies involvement of selected nuclei of the medulla oblongata, pons and midbrain, amygdala and nucleus basalis of Meynert. All cases showed marked loss of neurons in the substantia nigra pars compacta exceeding 60%, whereas moderate pathology occurred in the caudate and putamen. In no case did Lewy pathology involve the frontal cortex. No cases of dementia with Lewy bodies, according to the guidelines of the specialized international workshop (55,56), were included in the present series. AD-related pathology, including β-amyloid plaques and neurofibrillary tangles, was absent in the present series. Control subjects showed absence of neurological symptoms, metabolic and vascular diseases, and the neuropathological study disclosed no abnormalities including lack of PD- and AD-related pathology.

### Human tissues and sample preparation

Freshly frozen brain samples from deceased human subjects were collected at autopsy following informed consent from the next of kin under a protocol approved by the local ethics committee. Brain regions from each control and PD patient analyzed included cortex, cerebellum and caudate. For all samples, patient age, gender, time to postmortem tissue collection and postmortem pathological analysis was known.

Small sections of frozen tissue blocks were added to 700 μl of lysis buffer (1% SDS in PBS, 1 mM PMSF, 1 mM $Na_3VO_4$ and 5 mM NaF) and lysed using a 7-ml Dounce homogenizer with a Teflon pestle. Lysis was performed by applying slow strokes during maximally one minute. For cerebellar samples, an additional two pulses of sonication (1 s each) were needed to fully solubilize the tissue. Homogenates were subsequently centrifuged for 10 min at 13'500 rpm at 4°C. Protein concentration of the supernatants was estimated using a BCA assay (Pierce), and 0.2 volumes of 5× sample buffer added to extracts and boiling for 5 min at 95°C. Care was taken to ensure extracts were highly concentrated (>5 mg/ml) such that phospho-parkin levels could be determined using mini-gels. As mentioned above for transfected tissue culture cell extracts, samples were directly analyzed on western blots, as repetitive freeze–thaws were found to abolish detection of phospho-parkin levels and 80–100 μg of total protein extracts were loaded per lane to ensure sensitive detection of phospho-parkin levels.

### Western blotting

Proteins were resolved by SDS–PAGE, transferred onto polyvinylidene difluoride membranes (Hybond, GEHealthcare) and probed with primary antibodies overnight at 4°C. The following antibodies were employed: a rabbit polyclonal anti-parkin antibody (1:1000 for cell and brain extracts, ab6177, Abcam), a custom-made anti-parkin antibody 844 (1:1000 for recombinant proteins), an anti-phospho-S101-parkin antibody (1:200 for cell extracts and brain extracts) an anti-phospho-S378-parkin antibody (1:500 for cell extracts; 1:5000 for recombinant proteins), anti-p35 (1:100, C-19, Santa Cruz Biotechnology), anti-cdk5 (1:400, DC17, Santa Cruz Biotechnology), anti-casein kinase Iε (1:250, BD Transduction Laboratories), anti-casein kinase Iα (1:200, C19, Santa Cruz Biotechnology), anti-casein kinase Iδ (1:50, H60, Santa Cruz Biotechnology), anti-α-tubulin (1:5000, clone DM1A, Sigma) and anti-actin (1:100, Sigma). Membranes were washed and incubated with secondary antibodies [anti-rabbit HRP-conjugated antibody (1:2000) or anti-mouse HRP-conjugated antibody (1:2000) (Dako Cytomation)] for 90 min at room temperature, followed by detection using ECL reagents (Roche).

### Statistical analysis

Experiments were done the indicated amount of times, and the data were analyzed using a Student's paired *t*-test.

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*Conflict of Interest statement*. None declared.

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## 1.B- Annex 1:

### 1.B.1- Anti-parkin antibodies tested:

For studying parkin *in vitro* and *in vivo*, 3 antibodies were tested during this thesis. The first one used was from Abcam, generated against a peptide encompassing amino acids 305-323, situated between the RING1 and the IBR domain. The sequence of this peptide is quite conserved between human, mouse and rat parkin, but it is not present in other proteins.

The antibody was useful for Western blotting (WB) of recombinant purified proteins, human and rat cell line extracts, and human and rat brain extracts (Figure 17).

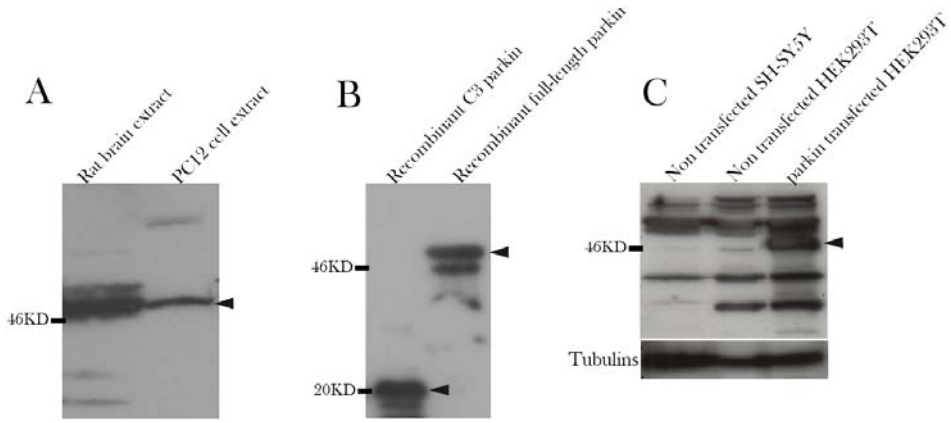


**Figure 17**: Anti-parkin Ab-cam antibody western blot. A. Endogenous parkin was detected in rat brain extract and PC12 cell extract. B. The antibody is also useful for recombinant purified human full-length parkin and its C3 fragment (IBR-RING2). C. Endogenous parkin was not detected with this antibody in SH-SY5Y and HEK293 cells but only upon transient overexpression.

Another commercial antibody tested was an Anti-parkin antibody from Cell Signalling. It was used for recombinant protein detection by WB, and immunofluorescence in HEK293T and PC12 cells. The antibody was not useful for WB of human and rat brain extract samples or of cell extracts overexpressing parkin.

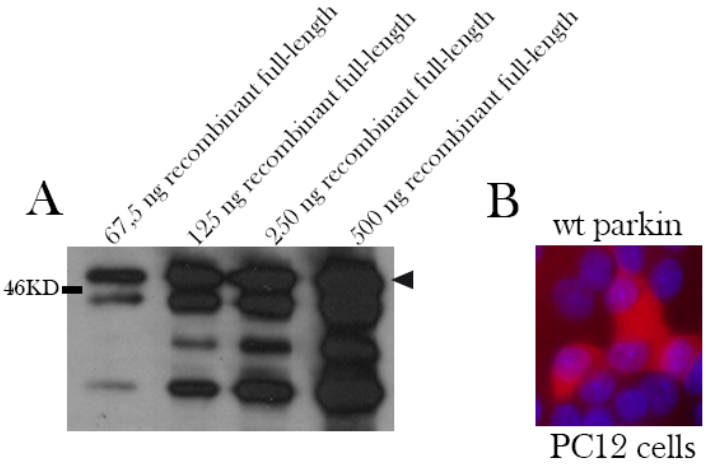


**Figure 18**: Anti-parkin antibody from Cell Signalling. A. The antibody was useful for recombinant purified human parkin detection. B. Non differentiated PC12 cells were transfected with wt parkin and stained with the Cell Signalling antibody. Nuclei were stained with DAPI.

844 antibody was generated in collaboration with PhosphoSolutions [22] and now is commercially available. The antibody gives a good signal by WB of recombinant proteins, but it does not recognize parkin from human and rat brain and cell extracts. The staining for immunofluorescence in cells expressing parkin is quite strong and has been really useful for aggresome detection.

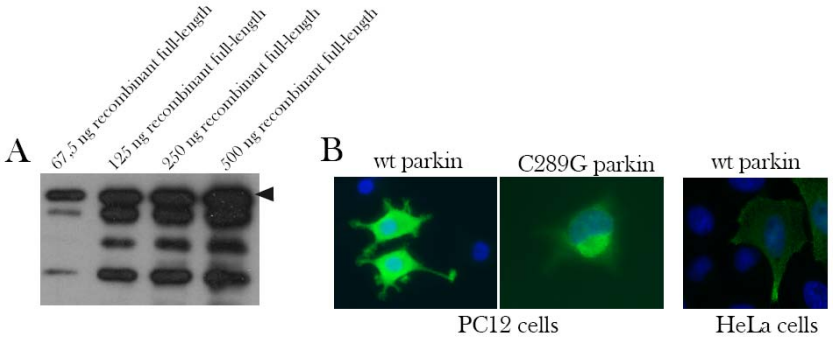


**Figure 19**: 844 anti-parkin antibody. A. The antibody was used for recombinant purified human parkin detection by Western blotting. B. Differentiated PC12 cells or HeLa cells were transfected with wt parkin or the C289G parkin mutant and stained with 844 antibody. Nuclei were stained with DAPI.

## 1.B.2- Caspase cleavage of parkin:

Caspases comprise a family of intracellular cysteine-proteases that are central initiators and executers of apoptosis. Parkin has been described to be processed by caspase-1 and 8, after aspartic residue 126 [299, 300]. Because of the proximity of this position to the CKI phosphorylation site serine 127, we decided to study whether parkin phosphorylation can modulate caspase cleavage.

Our results suggest that parkin phosphorylation by CKI can increase the cleavage of the protein by recombinant caspase 8 (Figure 20). However, these assays were not further pursued due to their largely irreproducible nature.

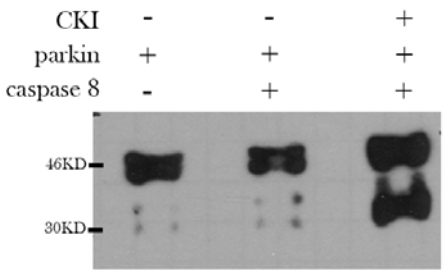


**Figure 20**: Parkin phosphorylation by CKI increses its cleavage by caspase 8 *in vitro*.

## 1.B.3- Parkin activity against synphilin-1:

We tried to study parkin-mediated ubiquitylation of a substrate (synphilin-1) in the HEK293T cell-based system, using wildtype as well as various mutant parkin proteins. For this purpose, we triple-transfected cells with parkin or parkin variants, HA-ubiquitin and myc-synphilin-1, and exactly followed published procedures (e.g. (Avraham, 2007 #143)). However, at least in our hands, we have been unable to detect significant and reproducible parkin-mediated ubiquitylation of synphilin-1 (Figure 21).

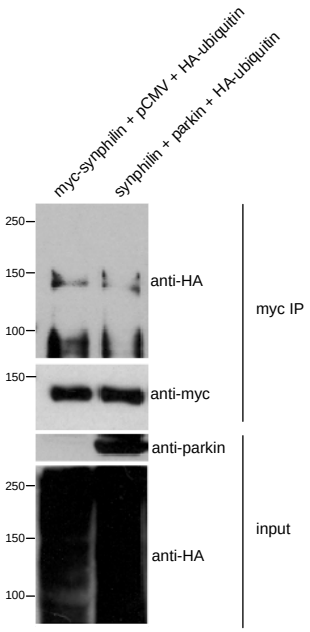


**Figure 21**: HEK293T cell extracts were triple-transfected as indicated, followed by immunoprecipitation using an anti-myc antibody. Ubiquitylated myc-synphilin was detected with an anti-HA antibody, and immunoprecipitation efficiency evaluated using an anti-myc antibody. Inputs (10%) were independently probed for parkin expression, as well as for expression and incorporation of HA-ubiquitin.

## 1.B.4- HEK293T cells as a model:

HEK293T cells were analyzed for kinase expression by WB, using specific antibodies against Cdk5, p35 and the different CKI isoforms, showing that this cell line is a good model for studying parkin phosphorylation. Note that we have not been able to analyse differences in the levels of the CKI β and γ isoforms, as two commercial antibodies (Santa Cruz) did not detect bands of the expected molecular weight for those isoforms, at least in our hands (Figure 22).

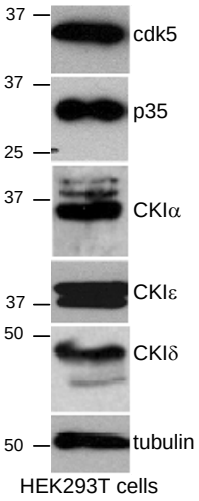


**Figure 22**: HEK293T cell extracts were analyzed by SDS-PAGE followed by WB for the presence of endogenous cdk5, p35, and the α, ε and δ isoforms of CKI.

## 1.B.5- Materials and Methods:

### 1.B.5.1- Tissue culture:

PC12 cell culture and transfections were carried out essentially as described [304]. PC12 cells (Riken Cell Bank) were cultured on 100 mm dishes coated with collagen (rat tail collagen type I; BD Biosciences). Cells were grown at 37 ºC in 5 % $CO_2$ in full medium (RPMI 1640 with heat-inactivated 10 % horse serum and 5 % fetal bovine serum, penicillin (50 units/ml) and streptomycin (50 units/ml)), and subcultured at a ratio of 1:2. Confluent cells were harvested using 0.25 % trypsin, 0.1 mM EDTA in PBS without $Ca^{2+}$ and $Mg^{2+}$. For immunocytochemistry of PC12 cells, confluent cells in 100 mm dishes were re-plated into 6-well plates at 80% confluency. The next day (90-95% confluency), cells were transfected with 3 µg plasmid of interest and 10 µl LipofectAMINE 2000 (Invitrogen) according to the manufacturer's specification, in Dulbecco's modified Eagle's medium without serum and antibiotics. After 4 hours, the transfection mixture was replaced by full medium. Transfected cells were harvested the next day and re-plated onto coverslips coated with poly-L-lysine (coated for 1 hour with 50 µg/ml poly-L-lysine, $M_r$ 30,000 – 70,000 (Sigma)) at a 1:4.5 ratio and grown in serum-reduced (1 %) medium containing 50 ng/ml NGF (2.5S NGF, Invitrogen) for 2 days before fixation.

Immuncytochemistry was performed as described for HEK293T cells, except that PC12 cells were fixed for 20 min in 4 % (w/v) paraformaldehyde in PBS containing 4% (w/v) sucrose.

HeLa cells were cultured in T75 flasks and grown at 37 ºC in 5% $CO_2$ in DMEM with 5% heat-inactivated fetal bovine serum, penicillin (100 units/ml) and streptomycin (100 units/ml) and non-essential aminoacid solution, and subcultured at a ratio of 1:10-1:15 three times per week. Confluent cells were harvested washing two times with PBS and using trypsin, 0.1 mM EDTA. For transfection, cells were diluted 1:10 and plated into 12 well plates containing coverslips. Next day, cells were transfected with 2 µg of DNA in 4 µl JetPeI transfection reagent per well, according to manufacturer's instructions. Immunocytochemistry was performed 2 days after transfection.

For immunocytochemistry, primary antibodies included anti-parkin (1:1000, Cell Signaling) or 844 anti-parkin (1:1000), and secondary antibodies goat anti-rabbit Alexa-Fluor-488 or Alexa-Fluor-594 (1:1000, Molecular Probes) were used.

SH-SY5Y cells were cultured in 100mm dishes and grown at 37 ºC in 5% $CO_2$ in DMEM/F12 50:50 mix (SIGMA) with 5% heat-inactivated fetal bovine serum, penicillin (100 units/ml) and streptomycin (100 units/ml) and non-essential aminoacid solution, and subcultured at a ratio of 1:5-1:6 once per week. Confluent cells were harvested using 0.25 % trypsin, 0.1 mM EDTA in PBS.

1.B.5.2- Caspase cleavage:

1 µg of recombinant purified human full-length parkin was incubated for 4 hours at 37 ºC with shaking in a buffer containing 5 mM PIPES, 100 mM NaCl, 10 mM DTT, 1 mM EDTA, 0,1% CHAPS and 1% saccarose. 200 ng of caspase 8 (Alexis) was added to the buffer prior to incubation at 37 ºC. For pre-phosphorylation, recombinant parkin was *in vitro* phosphorylated by CKI as previously described. Caspase cleavage buffer containing 200 ng of caspase 8 was added directly to the phosphorylated protein and the reaction incubated for 4 hours at 37 ºC. The reactions were stopped by addition of 5x sample buffer + β-mercaptoethanol.

1.B.5.3- Synphilin-1 ubiquitylation:

HEK293T cells were triple transfected with myc-synphilin, HA-ubiquitin and wt parkin or its mutants. Cells were expressing 72 hours and were treated with 5 µM of MG-132 during the last 12 hours. Cells were washed once with 2 ml of cold PBS and collected in 5 ml of cold PBS. They were centrifuged at 1500 rpm for 5 minutes and resuspended in 1 ml of cold IP buffer (50 mM Tris pH 7,4, 140 mM NaCL, 1% Tx-100, 0,1% SDS, 5 mM NaF, 1 mM PMSF, 1 mM $Na_3VO_4$, 5 µM MG-132 and proteases inhibitor cocktail). Extracts were incubated for 10 minutes at 4ºC with shaking and centrifuged at 13,200 rpm for 10 minutes at 4ªC. Supernatants were used as soluble extracts for IP. G Plus/ Protein A agarose beads (Calbiochem) were washed twice with 1 ml of cold IP buffer and incubated with 4 µg of Anti-c-myc antibody [75] in 200 µl of IP buffer for 1 hour at 4ºC. After binding, beads were washed twice with 1 ml of IP buffer. 500 µl of soluble cell extract was added to the beads and incubated with shaking at 4ºC for 3 hours. Beads were extensively washed with cold IP buffer and eluted with 100 µl of 2x Sample buffer + β-mercaptoethanol. 35 µl of eluate was loaded on a gel. Blotting was performed using anti-HA Ab (Ab-cam). For input, 50 µl of soluble input was loaded on a gel and blotted with anti-parkin (Ab-cam) and anti-c-myc (Sigma).

1.B.5.4- Primer sequences:

*Primers used for subcloning:*

| Name | Sequence: |
|---|---|
| PGEX-parkN1/N2/N3/Nfl-5' | TTA TGA ATT CAT ATA GTG TTT GTC AGG TTC AAC |
| PGEX-parkN1-3' | TTT AAA GCT TTT AGT TGT AGA TTG ATC TAC CTG C |
| PGEX-parkN2-3' | TTT AAA GCT TTT AAT TTG TTG CGA TCA GGT G |
| PGEX- parkN3-3' | TTT AAA CGT TTT AAC AGC CAG CCA CAC AAG GCA G |
| PGEX-parkNfl/C1/C2/C3-3' | TTT AAA GCT TTT ACA CGT CGA ACC AGT GGT CCC |
| PGEX-parkC1-5' | TTA TGA ATT CAT AGC TTT TAT GTG TAT TGC AAA GG |
| PGEX-parkC2-5' | TTA TGA ATT CAT AGT CGG AAC ATC ACT TGC ATT AC |

| | |
|---|---|
| PGEX-parkC3-5’ | TTA TGA ATT CAT CCC AAC TCC TTG ATT AAA GAG |
| Parkin-CMV-5’ | ATA AGA ATT CAT GAT AGT GTT TGT CAG GTT CAA |
| Parkin-CMV-3’ | TAA ATC TAG ATT ACA CGT CGA ACC AGT GGT C |

*Primers used for site-directed mutagenesis:*

| Name: | Sequence: |
|---|---|
| S101A-5’ | GAG CGG GAG CCC CAG GCA TTG ACT CGG GTG GAC |
| S101A-3’ | GTC CAC CCG AGT CAA TGC CTG GGG CTC CCG CTC |
| S101E-5’ | GAG CGG GAG CCC CAG GAG TTG ACT CGG GTG GAC |
| S101E-3’ | GTC CAC CCG AGT CAA CTC CTG GGG CTC CCG CTC |
| S127A-5’ | GTC ATT CTG CAC ACT GAC GCT AGG AAG GAC TCA CCA CC |
| S127A-3’ | GGT GGT GAG TCC TTC CTA GCG TCA GTG TGC AGA ATG AC |
| S127E-5’ | CAT TCT GCA CAC TGA CGA GAG GAA GGA CTC ACC ACC |
| S127E-3’ | GGT GGT GAG TCC TTC CTC TCG TCA GTG TGC AGA ATG |
| S131A-5’ | CTG ACA GCA GGA AGG ACG CTC CAC CAG CTG GAA GTC C |
| S131A-3’ | GGA CTT CCA GCT GGT GGA GCG TCC TTC CTG CTG TCA G |
| S131E-5’ | CTG ACA GCA GGA AGG ACG AGC CAC CAG CTG GAA GTC C |
| S131E-3’ | GGA CTT CCA GCT GGT GGC TCG TCC TTC CTG CTG TCA G |
| S131Aon127A-5’ | CAT TCT GCA CAC TGA CGC TAG GAA GGA CGC TCC AC |
| S131Aon127A-3’ | GTG GAG CGT CCT TCC TAG CGT CAG TGT GCA GAA TG |
| S131Eon127E-5’ | CGA GAG GAA GGA CGA GCC ACC AGC TGG AAG TC |
| S131Eon127E-3’ | GAC TTC CAG CTG GTG GCT CGT CCT TCC TCT CG |
| T240A-5’ | GAA CAT CAC TTG CAT TGC ATG CAC AGA CGT CAG G |
| T240A-3’ | CCT GAC GTC TGT GCA TGC AAT GCA AGT GAT GTT C |
| R256C-5’ | GTT TTC CAG TGC AAC TCC TGT CAC GTG ATT TGC TTA G |
| R256C-3’ | CTA AGC AAA TCA CGT GAC AGG AGT TGC ACT GGA AAA C |
| R275W-5’ | GAC AAG ACT CAA TGA TTG GCA GTT TGT TCA CGA C |
| R275W-3’ | GTC GTG AAC AAA CTG CCA ATC ATT GAG TCT TGT C |
| C289G-5’ | GGC TAC TCC CTG CCT GGC GTG GCT GGC TGT CCC |
| C289G-3’ | GGG ACA GCC AGC CAC GCC AGG CAG GGA GTA GCC |
| S378A-5’ | CAT GAA GGG GAG TGC GCA GCC GTA TTT GAA GCC |
| S378A-3’ | GGC TTC AAA TAC GGC TGC GCA CTC CCC TTC ATG |
| S378E-5’ | CAT GAA GGG GAG TGC GAG GCC GTA TTT GAA GCC |
| S378E-3’ | GGC TTC AAA TAC GGC CTC GCA CTC CCC TTC ATG |

## 1.C-Annex 2:

### 1.C.1- Patente: Uso de inhibidores de quinasas para el tratamiento de la enfermedad de Parkinson:

*Uso de inhibidores de quinasas para la elaboración de composiciones farmacéuticas para el tratamiento de la enfermedad de Parkinson, composiciones farmacéuticas y procedimientos de diagnóstico para la enfermedad de Parkinson.*

*Número de patente:* P200802696

*Solicitante:* Consejo Superior de Investigaciones Científicas (CSIC)

*Inventores:* Navarro Hilfiker, Sabine

Rubio de la Torre Gil, Elena

#### 1.C.1.1. Sector de la técnica:

Métodos para el diagnóstico, la prevención y tratamiento de trastornos neurológios asociados a cuerpos de Lewy, incluyendo enfermedad de Parkinson.

#### 1.C.1.2. Descripción breve de la invención:

Un aspecto de la siguiente invención lo constituye el uso de un compuesto inhibidor de la proteína Caseína quinasa I o de la proteína quinasa Cdk5, o de ambas, en adelante uso de un compuesto de la presente invención, para la elaboración de un medicamento o composición farmacéutica útil para el tratamiento de una enfermedad asociada a cuerpos de Lewy, preferentemente para el tratamiento de la enfermedad de Parkinson.

Un aspecto particular de la invención, lo constituye el uso de un compuesto de la invención en el que el compuesto inhibidor sea un inhibidor de la enzima Caseína quinasa I perteneciente, a título ilustrativo, y sin que limite el alcance de la invención, al siguiente grupo: IC261, CKI-7 y D4476.

Otro aspecto importante de la invención lo constituye el uso de un compuesto de la invención en que el compuesto inhibidor sea un inhibidor de la enzima Cdk5, perteneciente, a título ilustrativo, y sin que limite el alcance de la invención, al siguiente grupo: R-roscovitina, también conocido como CYC202 o seciclib, BMI-1026 (bis(amminopiridina)), Aloisina A (una pirrolopirazina sintética)

y GW8510. En el caso del inhibidor R-roscovitina, se ha iniciado un ensayo clínico en Fase II para el tratamiento del cáncer. Por otro lado, se ha descrito que roscovitina puede inhibir a Caseína quinasa I en ciertas condiciones, por lo que con este compuesto se podría producir la inhibición de ambas quinasas (lo cual es consistente con el leve efecto aditvo observado en células en cultivo).

Otro aspecto particular de la invención lo constituye el uso de un compuesto de la invención en el cual el compuesto inhibidor es un ácido nucleico o polinucleótido que impide o disminuye la expresión del gen codificante de , al menos, una secuencia de nucleótidos seleccionada entre:

a) una secuencia de nucleótidos antisentido específica de la secuencia del gen o del mRNA de la enzima Caseína quinasa I o Cdk5.
b) Una ribozima específica del mRNA de la proteína Caseína quinasa I o Cdk5
c) Un aptámero específico del mRNA de la enzima Caseína quinasa I o Cdk5
d) Un RNA de interferencia (shRNAi) específico del mRNA de la enzima Caseína quinasa I o Cdk5
e) Un microRNA específico de la enzima Caseína quinasa I o Cdk5.

Otro aspecto de la invención lo constituye una composición farmacéutica útil para el tratamiento de la enfermedad de Parkinson, en adelante composición farmacéutica de la invención, que comprenda una cantidad terapéuticamente efectiva de un compuesto o agente inhibidor de la enzima Caseína quinasa I o Cdk5, o de ambas, junto con, opcionalmente, uno o más adyuvantes y/o vehículos farmaceuticamente aceptables.

Finalmente, otro objeto de la presente invención lo constituye un procedimiento de diagnóstico y pronóstico de enfermedades asociadas con cuerpos o agregados de Lewy, preferentemente de la enfermedad de Parkinson, *ex vivo*, en adelante procedimiento de diagnóstico de la invención, basado en la determinación *in vitro* en células del sistema nervioso central, de la expresión de al menos una de

las siguientes proteínas: parkina fosforilada, p35 y Cdk5, y que comprende las siguientes etapas:

a) toma de una muestra biológica del sistema nervioso central
b) identificación o determinación de los niveles de, al menos, de una de las proteínas mencionada (parkina fosforilada, p35 o Cdk5), en la muestra del sistema nervioso central mencionada en el apartado a), y
c) comparación de dicha determinación observada en b) con una muestra control, donde la presencia incrementada de la proteína sea indicativa de una enfermedad asociada a cuerpos de Lewy.

Otro objeto particular de la invención lo constituye el procedimiento de diagnóstico de la invención, donde la muestra bilógica de a) se obtiene de neuronas pertenecientes a un plexo autónomo.

Otro objeto particular de la invención lo constituye el procedimiento de diagnóstico de la invención, donde la determinación de b) se realiza al menos con un anticuerpo específico de la proteína parkina fosforilada, más preferentemente, un anticuerpo fosfo-estado específico de residuos fosforilados en parkina, perteneciente, a título ilustrativo, al siguiente grupo: anticuerpo fosfo-estado específico fosfo-serina101, fosfo-serina378 y fosfo-serina127.

Una realización particular de la invención lo constituye el procedimiento de diagnóstico de la invención donde la muestra bilógica de a) se obtiene de neuronas pertenecientes al plexo autónomo y donde la determinación de b) se lleva a cabo mediante un anticuerpo fosfo-estado específico del residuo de parkina fosfo-serina101.

# 2- MECHANISTIC INSIGHT INTO THE DOMINANT MODE OF THE PARKINSON'S DISEASE G2019S LRRK2 MUTATION

# 2- Mechanistic insight into the dominant mode of the Parkinson's disease-associated G2019S LRRK2 mutation:

## 2.A- Resumen:

Las mutaciones patogénicas en el gen que codifica para la proteína LRRK2 (Leucine-rich repeat kinase-2) causan Parkinson autosómico dominante y algunas formas de Parkinson esporádico. La sustitución G2019S es el determinante genético más común hallado hasta la fecha. Se encuentra localizado dentro del dominio quinasa de la proteína, concretamente en el segmento de activación. En el siguiente trabajo mostramos como la autofosforilación de LRRK2 es una reacción intermolecular que implica dos residuos dentro del segmento de activación. La mutación patogénica G2019S altera la autofosforilación de la quinasa, incrementándola, así como la fosforilación de los sustratos, a través de un proceso que parece implicar una reorganización del segmento de activación. Nuestros resultados ofrecen una explicación molecular sobre cómo la mutación G2019S potencia la actividad catalítica de LRRK2, provocando así la enfermedad. Los hallazgos descritos son de gran importancia debido a las posibles implicaciones terapéuticas en el tratamiento de la enfermedad de Parkinson.

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# Mechanistic insight into the dominant mode of the Parkinson's disease-associated G2019S LRRK2 mutation

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**Pathogenic mutations in the leucine-rich repeat kinase-2 (*LRRK2*) gene cause autosomal-dominant and certain cases of sporadic Parkinson's disease (PD). The G2019S substitution in LRRK2 is the most common genetic determinant of PD identified so far, and maps to a specific region of the kinase domain called the activation segment. Here, we show that autophosphorylation of LRRK2 is an intermolecular reaction and targets two residues within the activation segment. The prominent pathogenic G2019S mutation in LRRK2 results in altered autophosphorylation, and increased autophosphorylation and substrate phosphorylation, through a process that seems to involve reorganization of the activation segment. Our results suggest a molecular mechanistic explanation for how the G2019S mutation enhances the catalytic activity of LRRK2, thereby leading to pathogenicity. These findings have important implications for therapeutic strategies in PD.**

## INTRODUCTION

Dominant mutations in the leucine-rich repeat kinase-2 (*LRRK2*) gene are associated with both familial and sporadic Parkinson's disease (PD) (1,2). One specific missense mutation in *LRRK2* (G2019S) comprises the most common genetic determinant of PD to date. While initially reported with a frequency of 5–6% for familial and 1–2% for sporadic PD cases (3–7), subsequent studies have revealed that mutation frequency is dependent on ethnicity (8–11). Extremely high frequency for the G2019S mutation has been reported in Ashkenazi Jews (overall around 14%) and North African Berber-Arabs (around 30%) (8,9), but low frequency in Chinese (10,11). In addition, the majority of G2019S carriers share a common haplotype dating several thousand years back (12,13). Even though the reported high frequency indicates a crucial role for mutant LRRK2 in PD susceptibility, incomplete penetrance (2,12,14–16) suggests that additional environmental and/or epigenetic factors may regulate the expression of the PD phenotype.

The *LRRK2* gene encodes for a large protein kinase (280 kDa) with multiple domains (17). These include different repeat sequences at the N-terminus such as ankyrin and leucine-rich repeats (LRR), a Roc GTPase domain, followed by a C-terminal of Roc (COR) domain, a kinase domain and WD40 repeats (18,19). While the presence of multiple protein–protein interaction domains hints towards a scaffolding function, the presence of GTPase and kinase domains suggests that LRRK2 may perform catalytic role(s). Indeed, biochemical efforts to characterize LRRK2 have revealed that it harbors kinase activity *in vitro*, which seems to be regulated by its GTP binding and/or GTPase activity (20,21), such that multiple signal transduction cascades may impact upon LRRK2 function.

The prevalent G2019S mutation lies within the activation segment of the kinase domain of LRRK2. Interestingly, the activity of a large variety of protein kinases is controlled by activation segment conformation, whereby phosphorylation of this segment leads to a conformational change switching the kinase from an 'off'- to an 'on'-state (22). Thus, the position of the G2019S mutation within the primary sequence suggests that it may directly modulate the catalytic status of LRRK2. This possibility has been addressed by overexpressing wild-type and mutant LRRK2 in tissue culture cells,

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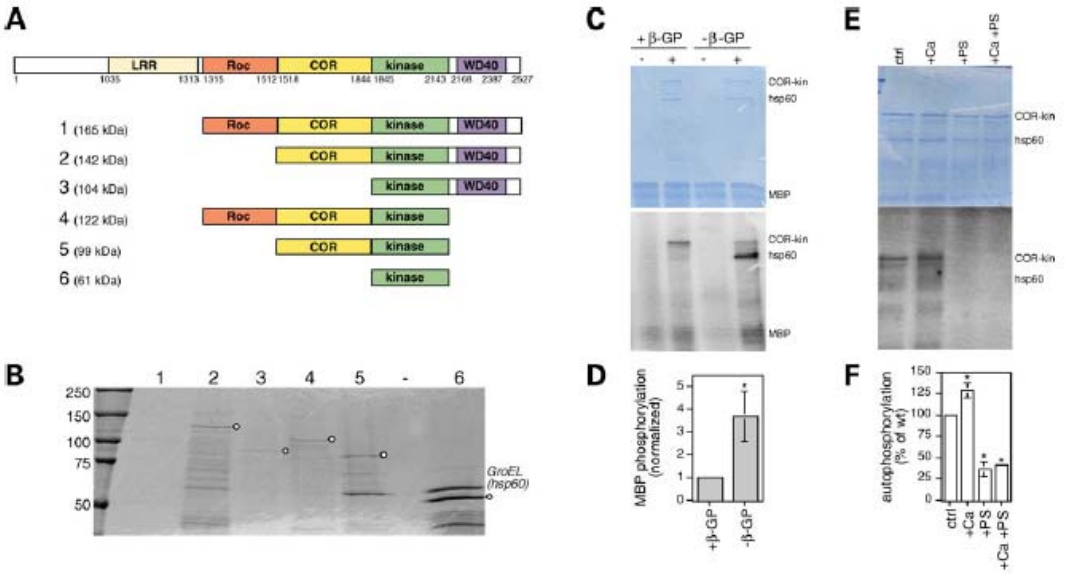


**Figure 1.** Characterization of the catalytic activity of recombinant LRRK2. (A) Schematic representation of LRRK2 domain structure, with domain boundaries shown by amino acid residue numbers beneath, and of recombinant LRRK2 domain combinations (constructs 1–6) analysed in the present study. The predicted molecular mass of each construct (including the GST moiety) is indicated in brackets. (B) The different recombinant GST-LRRK2 domains were purified as described in Materials and Methods, and analysed for purity by SDS–PAGE and Coomassie staining. While the largest fusion protein (construct 1) was entirely insoluble, other domain combinations (constructs 2–6) could be purified in soluble form (open circles). Significant amounts of GroEL (bacterial hsp60, as determined by mass spectroscopy) co-purified with all constructs containing the kinase domain. Positions of molecular weight markers are shown on the left (in kDa). (C) Autophosphorylation and model substrate phosphorylation of 1 μg of purified recombinant LRRK2 domain (COR-kinase) (COR-kin) and 10 μg of MBP. Top: Coomassie; Bottom: $^{32}$P autoradiograph. Phosphorylation reactions were performed in the presence or absence of 10 mM β-glycerolphosphate (±β-GP), respectively. Note that the presence of β-glycerolphosphate preferentially stimulated autophosphorylation over substrate phosphorylation. (D) Quantitation of MBP phosphorylation assays of the type depicted in (C) (mean ± SEM; $n = 4$). (E) Autophosphorylation of 1 μg of purified recombinant LRRK2 domain (COR-kinase) in the presence or absence of 1 mM $CaCl_2$ or 50 μg/ml phosphatidylserine (PS). Top: Coomassie; Bottom: $^{32}$P autoradiograph. (F) Quantitation of assays of the type depicted in (E) (mean ± SEM; $n = 4$). Samples were subjected to autophosphorylation reactions in phosphorylation buffer (50 mM HEPES pH 7.4, 5 mM $MgCl_2$, 5 mM $MnCl_2$, 0.5 mM DTT) for 30 min at 24°C. Reactions were stopped with sample buffer and boiling for 5 min, followed by SDS–PAGE and staining with Coomassie blue dye. Radioactive bands were quantified using ImageQuant (Molecular Dynamics) and corrected for background values. Differences in protein amounts were quantified on Coomassie stained gels using QuantityOne (Bio-Rad), and corrected for background values. Radioactivity values were corrected for differences in protein loading. Error bars represent SEM. *, $P < 0.05$.

followed by measurements of catalytic activity of immunoprecipitated protein. Indeed, the G2019S mutation was found to increase the kinase activity of LRRK2 (23–25), in line with an expected gain-of-function mechanism for its dominant transmission. Furthermore, increased kinase activity of G2019S-mutant LRRK2 resulted in increased apparent neurotoxicity (23–25). Although these studies suggest that enhanced kinase activity may underlie the mechanism of PD in G2019S carriers, the precise molecular basis by which this mutation enhances LRRK2 catalytic activity remains to be established.

In the present study, we have examined the effect of the G2019S mutation on the catalytic activity of the purified, recombinant LRRK2 kinase domain *in vitro*. For this purpose, we established conditions to purify recombinant kinase *in vitro* in its catalytically active form, and determined optimal buffer conditions for autophosphorylation and model substrate phosphorylation assays. We demonstrate that catalytic activity of recombinant LRRK2 kinase is regulated by intermolecular autophosphorylation at two residues within the activation segment. In addition, we find that the mutation generates a novel phosphorylation site within the activation segment. By mutating select other residues within the activation segment of wild-type and mutant kinase domain, we find that the increased catalytic activity of the G2019S mutant seems to involve reorganization of this segment to mimick the active state of the kinase. Together, our results suggest a molecular mechanism by which the G2019S mutation enhances the catalytic activity of LRRK2, which has important implications for the screening and design of kinase inhibitors as possible treatment option for PD.

## RESULTS

### Catalytic activity of recombinant LRRK2 kinase

To establish *in vitro* phosphorylation reactions using purified, recombinant LRRK2, we generated a series of GST-fusion protein constructs encoding for various domains (Fig. 1A). While the largest LRRK2 protein containing the Roc, COR, kinase and WD40 domains was entirely insoluble under our purification conditions, shorter domain combinations yielded soluble proteins (Fig. 1B). The GST moiety enhanced solubility, which greatly decreased when GST-fusion proteins were cleaved by thrombin to release the GST tag (data not shown). A prominent contaminant, identified as hsp60 by mass

spectroscopy, copurified with all fusion proteins (Fig. 1B). This contaminant copurified with fusion proteins containing the kinase domain either on its own or in combination (Fig. 1B), but not with recombinant proteins devoid of kinase domain (data not shown). These data indicate that hsp60 may perform a chaperone action in maintaining the proper folding of recombinant LRRK2 kinase domain in *Escherichia coli*, similar to that suggested for the reported hsp90 interaction with full-length LRRK2 in mammalian cells (26). Since reasonably large amounts of purified proteins could only be obtained with COR-kinase and kinase constructs, subsequent studies were performed with those GST-tagged LRRK2 proteins only.

To characterize the catalytic activity of recombinant LRRK2, the purified COR-kinase and kinase domains were subjected to autophosphorylation and model substrate phosphorylation assays *in vitro*. Recombinant LRRK2 kinase was capable of autophosphorylation and substrate phosphorylation using myelin basic protein (MBP) as a model substrate (Fig. 1C and D). While recombinant GST was not phosphorylated (data not shown), the copurifying hsp60 protein served as an additional model substrate for recombinant LRRK2 kinase activity (Fig. 1C). Using a variety of buffer conditions, catalytic activity was found to be dependent on the presence of $Mn^{2+}$, in contrast to previous reports using overexpressed full-length LRRK2 immunoprecipitated from mammalian cells (20,23,24,26). This dependency could not be overcome by increasing the concentration of $Mg^{2+}$, suggesting that recombinant LRRK2 kinase is an $Mn^{2+}$-preferring kinase *in vitro* (data not shown). The presence of β-glycerolphosphate favored autophosphorylation over substrate phosphorylation (Fig. 1C and D). Catalytic activity was thermolabile, such that all reactions had to be performed at 24°C, and enzyme activity was found to be slightly enhanced by $Ca^{2+}$ and profoundly inhibited by phospholipids (Fig. 1E and F). Together, these results establish the conditions necessary to measure the catalytic activity of recombinant LRRK2 kinase, which should aid in future studies aimed at screening LRRK2 kinase inhibitors *in vitro*.

To assure that the observed autophosphorylation and substrate phosphorylation were due to the catalytic activity of recombinant LRRK2, rather than the presence of a bacterial co-purifying kinase, we analyzed constructs predicted to decrease the kinase activity of LRRK2. For this purpose, we generated mutant D2017A, which alters the $Mg^{2+}$ binding loop, R1993A, a residue in the catalytic loop and K1906M, which abolishes ATP binding (22,27). Both D2017A ($54 \pm 7\%$; mean $\pm$ SEM; $n = 4$) and R1993A ($31 \pm 11\%$; mean $\pm$ SEM; $n = 4$) mutations decreased, whereas the K1906M mutation abolished autophosphorylation (Fig. 2A and B), indicating that we are measuring inherent catalytic activity of LRRK2. In addition, the ability of LRRK2 to phosphorylate both itself as well as a model substrate (Fig. 1C and D) suggests that basal LRRK2 activity *in vitro* is independent of an activating kinase.

### Intermolecular nature and sites of autophosphorylation

Autophosphorylation of a kinase can represent either a true intramolecular event, or transphosphorylation of one kinase molecule by another. To distinguish between the two processes, we performed kinase reactions using two electrophoretically distinct forms of LRRK2, one of which was catalytically competent (kinase domain only) and the other inactive (mutant COR-kinase domain) (Fig. 2C). In such mixed kinase reactions, the active kinase was able to phosphorylate a kinase-dead mutant (COR-kinase-K1906M mutant), indicating that autophosphorylation is an intermolecular reaction (Fig. 2D and E).

Next, we aimed to determine the autophosphorylation sites within recombinant LRRK2 kinase. The most common regulatory mechanism for kinase activity involves activation loop phosphorylation (22). In protein kinases whose crystal structures have been determined in both inactive and active forms, the non-phosphorylated activation loop usually binds to another region of the kinase domain, trapping it in an inactive conformation. Kinase activation is triggered by a critical change in the conformation of the activation loop, mostly induced by either autophosphorylation or exogenous phosphorylation by upstream activating kinases (22). LRRK2 kinase contains three putative residues (T2031, S2032 and T2035) within the activation loop which could serve as autophosphorylation sites (Fig. 3A). To determine whether autophosphorylation targets those residues, we constructed proteins with mutations of the Ser or Thr residue within the activation loop and determined their effects on LRRK2 autophosphorylation and activity. While single mutations of T2031A or S2032A caused a significant reduction in LRRK2 activity, these mutants did retain some basal activity (Fig. 3B and C). In contrast, when the two residues were simultaneously mutated to Ala (T2031A/S2032A), the activity of this mutant was almost completely lost (Fig. 3B and C), comparable to that of the kinase-dead mutant (Fig. 2A and B). Similarly, the T2035A mutant did not show detectable autophosphorylation (Fig. 3B and C). In transphosphorylation assays, the T2035A mutant incorporated phosphate comparable to wild-type, but almost no phosphate incorporation was detectable in the T2031A/S2032A double mutant LRRK2 (Fig. 3D and E). Together, these data establish T2031 and S2032 as the key regulatory phosphorylation sites required for LRRK2 activation, and indicate that T2035 is important for catalytic activity, but does not serve as a phosphate acceptor.

### Mechanistic underpinnings of G2019S mutation

The activation loop is anchored into the kinase structure at its N-terminus by a conserved DYG sequence (Fig. 3A). This sequence is part of the $Mg^{2+}$-binding loop and is altered by the prominent G2019S mutation. The G2019S mutant presumably did not prevent $Mg^{2+}$ binding, as it showed autophosphorylation *in vitro*. On the contrary, G2019S mutant LRRK2 kinase displayed around 3-fold higher autophosphorylation activity as compared to wild-type kinase (Fig. 4A). In contrast, the pathogenic I2020T mutation adjacent to G2019 within the N-terminal hinge region of the loop did not significantly affect activity. Similarly, a putatively pathogenic mutation in the kinase domain (R1941H) and a pathogenic mutation in the COR domain (Y1699C) were without effect (Fig. 4A). Intriguingly, the pathogenic I2012T mutation,

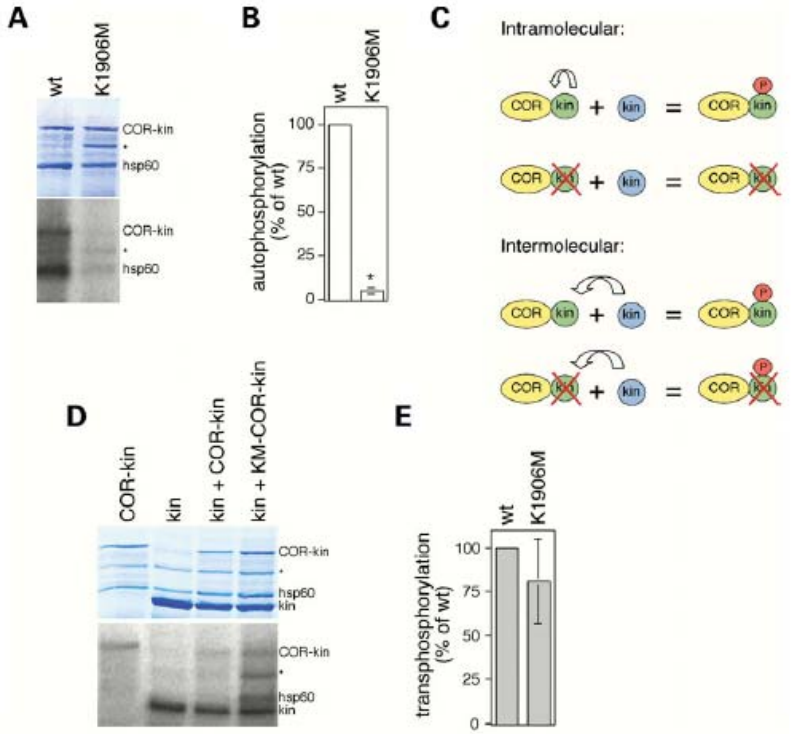


Figure 2. Intermolecular nature of autophosphorylation of recombinant LRRK2 kinase. (A) Autophosphorylation of wild-type or catalytically impaired COR-kinase (COR-kin) mutant. Top: Coomassie; Bottom: $^{32}P$ autoradiograph. Experiments and data analysis were performed as described in legend to Figure 1. *, contaminant observed in some protein preparations. (B) Quantitation of experiments of the type depicted in (A). The extent of autophosphorylation was compared to results with wild-type protein and charted as percent of the control measurement. Average values from eight independent experiments are shown. (C) Schematic diagram depicting the distinct outcomes of a transphosphorylation experiment. If autophosphorylation is an intramolecular reaction, mutant, catalytically inactive COR-kinase is not phosphorylated. If autophosphorylation is an intermolecular reaction, mutant COR-kinase can be phosphorylated by active kinase molecules in the reaction mixture, leading to phosphate incorporation. (D) Example of a transphosphorylation experiment using wild-type (COR-kin) or the K1906M point-mutant COR-kinase protein (KM-COR-kin), together with wild-type kinase domain protein (kin). Top: Coomassie; Bottom: $^{32}P$ autoradiograph. *, contaminant observed in some protein preparations. (E) Quantitation of experiments of the type depicted in (D). Experiments and data analysis were performed as described in legend to Figure 1. The extent of phosphate incorporation was compared to results with wild-type protein and charted as percent of the control measurement. Average values from four independent experiments are shown. Error bars represent SEM. *, $P < 0.05$.

situated within the $Mg^{2+}$ binding loop (Fig. 3A), displayed a small but significant decrease in catalytic activity (Fig. 4A). Thus, of all the mutations analyzed in this study, only G2019S enhanced LRRK2 catalytic activity. The enhanced autophosphorylation of the G2019S mutation was paralleled by enhanced substrate phosphorylation (Fig. 4B). These data indicate that the G2019S mutation results in a direct increase in catalytic activity of recombinant LRRK2, as previously shown for full-length LRRK2 (20).

To determine whether the substitution of the Gly for a Ser residue generated another phosphorylation site within LRRK2, we tested the G2019S mutation on the autophosphorylation-negative T2031A/S2032A mutation. Autophosphorylation assays showed that the triple mutant (G2019S/T2031A/S2032A), but not the double mutant (T2031A/S2032A) incorporated phosphate, indicating that the substituted Ser residue at position 2019 serves as an additional phosphorylation site (Fig. 4C).

To further analyze the mechanism by which the G2019S mutation enhances LRRK2 kinase activity, we mutated this residue to an Ala. The activity of the G2019A mutant was comparable to wild-type (Fig. 4D and E). Thus, the bulkier Ser substitution (and/or its phosphorylation) may selectively enhance catalytic activity by helping to stabilize the N-terminal hinge region of the activation loop, thereby mimicking the active state of the enzyme. To test this possibility, we analyzed residues at the C-terminal hinge region of the activation loop for their effects on catalytic activity. The C-terminal hinge in LRRK2 begins at T2035 (Fig. 3A), a highly conserved residue amongst Ser/Thr protein kinases which seems to play a structural role in maintaining close contact of the activation segment with the catalytic loop (22). The T2035A mutant LRRK2 did not show detectable autophosphorylation (Fig. 4D and E) nor model substrate phosphorylation (data not shown), but was phosphorylated in transphosphorylation reactions (Fig. 3D and E). These results indicate that T2035 is a structural determinant for LRRK2 enzymatic activity, rather than a regulatory phosphorylation site. To determine whether the G2019S mutation would bypass the requirement for T2035, we generated double mutant constructs. The G2019S/T2035A mutant was as hyperactive as G2019S, whereas the G2019A/T2035A mutant was catalytically dead (Fig. 4D and E). Thus, the

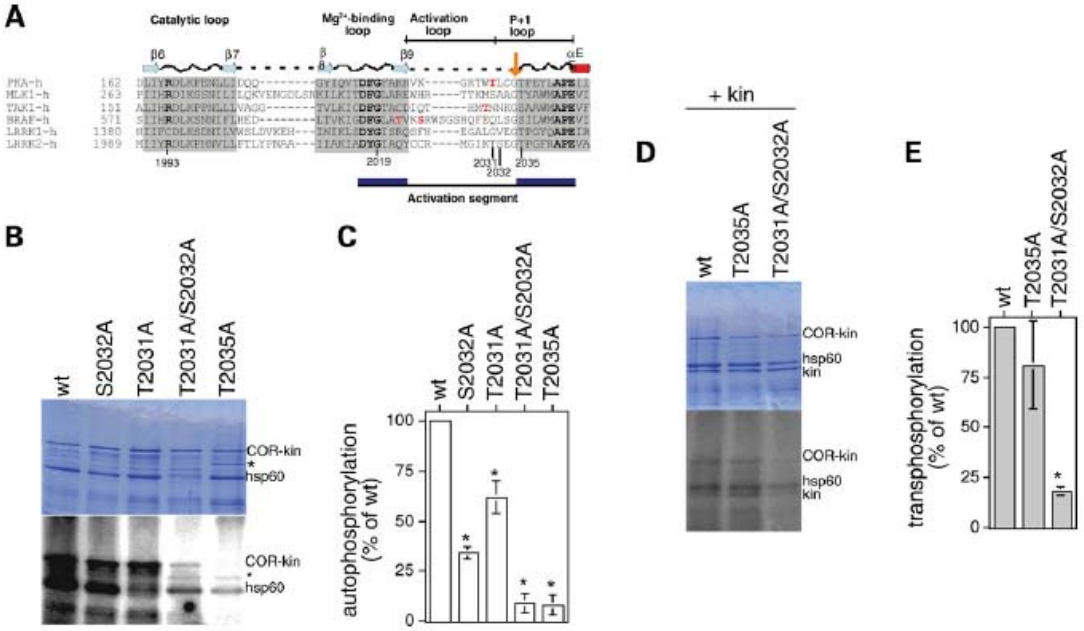


**Figure 3.** Determination of autophosphorylation sites within recombinant LRRK2 kinase. (**A**) Sequence alignment of the activation segment of LRRK2. The secondary structure of the kinase core of protein kinase A (PKA) is indicated above the alignment (22). Human LRRK2, LRRK1, MLK1, TAK1 and B-Raf were aligned against the sequence of PKA by maximizing alignment across structurally homologous regions of Ser/Thr protein kinases. These structurally conserved regions include the catalytic loop and its flanking β strands (β6 and β7), the $Mg^{2+}$ binding loop and its flanking β strands (β8 and β9), and a segment containing the $P+1$ loop and the $\pm\alpha$EF helix (gray boxes). All kinases regulated through activation segment phosphorylation have a conserved arginine preceding the catalytic aspartate in the catalytic loop (27) (bold). The primary sequence of the activation segment is defined as the region between and including two conserved tripeptide motifs, DF/YG and APE (bold). The N-terminal and C-terminal hinge regions of the activation segment are indicated in blue. For Ser/Thr kinases, the C-terminal hinge region begins at a conserved Ser or Thr residue (orange arrow). The residue immediately preceding the C-terminal hinge region often is a Gly, and its inherent flexibility may be important for kinase activity (22). Phosphorylation sites determined in PKA, TAK1 and B-Raf are indicated in red (35,43,44). The amino acid number of relevant residues in LRRK2 across the sequence alignment is indicated. (**B**) Example of an autophosphorylation experiment using wild-type or the indicated point-mutant COR-kinase proteins. Top: Coomassie; Bottom: $^{32}P$ autoradiograph. *, contaminant observed in some protein preparations. (**C**) Quantitation of autophosphorylation experiments of the type depicted in (B). Experiments and data analysis were performed as described in legend to Figure 1. The extent of phosphate incorporation was compared to results with wild-type protein and charted as percent of the control measurement. Average values from several experiments (S2032A, $n = 4$; T2031A, $n = 4$; T2031A/S2032A, $n = 3$; T2035A, $n = 6$) are shown. Error bars represent SEM. *, $P < 0.05$. (**D**) Example of a transphosphorylation experiment using wild-type or the T2035A or T2031A/S2032A-point-mutant COR-kinase proteins, together with wild-type kinase domain protein (kin). Top: Coomassie; Bottom: $^{32}P$ autoradiograph. (**E**) Quantitation of experiments of the type depicted in (D). Experiments and data analysis were performed as described in legend to Figure 1. The extent of phosphate incorporation was compared to results with wild-type protein and charted as percent of the control measurement. Average values from several independent experiments (T2035A, $n = 3$; T2031A/S2032A, $n = 4$) are shown. Error bars represent SEM. *, $P < 0.05$.

pathogenic G2019S mutant seems to reorganize the activation loop in a T2035-independent manner to enhance the catalytic activity of LRRK2. Further, this active-state mimickry is dependent on the bulkier nature (and/or phosphorylation) of the Ser residue, as it was not observed by an equivalent Ala substitution. In this manner, the G2019S mutation seems to modify the structure of the N-terminal hinge of the activation segment to mimick the active state.

## DISCUSSION

In the present study, we have examined the effect of select pathogenic mutations on the catalytic activity of purified, recombinant LRRK2 kinase *in vitro*, and the molecular mechanism by which the prominent G2019S mutation may enhance the catalytic activity of LRRK2. Amongst the pathogenic mutations analysed, only the G2019S mutation was found to enhance the kinase activity of LRRK2. Catalytic activity was regulated by intermolecular autophosphorylation on two sites within the activation segment, with the Gly to Ser mutation generating an additional autophosphorylation site. Importantly, wild-type but not G2019S mutant kinase activity was crucially dependent on a residue in the C-terminal hinge region of the activation segment. These data indicate that the G2019S mutation may lead to a conformational change in the activation segment structurally mimicking the active state of the kinase.

The present data describe for the first time conditions suitable for the purification of bacterially expressed, active recombinant LRRK2 kinase. This allows for detailed measurements of the enzymatic activity of recombinant kinase *in vitro*, which should aid in the design of high-throughput drug screening approaches. Indeed, while the activity of many kinases is modulated by separate regulatory domains and/or regulatory proteins (22,28), most inhibitors target the catalytic core of

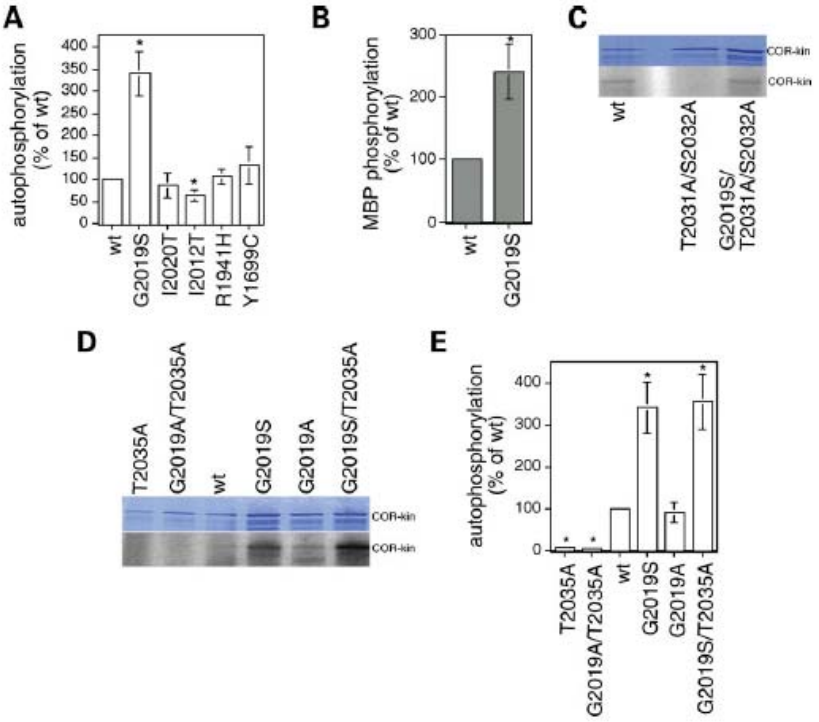


**Figure 4.** Effect of G2019S mutation on LRRK2 kinase activity. (**A**) Autophosphorylation of wild-type or various mutant COR-kinase proteins. Experiments and data analysis were performed as described in legend to Figure 1. The extent of phosphate incorporation was compared to results with wild-type protein and charted as percent of the control measurement. Average values from several experiments (G2019S, $n = 10$; I2020T, $n = 5$; I2012T, $n = 6$; R1941H, $n = 4$; Y1699C, $n = 5$) are indicated above the individual columns. Error bars represent SEM. *, $P < 0.05$. (**B**) Quantitation of MBP substrate phosphorylation assays with wild-type and G2019S-mutant LRRK2 protein (COR-kinase). Average values from five independent experiments are shown. Error bars represent SEM. *, $P < 0.05$. (**C**) Representative example of autophosphorylation of the phosphorylation-site mutant T2031A/S2032A in the presence or absence of the pathogenic G2019S mutation. Top: Coomassie; Bottom: $^{32}$P autoradiograph. (**D**) Example of an autophosphorylation experiment using wild-type or the indicated point-mutant COR-kinase proteins. Top: Coomassie; Bottom: $^{32}$P autoradiograph. (**E**) Quantitation of experiments of the type depicted in (D). Experiments and data analysis were performed as described in legend to Figure 1. The extent of phosphate incorporation was compared to results with wild-type protein and charted as percent of the control measurement. Average values from several independent experiments (T2035A; $n = 6$; G2019A/T2035A, $n = 3$; G2019S, $n = 10$; G2019A, $n = 9$; G2019S/T2035A, $n = 3$) are shown. Error bars represent SEM. *, $P < 0.05$.

the kinase (29,30). Therefore, inhibitor screens using the recombinant kinase domain of LRRK2 alone should be sufficient to identify compounds which selectively target LRRK2 kinase.

Recombinant kinase activity was found to be crucially dependent on the presence of $Mn^{2+}$, while most assays using full-length, immunoprecipitated LRRK2 have been performed in the absence of added $Mn^{2+}$ (20,21,23,24,26). Such discrepancy may be due to traces of endogenous $Mn^{2+}$ bound to immunoprecipitated material. In addition, catalytic activity was found to be profoundly inhibited by negatively charged phospholipids, suggesting that membrane association of LRRK2 (23,26,31,32) may further regulate its kinase activity.

The pathogenic G2019S and I2020T mutations lie within the N-terminal hinge region of the activation segment, which also forms part of the $Mg^{2+}$ binding loop of the kinase. Similarly, the pathogenic I2012T mutation maps to this metal binding loop. Based on such position, mutations in those residues have been hypothesized to abolish the correct positioning of $Mg^{2+}$ within the active site of the kinase, with concomitant decrease in catalytic activity (33). However, a multitude of studies, including ours, indicate that the G2019S mutation leads to enhanced kinase activity, thereby excluding that this mutation abrogates metal binding. On the contrary, our observation that the extent of enhanced catalytic activity of the G2019S mutant versus wild-type LRRK2 kinase is dependent on the concentration of $Mn^{2+}$ (data not shown) may suggest an increase in metal binding capacity, which needs further investigation.

The effect of other pathogenic mutations on LRRK2 kinase activity is more controversial. For example, the I2020T mutation has been documented to either increase (20,26) or decrease (34) the activity of full-length LRRK2, and in our assays had no effect on the kinase activity of recombinant protein. Thus, the mechanism by which this mutation affects LRRK2 function remains unknown. On the other hand, the I2012T mutation was found to decrease the activity of full-length LRRK2 in two independent studies (20,34). Similarly, we found that this mutation decreased the catalytic activity of recombinant kinase. Thus, all currently available data indicate that the I2012T mutation leads to a decrease in LRRK2 kinase activity, and the manner by which this mutation

induces PD thus seems to involve mechanisms unrelated to its kinase activity. Finally, a putatively pathogenic mutation (R1941H) in the kinase domain, as well as a pathogenic mutation in the COR domain (Y1699C) have been described to decrease or increase the activity of full-length LRRK2 kinase, respectively (20,34), but were without effect on the activity of recombinant kinase. Such discrepancy may reflect differences in assay sensitivity or the necessity to examine certain mutations in a full-length context. For example, the COR domain may be responsible for correct positioning of the catalytic Roc and kinase domains with respect to each other, and the effect of mutations in such a domain on catalytic activity may thus only be gauged from studying the full-length protein.

We find that LRRK2 activity is regulated by phosphorylation in the activation segment, as are a large variety of other protein kinases. The kinase-dead LRRK2 mutant (K1906M) was not phosphorylated, indicating that basal activity is required for the regulatory autophosphorylation. Our data show that the catalytic domain of LRRK2 is capable of intermolecular self-phosphorylation and activation in the absence of an upstream activating kinase. This is a feature shared with for example protein kinase A (35), but is distinct from the cascade phosphorylation demonstrated by MAP kinase modules (28). Indeed, recent studies also indicate that full-length LRRK2 expressed in tissue culture cells displays basal kinase activity (36). Thus, while phosphorylation of LRRK2 within regulatory domains by upstream kinases remains a possibility, we speculate that regulation of LRRK2 activity *in vivo* may be controlled by other mechanisms, such as its intrinsic GTP binding and/or GTPase activity (20,21) or its subcellular localization and/or clustering, which would serve to modulate the apparently intrinsic ability of LRRK2 to auto-activate. Phosphorylation-state-specific antibodies against active LRRK2 phosphorylated within the activation segment at the two identified sites will be required in future studies to address such hypothesis.

A conserved Thr residue (T2035) in the C-terminal hinge region of the activation segment seems to be essential for LRRK2 kinase activity, and mutation of the equivalent residue has been shown to affect the activity of other Ser/Thr kinases as well (22,37,38). Our data indicate that this Thr residue may play a crucial role for the rotational movement of the activation loop involved in switching between the active and inactive state of LRRK2. The G2019S mutation in the N-terminal hinge region seems to bypass the requirement for T2035 in the C-terminal hinge, suggesting that the G2019S mutation mimicks the constitutively active conformational status of the activation segment. In addition, this mimickry seems to be dependent on the hydrophilic nature and/or phosphorylation of the serine residue. Such 'active state mimickry' has been reported previously for other kinases implicated in human cancers. For example, some oncogenic mutations in B-RAF or BCR-ABL are situated within the activation segment as well, and have been suggested to destabilize the inactive conformation of the DFG motif/activation segment, mimicking the conformational changes normally promoted by activation segment phosphorylation (39,40). Consideration of such differential conformational states will be important when screening compounds for their ability to inhibit wild-type or G2019S mutant LRRK2 kinase activity (29,30).

The observed intermolecular nature of autophosphorylation and concomitant activation of LRRK2 kinase may explain the absence of a difference in phenotype between heterozygous and homozygous G2019S carriers (41), whereby mutant kinase molecules can phosphorylate wild-type kinase molecules, thereby increasing in a dominant manner the entire cellular and active pool of LRRK2. However, the reported incomplete penetrance (2,12,14–16) suggests that additional mechanism(s) besides regulation of kinase activity *per se* contribute to the pathogenesis of mutant LRRK2. The delineation of LRRK2 function holds great promise for our understanding of the molecular pathways underlying PD.

## MATERIALS AND METHODS

### Plasmid construction

Constructs encoding sequences for different human LRRK2 domain combinations, Roc-COR-kinase-WD40 (1315–2527), COR-kinase-WD40 (1518–2527), kinase-WD40 (1845–2527), Roc-COR-kinase (1315–2143), COR-kinase (1518–2143) and kinase (1845–2143) were generated by PCR using a human full-length LRRK2 construct and subcloned into the expression plasmid pGEX-KG (42). Domain boundaries were established based on BLASTP sequence alignments and the previously described domain architecture of Roco proteins (19). The kinase and Roc-COR-kinase constructs were subcloned via the XbaI/XhoI restriction sites, whereas the other constructs were subcloned non-directionally via the XbaI restriction site. The glycine linker encoded in the pGEX-KG plasmid, which separates the GST moiety and the thrombin cleavage site from the different LRRK2 fusion domains, was found to be crucial for efficient purification of active LRRK2 protein. The entire coding sequences of all constructs were verified by DNA sequencing.

Mutant constructs were generated using the QuickChange mutagenesis kit (Stratagene) according to manufacturer's instructions and resequenced as indicated above. The sequences of all primers used in the present study are available upon author's request.

### Protein purification

All recombinant proteins were expressed as N-terminally tagged GST fusion proteins in *E.coli* BL21 cells. Due to the limited solubility of the larger recombinant proteins, only COR-kinase and kinase domain proteins were analyzed in the present study. For this purpose, an overnight culture of cells was diluted 10-fold and grown at 16°C to an $OD_{600}$ of around 0.6, followed by induction with 0.1 mM IPTG at 16°C overnight. Cells were pelleted at 10 000*g* for 12 min, and the pellet was resuspended in 6 ml (per liter culture) of resuspension buffer [PBS containing 1% Triton-X100, 100 μM phenylmethylsulfonyl fluoride (PMSF), 1 mM DTT, 50 μg/ml RNase, 5 μg/ml DNase and 100 μg/ml lysozyme]. The cell resuspension was incubated for 30 min at 4°C on a rotary shaker, followed by four sonication pulses (30 s each, separated by 15 s intervals) on ice. Note that the gentle

(albeit inefficient) disruption of cells by short sonication pulses yielded less, but significantly more active recombinant LRRK2 protein than that obtained by the use of a French Press. Upon centrifugation at 16 000*g* for 30 min at 4°C, the soluble fraction was filtered through a 0.22 μm filter and diluted with an equal volume of resuspension buffer. Proteins were bound to glutathione Sepharose beads (Pharmacia) (500 μl of packed bead volume per liter culture) for 2 h at 4°C in binding buffer (PBS containing 1% Triton-X100, 100 μM PMSF and 1 mM DTT), followed by 12 washes in binding buffer and 6 washes in elution buffer (50 mM Tris–HCl pH 8.0, 1% Triton-X100, 100 μM PMSF and 1 mM DTT). Proteins were eluted with 20 mM glutathione in elution buffer (six times 500 μl for 15 min at 4°C on rotary shaker). Note that neither the presence of Triton-X100 (as compared to proteins purified in the absence of detergent) nor the GST moiety (as compared to proteins eluted by the addition of thrombin) adversely affected LRRK2 kinase activity. Protein concentrations were estimated using the Bradford (Bio-Rad) assay with bovine serum albumin as a standard. Small aliquots of recombinant LRRK2 proteins were flash-frozen in liquid $N_2$, stored at −80°C and only thawed up once.

### Phosphorylation reactions

Unless otherwise indicated, the buffer used for phosphorylation reactions contained 50 mM HEPES pH 7.4, 5 mM $MgCl_2$, 5 mM $MnCl_2$ and 0.5 mM DTT. LRRK2 recombinant protein (1 μg) was preincubated in phosphorylation buffer for 1 min at 24°C, and reactions were initiated by the addition of [γ-$^{32}$P]ATP (final concentration, 100 μM). Reactions were carried out in a final volume of 40 μl. After 30 min at 24°C, reactions were terminated by the addition of 0.2 volumes of 5× sample buffer and heating for 5 min at 95°C. Transphosphorylation reactions contained 0.5 μg of COR-kinase and 2 μg of kinase proteins. Substrate phosphorylation assays contained 10 μg MBP.

Proteins were separated by SDS–PAGE on 7.5% polyacrylamide gels (or 12.5% gels for assays containing MBP), followed by staining with Coomassie blue dye. Incorporation of $^{32}$P was quantitated by using a PhosphorImager (Molecular Dynamics). Radioactive bands were quantified using ImageQuant (Molecular Dynamics), and corrected for background values. Differences in protein amounts were quantified on Coomassie stained gels using QuantityOne (Bio-Rad), and corrected for background values, and radioactivity values were corrected for differences in protein loading. Experiments were done the indicated amount of times, and the data were analyzed using the paired Student's *t*-test.

### Materials

Phosphatidylserine was from Avanti Polar Lipids. [γ-$^{32}$P]ATP (specific activity, 150mCi/ml) was from *GE*Healthcare. MBP was a gift from Prof. Paul Greengard. All other chemicals were of reagent grade or better.

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*Conflict of Interest Statment*. The authors declare no conflicts of interest.

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## 2.B- Annex 3:

2.B.1- parkin and LRRK2 interaction:

It has been published that parkin and LRRK2 interact (COR domain of the Kinase and RING domain of parkin) [119]. We decided to test this interaction *in vitro* using recombinant purified proteins.

For that purpose, LRRK2 domains were bound to beads and incubated with recombinant full-length purified parkin protein.

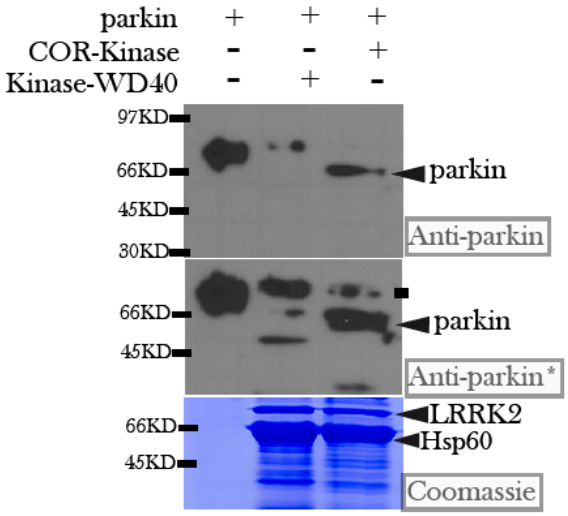


**Figure 23:** Parkin and LRRK2 pull-down. 1 µg of parkin was incubated with beads as a control or with beads bound to COR-Kinase LRRK2 protein or Kinase-WD40 LRRK2 protein, washed and eluted. Half of the sample was run on a gel and stained with Coomassie for LRRK2 domain input. The other part of the sample was run on a gel and blotted with anti-parkin Ab from Ab-cam (1/1000 dilution). Black square indicate a cross-reacting band, also present in the control sample, * Overexposed blot showing the presence of the cross-reacting band in the three samples.

Given the huge amount of recombinant LRRK2 domain and parkin proteins used in the pull-down, a strong band should be visible by WB, even if the described interaction were weak. The experiment showed a weak interaction of parkin with the COR-kinase construct, but not with the kinase-WD40 construct, indicating that the COR domain may interact with parkin. However, at best, such interaction is negligibly weak, at least in our hands.

Our data showed that LRRK2 is a kinase with auto-phosphorylation activity, so we decided to test if parkin is a LRRK2 substrate *in vitro*. Our data using wildtype or G2019S-mutant COR-Kinase and Kinase-WD40 domains did not show specific phosphorylation of parkin (data not shown). Together, these albeit preliminary results indicate that there is no relevant interaction between parkin and LRRK2, and that parkin is not a substrate for LRRK2.

## 2.B.2- Materials and Methods:

For pull-down experiments, LRRK2 constructs were purified as described above and bound to glutathione-sepharose beads for 1 hour at 4 ºC in PBS/1% Tx-100. After binding, beads were washed once with PBS/1% Tx-100 and once with lysis buffer (20 mM Hepes pH 7,4, 2 mM EGTA, 50 mM β-glycerophosphate, 1% Tx-100, 10% Glycerol, 1 mM DTT, 1 mM PMSF, 1 mM $Na_3VO_4$, 5 mM NaF) and incubated in 300 µl of lysis buffer containing 1 µg of recombinant full-length parkin, for 4 hours at 4 ºC with shaking. Beads were extensively washed with lysis buffer and eluted with 5x sample buffer + β-mercaptoethanol.

*In vitro* phosphorylation was performed as described above using COR-Kinase, Kinase-WD40, COR-Kinase-G2019S and 1µg of recombinant full-length parkin.

# DISCUSSION

# V. DISCUSSION

## 1) Parkin phosphorylation:

Our results report that parkin, a protein essential for the sustained survival of dopaminergic neurons, is subject to compound phosphorylation *in vitro* and in cultured cells. We show that parkin was phosphorylated *in vitro* by CK1 at serines 101 and 378, whereas serine 131 was not a phosphorylation site for this kinase, as previously described [303]. An additional site for CK1 was identified as serine 127, these three positions (101, 127 and 378) being the major phosphorylation sites. Parkin was neither an *in vitro* substrate for other kinases such as CK2 and GSK-3β, as previously shown [232, 303] nor for kinases not tested until now, such as CaMKII or ASK-1. Parkin further was found to be a substrate for cdk5 phosphorylation at serine 131, as previously described [232]. We used commercially available protein kinases for our *in vitro* phosphorylation assays, and the kinases used were highly pure and active, as demonstrated by using positive controls in each phosphorylation experiment. It also allowed us to compare the efficiency of phosphate incorporation into parkin versus other well described substrates. Using such controlled conditions, we could demonstrate that parkin is not a real substrate for PKA and PKC *in vitro*, in contrast to published results [303].

Parkin has also been reported to be phosphorylated in tissue culture cells [232, 303]. Importantly, using phospho-state-specific antibodies, we could directly demonstrate phosphorylation of parkin at the S101 and S378 sites *in situ.*

We discovered a cross-talk between the CK1 and Cdk5 phosphorylation events. *In vitro*, we observed that phosphorylation of parkin by CK1 increases subsequent phosphorylation by Cdk5 and vice versa, thereby generating a multiple-phosphorylated state. In cultured cells, mimicking phosphorylation on the Cdk5 site led to an increase in phosphorylation on the CK1 sites as assessed using the phospho-state-specific Abs. The converse experiment, mimicking phosphorylation on the CK1 sites and assessing phosphorylation on the Cdk5 site could not be performed due to the absence of a phospho-state-specific Ab for the S131 site. However, blocking phosphorylation of the Cdk5 site using Roscovitine, a specific Cdk5 inhibitor (Meijer, Borgne et al., 1997) led to a decrease in the phosphorylation on the CK1 sites as assessed with the phospho-state-specific antibodies.

A future approach to demonstrate the CK1-Cdk5 cross-talk could involve experiments using RNA interference. Suppression of Cdk5 levels should decrease parkin phosphorylation by CK1 and conversely, suppression of CK1 should decrease Cdk5 phosphorylation. Again, for the latter experiment, a p-S131 Ab would be needed. Importantly, the siRNA approach would be useful for establishing which CK1 isoform(s) phosphorylate parkin in intact cells. In addition, future generation of a double-phospho-state-specific antibody (against p-S127 and p-S131) may allow detection of the hyperphosphorylated form of parkin *in situ*.

## 2) Parkin catalytic activity:

Phosphorylation experiments aimed at determining the kinases which phosphorylate parkin, and the sites within parkin being phosphorylated by a given kinase, were initially performed using recombinant purified parkin protein. Such experiments are only valid if the purified recombinant protein is properly folded. If parkin exists in its native condition, it should display catalytic activity. Therefore, we subjected the protein to *in vitro* autoubiquitylation assays. To determine if parkin autoubiquitylation is multiple-mono or poly, we used a lysine-less Ub derivative, which lacks the lysine residues required for Ub chain elongation. An enhanced signal was observed when using normal Ub as compared to lysine-less Ub, indicating that parkin may perform both multiple-mono as well as polyubiquitylation. However, the observed differences may equally be due to differences in the efficiency by which parkin can incorporate Ub as compared to lysine-less Ub, a possibility which in the future can be addressed using Abs that only recognize polyubiquitin chains (like FK1 Ab, Biomol), especially since published reports largely suggest that parkin performs multiple-monoubiquitylation activity [212, 213], at least *in vitro*. It will be important to extend these assays to determine which kind of Ub chains can be added by parkin *in vitro*. It has been previously published that parkin can add Ub through K48 [194] and K63 linkage [194, 195, 211], but these experiments were performed by measuring ubiquitylation of distinct substrates. For testing the auto-ubiquitylating activity of parkin, *in vitro* ubiquitylation assays can be performed using K48 and K63 Ub mutants. The use of these Ub mutants in conjunction with different E2 enzymes will report if parkin displays different activities depending on the E2 used.

Post-translational modifications within parkin may modulate its E3 Ub ligase activity. Using phospho-mimetic parkin mutants, and non-phosphorylatable counterparts, we did not find differences in parkin activity using *in vitro* autoubiquitylation assays. These data are in disagreement with other results reporting a decrease in parkin activity upon CK1 phosphorylation [303]. However, these experiments were done in a different manner, by using recombinant purified parkin protein and subjecting it to *in vitro* phosphorylation assays prior to autoubiquitylation [303]. It is possible that *in vitro* phosphorylation experiments may lead to secondary events, such as kinase binding to parkin, which may enhance parkin autoubiquitylation activity. In either case, the enhancing effects on activity were very slight [303], suggesting that parkin phosphorylation does not lead to drastic changes in activity, at least towards itself.

Finally, we detected a slight increase in parkin activity using the non-phosphorylatable Cdk5 mutant 131A, and these data are in agreement with previous results [232]. Importantly, no difference in monoubiquitylation was observed when mimicking phosphorylation on both the CK1 and Cdk5 sites. A slight increase in polyubiquitylation was observed with this phosphomimetic mutant, but this increase was also observed with its non-phosphorylatable counterpart, indicating that it is not a direct consequence of phosphorylation. Together, the data suggest that compound phosphorylation of parkin does not lead to drastic changes in its catalytic activity, at least towards itself.

All our measurements of parkin activity have been performed using *in vitro* autoubiquitylation experiments. Whilst this is a reasonable measure of net activity, it is quite crude and has not been demonstrated to be physiologically relevant. We tried to assess the ability of parkin to ubiquitylate a previously published heterologous substrate, synphilin-1 [211, 240]. As shown in Figure 21, Annex 1, we were unable to detect significant and reproducible parkin-mediated ubiquitylation of synphilin-1, even though we precisely followed published procedures [232]. Thus, at least in our hands, autoubiquitylation has been the most robust and reproducible method for analysis of catalytic activity.

## 3) Parkin aggregation:

Here we report that parkin phosphorylation increases its aggregation and decreases the amount of soluble parkin. To obtain these data, we over-expressed wt parkin and its phospho-mimetic or non-phosphorylatable counterparts in a cell line that has been established as a good model system for studying aggresome formation, HEK293T cells [228]. We and others have shown that parkin is a largely soluble protein, mainly cytosolic when overexpressed in cells [231, 233, 234, 294]. Endogenous parkin has an increased tendency to form aggresomes after proteasome inhibition in neuroblastoma cell lines [229, 230], and this increase has also been detected in other cell lines when parkin is overexpressed [229, 231, 234]. Indeed, when over-expressing wildtype parkin or the phosphomimetic or non-phosphorylatable mutants, differences in the number of cells with aggresomes, or changes in detergent solubility were obvious only upon proteasome inhibition, further indicating that inhibition of proteasomal degradation is generally required to uncover differences in parkin solubility and aggregative properties. A better cell culture system to study parkin solubility and aggregation may be a dopaminergic cell line, as studies in such cells have a more direct impact on our understanding of dopaminergic dysfunction than human embryonic kidney cells (HEK293T). We used PC12 cells, a rat dopaminergic cell line [305], but endogenous parkin levels were very low. When overexpressed in PC12 cells, exogenous parkin levels were low as well, which made it impossible to determine parkin detergent extractability properties and differences in parkin phosphorylation levels. Given that transfection efficiencies were low (less than 15%), quantification of cells with inclusions upon wildtype or mutant parkin overexpression was not feasible.

In the future, a new technique, Bimolecular fluorescence complementation (BiFC), will be optimal for the quantification of the number of cells containing parkin inclusions. In this technique, non fluorescent fragments of GFP (or a different fluorescent protein) can reconstitute the fluorophore when brought together by interactions between proteins covalently linked to each fragment [306, 307]. In this manner, fluorescence will be visible only upon parkin aggregation, making quantification easier, as has been shown for quantification of α-SN oligomerization [308].

## 4) Parkin phosphorylation *in vivo*:

We made use of our phospho-state specific Abs to check if parkin phosphorylation takes place in human brain extracts. The reliability of studies using human post-mortem brain tissue largely depends on protein preservation. After death, several factors may interfere, such as long post-mortem delay between death and sample processing for storage, or temperature of storage [309, 310]. For that reason, we matched the samples according to post-mortem interval, tissue-handling and storage conditions for protein phosphorylation determination.

We found parkin protein levels to be similar in the three brain areas analyzed (cortex, caudate, cerebellum), and no differences in total parkin levels between control and PD patients were detected, as previously shown for cortical samples [311].

Importantly, we found a significant increase in parkin phosphorylation in the caudate of PD patients as compared to controls, whilst no differences were observed in the cortex, and no detectable phosphorylation was identified in the cerebellum. Interestingly, differences in parkin phosphorylation correlated with the relative extent to which these brain areas are affected by LB pathology, namely much LB pathology in caudate, little in cortex, and none in cerebellum [12].

In the future, it will be important to extend those findings using a larger sample size to potently establish the observed correlation. In addition, it will be interesting to test for possible differences in parkin phosphorylation in other neurodegenerative diseases such as diffuse LB disease, Huntington's or AD. Studies of this type may reveal whether enhanced parkin phosphorylation correlates with other protein aggregation diseases.

In addition, it would be useful to analyze whether there are quantitative differences in the phosphorylation levels of parkin across different brain areas, e.g. between cortex and caudate, in control patients. If performed with large enough sample sizes to obtain statistically significant results (given the inter-patient variability), such experiments may reveal whether parkin is differentially phosphorylated in distinct brain areas under control conditions, a finding which would correlate with the distinct sensitivity of those brain areas to be affected by LB pathology.

Furthermore, it would be informative to establish whether phosphorylated parkin is present in the detergent soluble or insoluble fraction of the brain samples analyzed. Parkin seems to be more insoluble in caudate compared to cortex in control patients [8], and is also more insoluble in the caudate of PD patients compared to controls, whereas no differences in the cortex have been detected [8]. Whilst such changes in solubility may be due to covalent dopamine modification of parkin [8], a correlation between phosphorylation and solubility may further support the finding that differential phosphorylation, another covalent parkin modification, contributes to changes in parkin solubility, as we have determined in cultured cells.

Future experiments should also be aimed at evaluating our phospho-state-specific Abs by immunohistochemistry in human brain samples. Preliminary data indicate that phospho-parkin can be prominently detected in non-neuronal cells as well as in the peripheral nervous system. Thus, data of this type may indicate other cellular contexts in which phosphorylation of parkin may play a physiological role, and the phospho-state-specific Abs may even be useful for future diagnostics purposes [312, 313].

## 5) Kinases involved in parkin phosphorylation:

Protein phosphorylation has emerged as one of the major mechanisms involved in PD [314] and our data indicate that CK1 and Cdk5, through their phosphorylation of parkin, may both be involved in the development of PD.

Cdk5 is a proline directed, serine-threonine protein kinase member of the cyclin dependent kinase family [315], but is neither activated by cyclins, nor directly involved in cell cycle regulation [316]. It is highly conserved and ubiquitously present, with maximal expression in postmitotic neurons and glial cells [317-319]. Cdk5 does not seem to have any specific pattern of distribution in the cell but tends to co-localize with its activators. Cdk5 is activated by p35 and p39 [320, 321], which are highly expressed in neuronal cells [321, 322] and localized to the plasma membrane [323] (Figure 1). p25 is a proteolytic fragment of p35, generated by the calcium activated protease calpain [324], that is activated under neurotoxic conditions, such as oxidative stress and excitotoxins [324]. p35 is short-lived, while p25 has a half-life two-threefold more than p35, thus releasing the kinase to the cytosol and maintaining it in a hyperactive state [323, 325] (Figure 24).

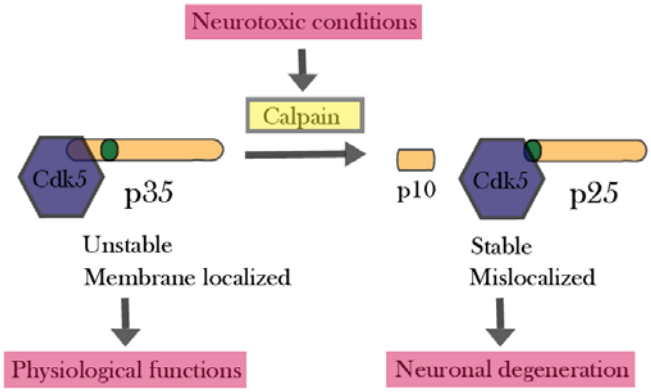


**Figure 24**: Cleavage of p35 into p25. Several neurotoxic conditions, including ischemic brain damage, oxidative stress and excitotoxicity activates calpain, leading to p25 generation [316].

Whilst clearly implicated in the development of the central nervous system [326], Cdk5 also seems to be involved in the development of several neurodegenerative diseases. For example, it has been published that p25 accumulates in the brain of patients with AD [323], that correlates with an increase in Cdk5 kinase activity [323].

Cdk5 hyperphosphorylates tau [323, 327], reducing its ability to bind microtubules, leading to cytoskeletal disruption, degeneration and apoptosis [323]. The kinase may also be involved in the pathology of AD through phosphorylation of β-amyloid precursor protein (APP) [328] and presenilin-1 [329] [330]. Deregulated Cdk5 activity has also been implicated in PD. For example, the kinase and p35 are present in LB of PD patients [331, 332]. Induction of dopaminergic cell loss in a mouse model of PD (MPTP treatment), increases Cdk5 expression and activity, whereas Cdk5 inhibition attenuates the loss of dopaminergic neurons in this model [333].

Casein Kinase I comprises a family of serine/threonine protein kinases [334]. Nowadays, seven mammalian CK1 isoforms have been described, designated α, β, γ1, γ2, γ3, δ, and ε [335-338].

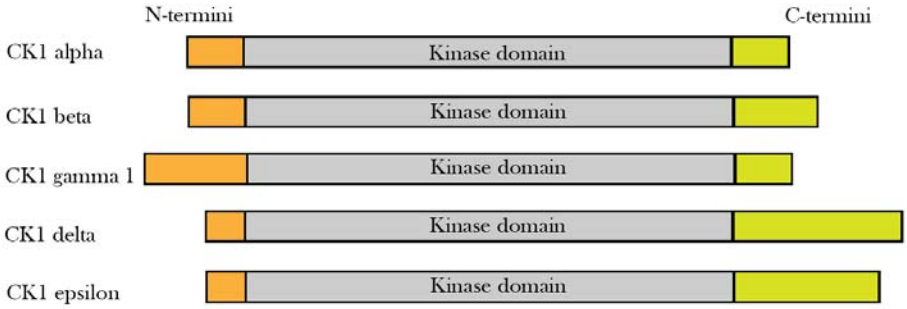


**Figure 25**: CK1 isoforms. All family members contain a highly related (53-98% Identical) central kinase domain which is flanked by divergent amino- and carboxyl-terminal extensions of variable length. The extensions are important for kinase activity regulation, cellular localization and interaction with substrates [339].

CK1 isoforms are found in all tissues and cellular compartments analysed, including human and rodent brain [340] and they appear to be constitutively active [339]. It is therefore interesting that despite high sequence similarity and common expression patterns, CKI family members have different targets. CKIα is associated with distinct structures throughout the cell, including association with cytosolic vesicles, linking the kinase to the regulation of vesicular trafficking and neurotransmitter release [341, 342], but it is also speculated to play a role in cell cycle progression, spindle dynamics and chromosome segregation [342]. CKIβ is currently known to exist only in bovine brain [341] and CK1ε has been shown to be in involved in circadian rhythm regulation, controlling the stability and localization of several proteins implicated in these processes [343]. Very little is known regarding CKIγ (1, 2 and 3), but it is probably involved in specific receptor tyrosine kinase-mediated signal transduction events, through its association with adaptor proteins [339]. In addition, CKIα, ε and δ have also been described as regulators of the Wnt-signalling, which plays an important regulatory role in cell proliferation processes [342].

Amongst all those distinct CK1 isoforms, CK1δ has been implicated in neurodegenerative diseases. Elevated CK1δ mRNA and protein levels have been detected in the brain of AD patients [344]. This isoform was found to phosphorylate

the microtubule-associated protein tau *in vitro* [345, 346], disrupting its binding to microtubules [346] and inducing cell death [347]. CK1 isoforms accumulate in the AD lesions, but the pattern varies with isoform. CKIα colocalizes with neurofibrillary tangles, whereas CK1δ colocalizes with granulovacuolar lesions [348]. CK1 isoforms are also implicated in AD through phosphorylation of β-secretase [349] and presenilin-2 [350], and have also been associated with the production of the neurotoxic peptide amyloid-β [351]. Whilst there is a clear link between CK1 and AD, an involvement of CK1 in PD has not been established yet. The isoform δ does not seem to be present in LB of patients with PD or DLB [352], but additional studies aimed at evaluating the presence of all CK1 isoforms will be necessary to determine a potential involvement for CK1 in LB pathology.

The generally high basal activities of CK1 and Cdk5 suggest a very active role for protein phosphatases in controlling the steady-state levels of parkin phosphorylation, as our and other data suggest [232, 303]. Phosphatases are divided into two major families, those that dephosphorylate phosphotyrosine residues (PTP) and those that dephosphorylate phosphoserine and/or threonine residues (PSTP). According to the aminoacid sequence of the catalytic region, PTPs can be classified into four families [353]. The class I constitutes the "classical" PTPs and the "dual specificity" protein phosphatases (DSPs). The DSPs can hydrolyze phospho-serine and phospho-threonine groups as well as phospho-tyrosine, and molecules that are not phospho-proteins, such as phospholipids. Class II are largely found in bacteria and are tyrosine specific. Class III are tyrosine/threonine specific. Class IV has a different catalytic mechanism with dependence on an aspartic residue instead of a cysteine residue in the catalytic region [353]. The family of PSTP are classified into PPP (phospho-protein phosphatase) and PPM ($Mg^{2+}$-dependent protein phosphatase) [354]. The PPP family includes the most abundant protein phosphatases, PP1, PP2A, PP2B (or calcineurin) and PP2C. They are classified based on substrate specificity (α or β-subunit of the phosphorylase kinase), sensitivity to inhibitors (heat-stable inhibitor proteins) and cation requirements (Figure 27) [355].

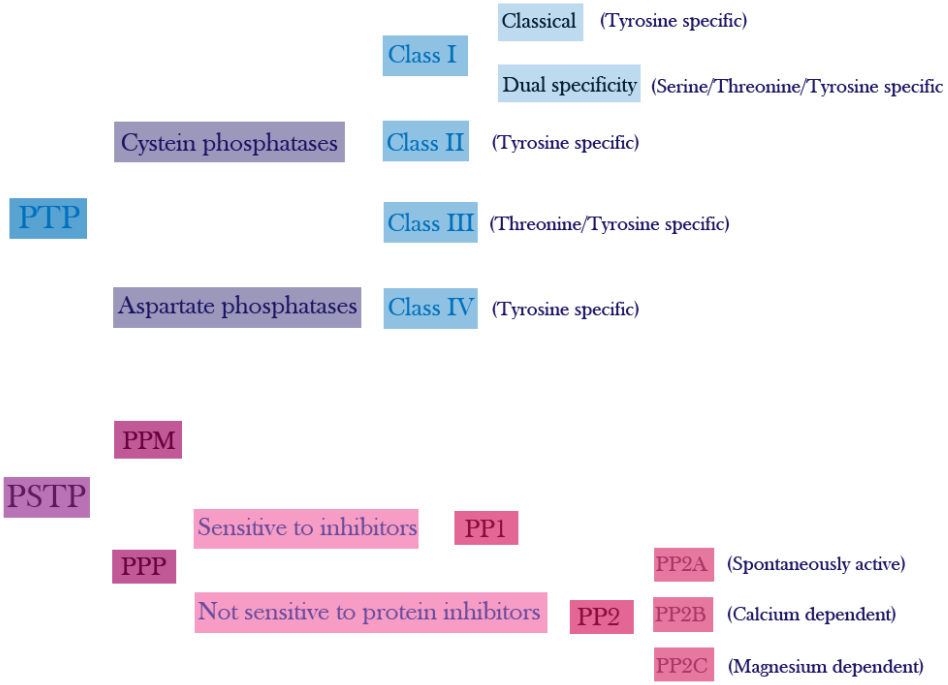


**Figure 27**: Phosphatase classification and specificity.

Protein phosphatases have also been implicated in neurodegeneration. Decreased phosphatase activity has been related with the pathology of AD [356, 357]. It has been shown that inhibition of protein dephosphorylation induces neuronal stress and neurodegeneration *in vivo* [358, 359], and that DA depletion in a rat model of PD inhibits PP1 [358], contributing to hyperphosphorylation of synaptic proteins and disruption of synaptic plasticity [358]. However, a recent paper suggests that phosphatase inhibition can also be protective in the same rat model (6-OHDA) [360]. The differences may be due to the phosphatases inhibited (serine-threonine or tyrosine specific), leading to distinct cellular outcomes.

For parkin phosphorylation detection using the phospho-state specific Abs, we used a phosphatase inhibitor, Okadaic acid, which has been shown to inhibit PP1, PP2A and PP2B [361]. This indicates that at least one of these phosphatases is involved in parkin dephosphorylation. It will be important to determine which phosphatase(s) are involved in this process, and which circumstances may lead to their inactivation, thereby increasing parkin phosphorylation and aggregation.

Knowing the kinases involved, we further analyzed if the enhancement of parkin phosphorylation in PD brains compared to controls may correlate with increases in the levels of the kinases or its activators. CK1 α and ε isoform levels did not change between control and PD. Whilst the levels in cortex and caudate of CK1δ did not change between control and PD, this isoform was non-detectable in the cerebellum as previously shown [344]. Interestingly, this is the brain area were parkin phosphorylation was non-detectable. Thus, the data suggest that this may be the CK1 isoform involved in parkin phosphorylation. Cdk5 and p35 levels did not change between control and PD samples in the three brain areas analyzed, and low levels of p35 were detected in the cerebellum, as previously published [362]. We could detect a significant increase in p25 levels in the caudate of PD patients as compared to controls, with no changes in the cortex and undetectable levels in the cerebellum. These data suggest that the changes in the levels of p25 in the distinct brain areas analyzed may lead to changes in Cdk5 activity, followed by compound parkin phosphorylation and aggregation.

As mentioned above, an increase in p25 levels in the brains of patients with AD as compared to controls has been reported [323]. This finding has been highly controversial, as samples with longer post-mortem delays display protease activity able to degrade p35 to p25 [363-366]. [365, 367]. Our analysis has been performed with samples subject to only short post-mortem interval, and we matched control and PD patient samples accordingly.

Our data suggest a model by which different cellular pathways converge to control parkin aggregation-solubility (Figure 28). CK1 phosphorylation of parkin enhances Cdk5 phosphorylation and *vice versa*, leading to a hyperphosphorylated state that promotes parkin aggregation and inactivation.

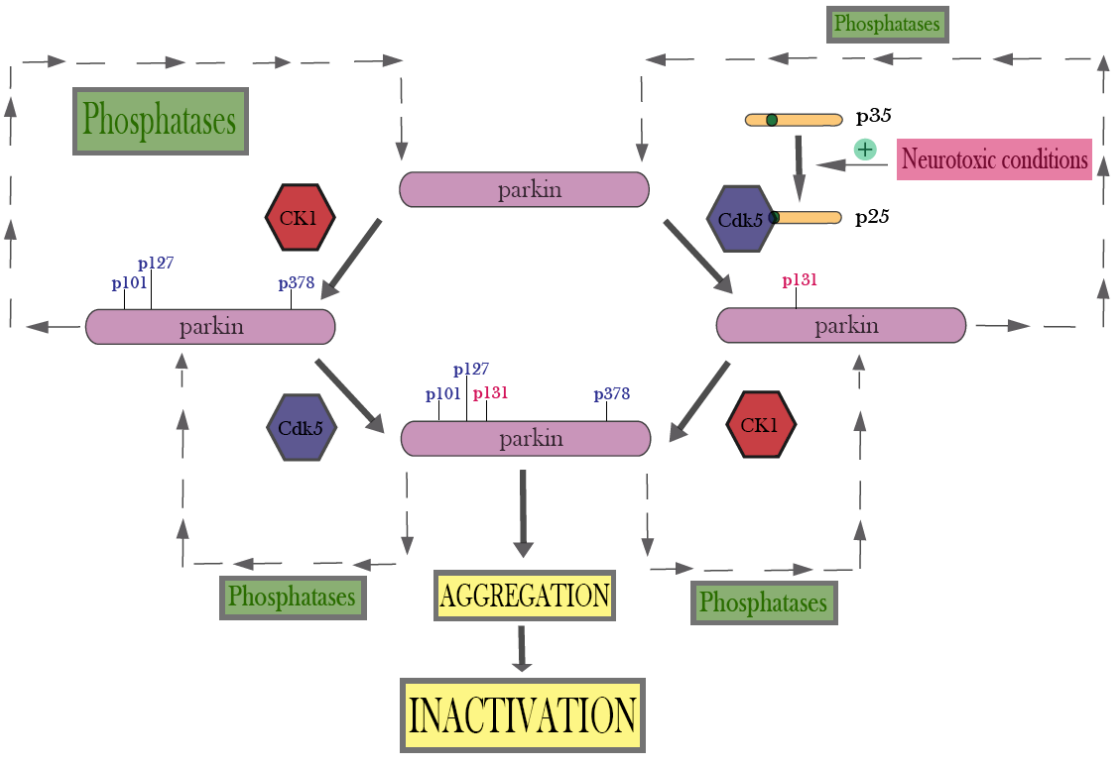


**Figure 28:** Schematic model depicting different processes that lead to parkin hyperphosphorylation. (comment: put neurotoxic conditions also on cdk5 on left side)

Cdk5 activity is usually kept at a low level. However, some neurotoxic insults like excitotoxicity lead to an increase in calcium concentration [281], which activates calpain and promotes the generation of p25 [324]. In that manner, activated cytosolic Cdk5 may phosphorylate parkin, making the protein a better substrate for CK1 to generate hyperphosphorylated parkin. On the other hand, CK1 isoforms constitutively phosphorylate parkin, but the CK1 sites are rapidly dephosphorylated by protein phosphatase(s). Deregulation of the phosphatase activities involved in this process may lead to an increase in CK1-phosphorylated parkin, which subsequently is phosphorylated by Cdk5, generating hyperphosphorylated parkin. Such decrease in protein phosphatase activities may worsen the situation not only by inhibiting CK1 dephosphorylation, but also by promoting accumulation of Cdk5-phosphorylated and hyperphosphorylated parkin. A decrease in the synthesis of protein phosphatases or an increase in the endogenous inhibitors of these proteins [368] may lead to neuronal cell death through parkin aggregation and inactivation (Figure 28). In summary, two signal transduction cascades are regulating this

event, Cdk5 overactivation and phosphatase(s) activity inhibition. Deregulation of one of these cascades may lead to parkin aggregation, and deregulation of both may be needed for further aggregation and inactivation.

### 5) Inhibition of parkin phosphorylation:

To determine if inhibition of CK1 and Cdk5 activities would display beneficial effects in decreasing the aggregative properties of pathogenic parkin mutants, we analyzed three pathogenic point mutants, R256C, R275W and C289G, all of them located in the RING1 domain. We chose these mutants as previous reports have indicated that they display ubiquitylation activity similar to wildtype, but have an increased tendency to aggregate. In this manner, they reflect the phenotype of pseudophosphorylated parkin, but to a bigger extent such that measurements of aggregates were more feasible. We confirmed that these mutants were overexpressed to similar degrees, displayed autoubiquitylation activity *in vitro*, and had a tendency to aggregate when overexpressed in cells.

Additional biochemical evidence further supports the decreased solubility of those mutants as compared to wildtype parkin. For example, whilst all mutants were easily expressed and purified from bacteria, they precipitated faster than wt parkin protein purified under the same conditions. In addition, the amount of bacterial Hsp70 that co-purified with the mutants was higher than the one that co-purified with wt parkin, indicating that chaperone activity is required for proper parkin folding, as suggested by Winklhofer and co-workers [235, 293]. All three mutants showed an enhanced propensity to form intracellular inclusions in the absence of proteasome inhibition, with C289G > R275W > R256C. These inclusions have been previously reported to be aggresomes [213, 231, 233, 294, 369], and we also identified them as aggresomes (co-staining with vimentin and disruption of formation upon nocodazole treatment) (not shown).

To determine the number of transfected cells containing inclusions, we quantified the percentage of transfected cells displaying at least one big perinuclear inclusion typical of an aggresome. However, additional small aggregates within the same cell were observed as well with some of the mutants. R256C mainly showed medium-sized perinuclear inclusions, with additional high soluble parkin staining in the cytosol. Inhibition of proteasome activity increased the percentage of cells displaying aggregates, and the percentage of cells displaying

a large perinuclear aggregate (not shown). As compared to R256C, R275W had a higher tendency to aggregate and parkin cytosolic staining was lower. This mutant displayed big perinuclear inclusions, in addition to small inclusions scattered throughout the cytosol and very small nuclear inclusions (not shown). Nuclear inclusions after parkin overexpression in HEK293 cells have previously been reported [294] and have also been observed in other cell lines [369]. The C289G mutant had a giant tendency to form inclusions. No soluble, cytosolic parkin staining was observed, inclusions were big or small scattered throughout the cytosol, big perinuclear, or small or medium-sized in the nucleus (not shown). Addition of MG-132, a proteasome inhibitor, concentrated the small cytosolic inclusions into one big perinuclear aggregate (not shown). Whilst we only quantified the big perinuclear aggregates typical of aggresomes in our experiments, the different phenotypes observed further highlight the differential tendency (C289G>R275W>R256C) of these mutants to aggregate. Interestingly, whilst all three mutants were phosphorylated by CK1 and Cdk5, some experiments indicated that C289G may be a better substrate for phosphorylation than wildtype parkin (not shown). Thus, it will be interesting to determine in the future whether these point mutations turn the protein into a better substrate for phosphorylation. Such experiments could be performed *in vitro* as well as in intact cells using our phospho-state-specific antibodies. If indeed the case, such results would indicate that the differential aggregative properties of these three mutants (C289G>R275W>R256C) may be related to differences in their phosphorylation status.

The pathogenic parkin mutants studied displayed differences in detergent extractability properties as well. Whilst the detergent solubility of the mutants were C289G < R275W < R256C, in agreement with previous studies [213, 233, 294, 295, 369], we found, using the phospho-specific Abs, that they were heavily phosphorylated in the insoluble fraction, indicating that parkin phosphorylation may increase its insolubility and promote its aggregation. Alternatively, since these mutants have an increased tendency to aggregate, their aggregation may prevent subsequent dephosphorylation by phosphatases. However, preliminary data obtained in the laboratory show that a quadruple pseudo-phosphorylated mutant on top of the pathogenic parkin mutation R256C displays a significant increase in the number of cells containing inclusions, as compared to the R256C mutant, supporting the notion that enhanced compound parkin phosphorylation promotes aggregation.

To inhibit the kinases involved in parkin phosphorylation, we used two Cdk5 and three CK1 inhibitors, all of which were structurally dissimilar. A combination of inhibitors was found to reduce the number of cells containing inclusions and the number of cells displaying big inclusions. It remains to be determined whether this results in increased amounts of soluble mutant parkin protein. This could be determined by assessing detergent solubility properties of the mutants before and after treatment.

Once this question is ready, an increase in parkin protection may be demonstrated. For that purpose, stress conditions like oxidative stress or excitotoxicity, where parkin exerts a neuroprotective role [9, 225, 236, 269], should be induced in a neuronal cell line. Transfection of wt parkin or the non-phosphorylatable parkin mutant should decrease the cell death/apoptosis induced by these stressors, while transfection of the pseudophosphorylated parkin mutant should worsen the phenotype. Finally, transfection of parkin combined with kinase inhibitor treatment should increase parkin neuroprotection.

Our findings indicate that CK1 and Cdk5 may represent novel combinatorial therapeutic targets for treating PD. The CK1 inhibitors we used were CK1-7, IC261 and D4476, which are all ATP competitive inhibitors but structurally dissimilar. CK1-7 (N-(2-amino-ethyl)-5-chloroisoquinoline-8-sulfonamide) was the first CK1 inhibitor reported [370]. It was found to specifically inhibit CK1, but not CK2 [370], and is the least potent inhibitor of the three used [371]. CK1-7 has been shown to inhibit CK1ε [335], CK1α, CK1δ, and CK1γ, but the last one with less efficiency than the other isoforms [338]. IC261 (3-[(2,4,6-trimethoxyphenyl)methylidenyl]-indolin-2-one) [372] has been reported to be more potent than CK1-7 [371] and seems to be more effectively inhibiting CK1δ than CK1α [372]. D4476 (4-[4-(2,3-dihydrobenzo[1,4]dioxin-6-yl)-5-pyridin-2-yl-1H-imidazol-2-yl]benzamide) [373] has been shown to be the most potent and specific CK1 inhibitor [371]. The specificity has not been tested against the different CK1 isoforms, but it seems to inhibit, at least CK1δ [371] and CK1ε [374]. The three inhititors used are highly specific and covered all CK1 isoforms. Whilst they are useful tools for studying the physiological roles of this family of protein kinases, they are quite insoluble (with CK1-7 displaying the better solubility amongst the three) (personal observation). This makes their therapeutic potential questionable.

GW8510 (4-{[( 7-Oxo-6,7-dihydro-8H - [1,3] thiazolo [5,4-e]indol-8–ylidene) methyl]amino}-N-(2-pyridinyl )-benzenesulfonamide) is a 3-substituted indole that inhibits CDKs and is structurally different from the other known CDK inhibitors [375]. Its specificity has not been extensively tested, but it seems to be more selective for Cdk5 than for other CDKs [376].

Roscovitine ([6-Benzylamino-2-[(R)-(1'-ethyl-2'-hydroxyethylamino)]-9-isopropylpurine]) is a purine analogue and an ATP competitive [377] potent inhibitor of cdk5 [378]. Other related kinases like cdk4 and cdk6 are poorly inhibited, while erk1 and 2 are only inhibited at higher $IC_{50}$ concentrations [378]. Cdc2 and cdk2 (A and B) are inhibited at the same concentration than Cdk5 [378], and erk8 is also inhibited, but not as efficiently as Cdks [371]. It has been described that CK1 isoforms are not inhibited by roscovitine at low concentrations [371, 379].

Roscovitine (or CYC202) is currently entering phase II clinical trials against cancer and phase I clinical tests against glomerulonephritis, and is also under evaluation, at the preclinical level, for the therapeutic use against various neurodegenerative diseases [380]. Given that it seems to be well tolerated in patients, this inhibitor may be a promising compound for the treatment of PD. Interestingly, novel roscovitine-derived compounds can simultaneously inhibit CDKs and CK1 [381], and such compounds may be used in the future to assess their potential for regulating parkin solubility and aggregation as performed in the present study.

In summary, we report that compound parkin phosphorylation by CK1 and cdk5 decreases parkin solubility, leading to its aggregation and inactivation. As parkin plays a clear neuroprotective role, increasing the amount of soluble active parkin protein in cells by combined kinase inhibition may be a feasible novel therapeutic treatment option for PD.

# CONCLUSIONS

# VI. CONCLUSIONS:

1) Casein Kinase 1 phosphorylates parkin at S101, S127 and S378, and Cdk5 phosphorylates parkin at S131.
2) Parkin phosphorylation by Casein Kinase 1 promotes phosphorylation by Cdk5 and *vice versa*.
3) Compound parkin phosphorylation enhances its insolubility leading to aggregation.
4) The increase in parkin phosphorylation in the different brain areas correlates with the relative extent to which these distinct brain areas are affected by disease pathology.
5) p25 levels are higher in caudate of Parkinson's disease patients as compared to control, whilst no differences are detected in the cortex, with the absence of detectable p25 in the cerebellum.
6) Combined kinase inhibition decreases the aggregative properties of some pathogenic parkin mutants in cultured cells.
7) Casein Kinase 1 and Cdk5 may represent novel combinatorial therapeutic targets for treating Parkinson's disease.

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# VII. REFERENCES


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