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The role of RAB10 and RAB29 in endolysosomal trafficking alterations mediated by pathogenic LRRK2

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El papel de RAB10 y RAB29 en las alteraciones del tráfico endolisosomal mediadas por LRR2 patogénica

Memoria presentada por la doctoranda María Romo Lozano, graduada en Biología, para optar al título de Doctora por la Universidad de Granada dentro del programa en Bioquímica y Biología Molecular

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Abbreviations

α-syn: α-synuclein

AD: Alzheimer Disease

CIE: clathrin-independent endocytosis

CME: clathrin-mediated endocytosis

CNS: Central Nervous System

COR: C-terminal of Roc

DA: Dopamine

DBS: Deep brain stimulation

DLB: Dementia with Lewy bodies

EE: early endosome

EGF: epidermal growth factor EGFR:

EGFR: epidermal growth factor receptor

ER: endoplasmic reticulum

ERC: early recycling compartment

GA: Golgi Apparatus

GAP: GTPase-activating proteins

GBA: *β-glucocerebrosidase*

GCase: β-glucocerebrosidase

GDI: GDP dissociation inhibitor

GDP: guanosine diphosphate

GEF: guanosine diphosphate

GGtase II: RAB geranylgeranyl transferase

GST: glutathione S-transferase

GTP: guanosine triphosphate

GWAS: genome wide association studies

ILV: intraluminal vesicle

iPSCs: induced pluripotent stem cells

KD: knockdown

KI: knock-in

KO: knockout

LBs: Lewy's bodies

LE: late endosome

LID: levodopa-induced dyskinesia

LN:Lewy's neurites

LRRK2: leucine-rich repeat kinase 2

M6PR: mannose 6-phosphate receptor

MAPT: Microtubule associated protein Tau

MPTP: 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine

MTOC: microtubule-organizing center

MVB: multivesicular body

PAK: p21-activated kinases

PD: Parkinson Disease

PM: plasma membrane

PNS: Peripheral nervous system

RAB: RAB interacting lysosomal protein-like ½

RBD:REM sleep behavior disorder

RE: recycling endosome

REP: RAB escort proteins

RILP: RAB-interacting lysosomal protein 1

RILPL1/2: Rab-interacting lysosomal protein-like ½

RIPKs: Receptor-interacting serine/threonine-protein kinase 1

Roc: Ras of complex

ROS: reactive oxygen species

SE: sorting endosome

SNCA: *α-synuclein*

SNpc: Substantia Nigra pars compacta

SVE: synaptic vesicle endocytosis

TFEB: Transcription Factor EB

TfR: Transferrin Receptor

TGN: trans-Golgi network

VPS35: *Vacuolar protein sorting-associated 35*

WT: wild-type

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Summary/Resumen

Summary

Point mutations in the gene encoding the Leucine-Rich Repeat Kinase 2 (LRRK2) are the most common cause of autosomal dominant familial Parkinson's Disease, while certain variants can also increase risk of developing sporadic PD. LRRK2 has been implicated in several cellular processes including mitochondrial function, cytoskeletal dynamics and vesicular trafficking events. The latter include endocytosis, autophagy, retromer-mediated recycling and endolysosomal trafficking pathways. The exact molecular mechanisms responsible for the LRRK2-mediated defects in these trafficking pathways remain largely unknown.

A subset of the RAB family of small GTPases including RAB8A and RAB10 have been identified to act as LRRK2 substrates, and RAB29 is an important LRRK2 interacting protein. Previous work showed that pathogenic LRRK2 mediates defects in the endolysosomal degradation and recycling of the Epidermal Growth Factor Receptor (EGFR) by phosphorylating and inactivating RAB8A, which causes a decrease in RAB7A activity. Here, we analyzed the role of the most prominent LRRK2 kinase substrate RAB10 and the role of the LRRK2 interactor RAB29 in the trafficking defects mediated by pathogenic G2019S LRRK2. We find that expression of active RAB10 rescues the G2019S LRRK2-mediated phenotypes on endolysosomal trafficking, while RAB10 *knockdown* mimics those defects which correlates with a decrease in RAB7A activity, identical to that previously described for RAB8A.

The EGFR endolysosomal trafficking impairments caused by either G2019S LRRK2 expression or RAB10 knockdown results in the accumulation of EGF in a RAB4-positive endocytic recycling compartment, which is reversed by active RAB7A, active RAB8A or active RAB10 expression but not by either of their WT or inactive versions.

Furthermore, we find that WT RAB29, previously reported to recruit LRRK2 to the Golgi Apparatus (GA), efficiently rescues G2019S LRRK2-caused impairments in EGFR degradation and reverts the accumulation of EGF in a RAB4-positive endocytic recycling compartment, without causing LRRK2 recruitment to the Golgi apparatus. Inactive variants of RAB29 are unable to rescue the described impairments. Upon higher expression levels, WT RAB29 is able to recruit LRRK2 to the Golgi apparatus but fails to rescue the G2019S LRRK2-mediated deficits in EGFR trafficking.

Finally, WT RAB29 but not its inactive variants reverts the described EGFR trafficking impairments mediated by not only G2019S LRRK2 expression, but also by

knockdown of either RAB8A or RAB10 or dominant-negative RAB7A expression, in a manner which is independent of Golgi apparatus structural integrity.

Together, these data suggest a mechanism underlying the endolysosomal trafficking deficits mediated by pathogenic LRRK2 by which it phosphorylates RAB8A and RAB10 thus causing their inactivation, which also leads to a decrease in RAB7A activity. We find that RAB8A and RAB10 play redundant roles in regulating EGFR recycling and endolysosomal trafficking events. Lastly, we report a novel role for RAB29 in regulating endocytic recycling and endolysosomal trafficking events which is independent on its role at the Golgi complex.

Resumen

Mutaciones puntuales en el gen que codifica la quinasa 2 rica en repeticiones de leucina 2 (*Leucine-Rich Repeat Kinase* 2, LRRK2) son la causa más común de la enfermedad de Párkinson (EP) familiar autosómica dominante, mientras que ciertas variantes también pueden aumentar el riesgo de desarrollar EP esporádica. LRRK2 se ha visto implicada en varias funciones celulares, incluyendo la función mitocondrial, las dinámicas del citoesqueleto y los eventos de tráfico vesicular. Estos últimos incluyen endocitosis, autofagia, reciclaje mediado por retrómero y las rutas de tráfico endolisosomal. Los mecanismos moleculares exactos responsables de los defectos mediados por LRRK2 en estas vías de tráfico siguen siendo en gran parte desconocidos.

Se ha identificado que un subconjunto de pequeñas GTPasas pertenecientes a la familia RAB, incluyendo a RAB8A y RAB10, actúan como sustratos de LRRK2. Tambien se ha descrito que RAB29 es una importante proteína que interactúa con LRRK2. Trabajos previos han demostrado que LRRK2 patogénica media defectos en la degradación endolisosomal y en el reciclaje del receptor del factor de crecimiento epidérmico (*Epidermal Growth Factor Receptor*, EGFR) fosforilando e inactivando RAB8A, lo que provoca una disminución de la actividad de RAB7A. En este trabajo analizamos el papel del sustrato más prominente de la quinasa LRRK2, RAB10, y el papel del interactor de LRRK2, RAB29, en los defectos de tráfico mediados por LRRK2 patogénica, G2019S. Encontramos que la expresión de RAB10 activo rescata los fenotipos mediados por LRRK2 G2019S en el tráfico endolisosomal, mientras que el silenciamiento de RAB10 mimetiza esos defectos que se correlacionan con una disminución de la actividad de RAB7A, idéntica a la descrita previamente para RAB8A. Las deficiencias en el tráfico endolisosomal del EGFR causados tanto por LRRK2 G2019S como por el silenciamiento de RAB10 resultaron en la acumulación de EGFR en un compartimento alternativo del reciclaje endocítico positivo para RAB4, la cual fue revertida por la expresión de RAB7A, RAB8A y RAB10 activas pero no por ninguna de sus versiones silvestres o inactivas.

Las alteraciones en el tráfico endolisosomal del EGFR causadas por la expresión de LRRK2 G2019S o por el silenciamiento de RAB10 resultan en la acumulación de EGF en un compartimento de reciclaje endocítico positivo para RAB4, que se revierte por la expresión de RAB7A activo, RAB8A activo o RAB10 activo pero no por ninguna de sus versiones silvestres o inactivas.

Además, encontramos que RAB29 silvestre, previamente reportada como reclutadora de LRRK2 al aparato de Golgi, rescata eficientemente los defectos causados por

LRRK2 G2019S en la degradación de EGFR y revierte la acumulación de EGF en un compartimiento de reciclaje endocítico positivo para RAB4, sin causar el reclutamiento de LRRK2 en el aparato de Golgi. Las variantes inactivas de RAB29 son incapaces de rescatar las deficiencias descritas. A mayores niveles de expresión, RAB29 silvestre es capaz de reclutar LRRK2 en el aparato de Golgi pero no consigue rescatar los déficits en el tráfico de EGFR mediados por LRRK2 G2019S.

Por último, RAB29 silvestre, pero no sus variantes inactivas, revierte las deficiencias descritas en el tráfico de EGFR mediadas no sólo por la expresión de LRRK2 G2019S, sino también por la eliminación de RAB8A o RAB10, o la expresión de RAB7A negativa dominante, de una manera que es independiente de la integridad estructural del aparato de Golgi.

En conjunto, estos datos sugieren un mecanismo subyacente al déficit de tráfico endolisosomal mediado por LRRK2 patogénica por el cual fosforila a RAB8A y RAB10, causando así su inactivación, lo que también conduce a una disminución de la actividad RAB7A. Descubrimos que RAB8A y RAB10 juegan papeles redundantes en la regulación de los eventos de reciclaje y tráfico endolisosomal del EGFR. Por último, reportamos un nuevo papel de RAB29 en la regulación del reciclaje endocítico y el tráfico endolisosomal, que es independiente de su papel en el complejo de Golgi.

Introduction

1. Parkinson's Disease

Parkinson's Disease (PD) was first thoroughly described in 1817 by a British medical practitioner called Dr. James Parkinson in his monograph "An Essay on the Shaking Palsy". In his publication, Parkinson described and compiled six different clinical cases of what was called *paralysis agitans* at the time with the aim of defining a disabling and largely unknown disease (Jost & Reichmann, 2017). His work was barely acknowledged for years until Jean-Martin Charcot, a French neurologist, added his observations and brought attention to Parkinson's essay published from England and proposed the renaming of the sickness as Parkinson's Disease in honor of the clinician who first described it in detail (Goldman & Goetz, 2007).

PD is a common prevalent age-related neurodegenerative disease (Dorsey et al., 2018) and currently affects around 8.5 million people, which represents roughly 0.1% of the global population (Abbafati et al., 2020). The prevalence of PD is predicted to double by 2040 (Ray Dorsey et al., 2018) which is consistent with the progressive ageing of the world population. This, together with the fact that advanced stages of the disease highly disable the patients and preclude them from having an independent life, PD is a major health and economic concern not only for the occidental society but for the entire global population (Abbafati et al., 2020).

1.1. Pathology

One of the main pathological hallmarks for PD are the loss of dopaminergic neurons in the ventral Substantia Nigra pars compacta (SNpc). SNpc neurons project their axons and release dopamine (DA) mainly to the dorsal striatum, specifically the putamen, regulating its activity and contributing to fine motor control (**Fig. 1 a**, (Pioli et al., 2008; Shulman et al., 2011)). Thus, dopaminergic neurodegeneration leads to the presence of motor symptoms which are the most characteristic features of the disease and include resting tremor, bradykinesia or slowness of movement, rigidity and postural instability. The second characteristic pathological feature of PD is the presence of intraneural protein inclusions in surviving neurons called Lewy's bodies (LBs) in the cytoplasm or Lewy's Neurites (LNs) in neurites, respectively (**Fig. 1 b** and **c**, (Chartier & Duyckaerts, 2018; Goedert et al., 2013)). LBs an LNs are mainly composed of aggregated α-synuclein (α-syn) and ubiquitin, but their composition can be rather assorted and complex including even lipids (Wakabayashi et al., 2013). This feature is shared with various diseases called synucleinopathies, which include

Dementia with Lewy Bodies (DLB), Multiple System Atrophy and PD. By the time motor symptoms become evident in PD, DA neuronal loss is estimated to be around 60% (Gibb & Lees, 1991). Before such massive dopaminergic cell loss, PD develops over years and is associated with a variety of more subtle motor and non-motor features. In an attempt to explain the causative association between pathogenic mechanisms and the clinical manifestations of the disease, the Braak hypothesis states that LB pathology occurs in localized areas and in a progressive manner. According to this hypothesis, LB pathology starts in the peripheral nervous system (PNS) and then expands to the central nervous system (CNS) in such a way that the presence of LBs in certain brain regions is used to determine disease stage (Braak et al., 2003). Although there is much published evidence supporting this hypothesis (Rietdijk et al., 2017), it has also been widely debated because it does not adjust to a sizeable fraction of PD cases (Beach et al., 2021; Burke et al., 2010;

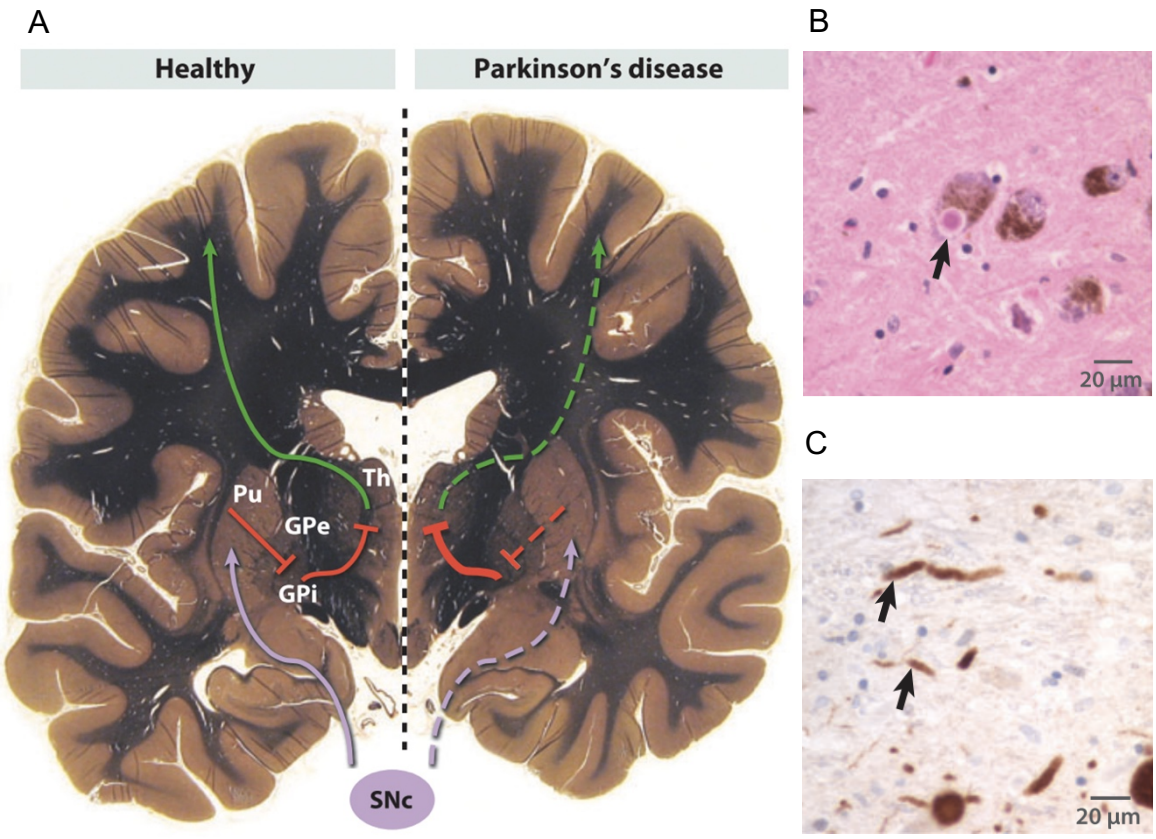

Fig. 1. Main pathological hallmarks of PD*. a) Anatomy and physiology of PD neurodegeneration. A simplified schematic of the neuronal circuits affected in PD. Substantia nigra pars compacta (SNc) provides dopaminergic input to the putamen (Pu), which indirectly results in the activation of the voluntary movement-implicated pathway. In a healthy condition, the putamen inhibits (red) the globus pallidus interna (GPi), which inhibits the thalamus (Th). The thalamus projects excitatory input (green) to the motor cortex, initiating movement. In PD, SNc dopaminergic neurodegeneration increases inhibition of the thalamocortical projection, impeding fine movement control. b & c) Immunohistochemical stainings of α-Synuclein aggregates, namely, b) Lewy bodies and c) Lewy neurites. Taken from (Shulman et al., 2011) and (Chartier & Duyckaerts, 2018).*

Parkkinen et al., 2008; Rietdijk et al., 2017). For example, there are patients who present LB pathology corresponding to stages 4 to 6 but show no neurological manifestations (Beach et al., 2021) and also patients who clinically present PD in the absence of LB pathology (Calne & Mizuno, 2004), especially patients with LRRK2-related PD (Gaig et al., 2007; Kalia et al., 2017). Indeed, Blesa and colleagues in an extensive and thorough review have recently proposed a PD progression model that aims to directly correlate the severity of the neuronal degeneration (cell loss) with the observable motor phenotypes which not necessarily correlates with LBs pathology (Blesa et al., 2022).

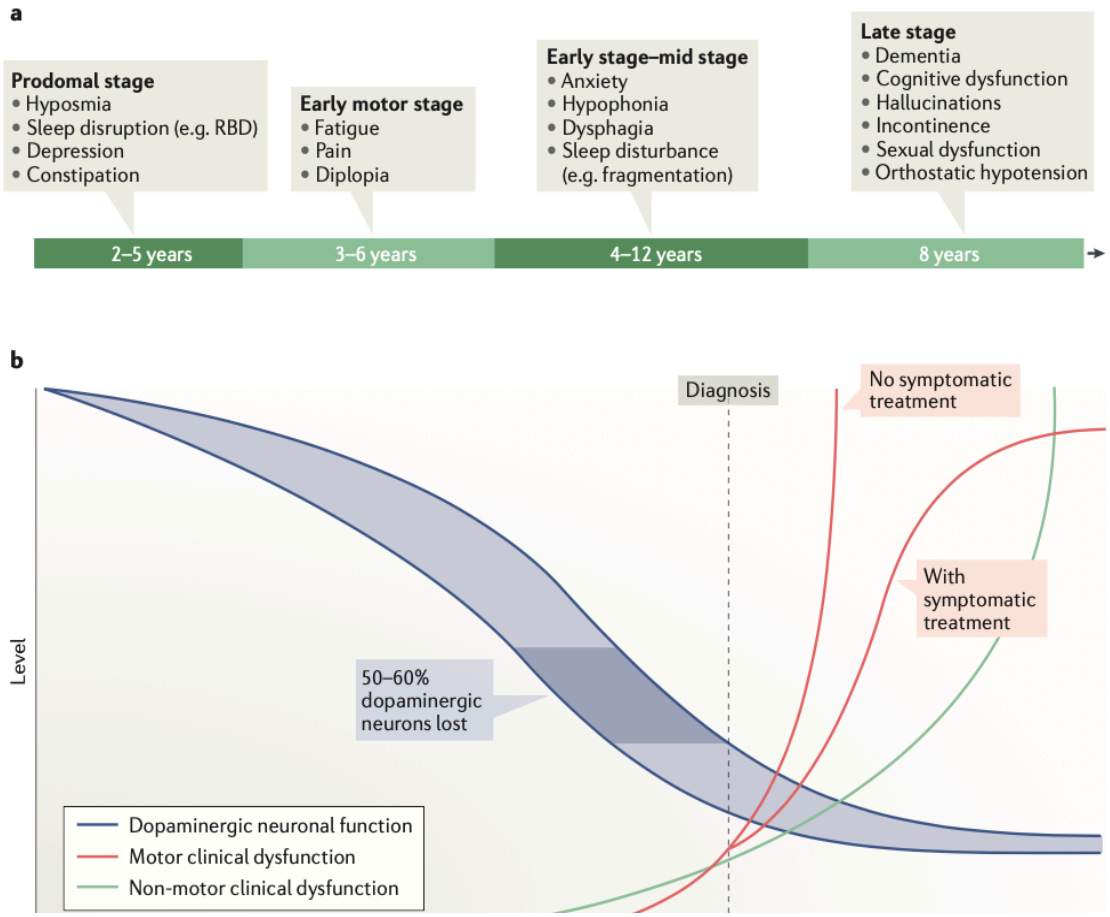

Fig. 2. Relative motor and non-motor onset of PD*. a) A timeline of the potential spawn time of the different non-motor symptoms. Some of them often develop years before diagnosis is made. Timing, sequence and development of these symptoms vary between patients. b) Relative onsets of motor and non-motor features of PD compared to the progression of dopaminergic neural loss. Treatment with dopamine replacement drugs improves motor function and slows PD progression rate, reaching a disability ceiling effect. All variables depend on the etiology of each patient. RBD, REM sleep behavior disorder. Taken from Schapira et al., 2017.*

Apart from the distinctive motor symptoms, PD can manifest with several non-motor features. These include olfactory dysfunction (Monje et al., 2021; Obeso et al., 2008), REM sleep behavior disorder (Postuma et al., 2011; Stiasny-Kolster, 2004), gastrointestinal disorders (Cersosimo & Benarroch, 2008; Wakabayashi et al., 2010), genitourinary dysfunctions (Fumimura et al., 2007), cardiovascular alterations (Orimo et al., 2008), pain manifestations (Ikemura et al., 2008; Nolano et al., 2008), visual symptoms such as reading difficulties or diplopia, neuropsychiatric symptoms, mood symptoms such as anxiety, psychosis, mood changes (Jellinger, 2015), dementia (Sonnen et al., 2010) or depression (Braak et al., 2005; Jellinger, 2010; Kalaitzakis & Pearce, 2009). Some of these symptoms may develop in the prodromal stage of the disease (**Fig. 2**). LBs as well as non-LB-related α-syn aggregates have been observed in multiple of those peripheral systems, but if and how LB pathology may cause the various non-motor features as well as its exact relation with neurodegeneration remains unknown. In either case, the progressive LB pathology follows a pattern of synaptic connectivity and indicates that the pathology may be transmitted in a prion-like and retrograde manner from the periphery to more central areas of the brain.

In addition to dopaminergic neurodegeneration, cell loss is also observed in various regions outside the SNpc and across the central and peripheral nervous systems (Braak & Del Tredici, 2017). This is specific to the loss of cholinergic neurons in the pedunculopontine nucleus, the nucleus basalis of Meynert and the dorsal motor nucleus of the vagus, the loss of noradrenergic neurons in the locus coeruleus and the loss of serotonergic neurons in the raphe nuclei (Giguère et al., 2018). A possible explanation for this selective vulnerability is that these neurons share a set of anatomical and physiological properties such as long and highly branched axons with numerous neurotransmitter release sites, pacemaker-like spiking, cytosolic calcium oscillations and basal mitochondrial oxidant stress (Surmeier et al., 2017) that could make them especially sensitive to changes in their environment. However, further investigations are necessary to understand cell death associated with PD which may help with future therapies to halt PD progression.

1.2. Diagnosis and treatment

The existing PD treatments are strictly symptomatic and involve pharmacological and nonpharmacological approaches (Arjunan & Kant, 2022). The latter englobes physical, occupational and speech therapies and the former group is composed by different drugs that essentially aim to increase DA concentration. Levodopa (l-3,4-dihydroxyphenylalanine) is the precursor of DA in its biosynthetic pathway and is the most used and effective drug in

reducing parkinsonism motor symptoms (Sivanandy et al., 2022). However, in the long term, levodopa treatment leads to levodopa-induced dyskinesia (LID) which significantly reduces the quality of life of the patients (Hechtner et al., 2014) and requires the treatment to be interrupted. Deep brain stimulation (DBS) of the subthalamic nucleus and the GPi (Weaver et al., 2012) and amantadine, an anti-dyskinetic drug (Wolf et al., 2010) are used in advanced stages of the disease, and are quite effective in managing LID and PD motor symptoms (Schrag et al., 2000). However, motor improvements diminish over time, non-motor symptoms are not attenuated and other complications may appear such as hallucinations, gradual cognitive decline, development of impulsive-compulsive behaviors, and personality changes among others (Cao et al., 2022; Malvea et al., 2022; Pahwa et al., 2017; Perez-Lloret & Rascol, 2018).

Even though none the aforementioned therapies modify the course of the disease they notably increase the quality of life of the patients, at least temporarily (Axelerad et al., 2022; Collett et al., 2017; Ellis et al., 2021; Sturkenboom et al., 2014) but for this to occur their prompt application is crucial. Therefore, there is an urgent need for early diagnostic criteria which allow for early and quick interventions. The latest accepted clinical criteria for diagnosis (Postuma et al., 2015) are highly specific but are designed for identification of PD when motor symptoms are already evident, thus not allowing for an early diagnosis. Nonetheless, in the past decade, noteworthy advances such as publication of prospective studies in target population cohorts, identification of prodromal non-motor and motor symptoms, advances in neuroimaging and high availability of biosamples for analysis have taken place (Postuma & Berg, 2019). These advances together with those yet to come pave the way towards prodromal PD identification, early diagnosis and the possibility of early-starting treatments (Postuma & Berg, 2019).

1.3. Etiology

Approximately 5-10% of PD cases are classified as familial PD, in which autosomal-dominant or autosomal-recessive mutations in select genes cause the disease. However, the immense majority of PD patients suffer from idiopathic PD. The first event that shed a light on possible underlying causes for PD occurred in 1982 in San Diego with the intoxication of 4 intravenous heroin users. As a result of high concentrations of a toxic by-product in the synthesis of heroin, these people developed severe and irreversible PD in a matter of days. The substance was identified as 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and thus it became clear that the exposure to environmental toxins was an important risk factor

for PD (Langston et al., 1982). MPTP has been shown to cross the Blood Brain Barrier, be processed by and extruded from astrocytes in a form capable of entering the dopaminergic neurons and cause high cellular stress by inhibiting mitochondrial Complex I. This causes reduced ATP production and increased production of free radicals which ultimately causes neurodegeneration (Goldman, 2014). Exposure to other toxic substances such as rotenone, a pesticide, (Sherer et al., 2007) and paraquat, a herbicide, (X. F. Zhang et al., 2016) have been shown to cause these same disturbances but through slightly different mechanisms.

In addition to these toxic compounds, other risk factors have been identified over the years, with age being the most important one (Reeve et al., 2014). These include exposure to other toxic compounds such as solvents or heavy metals or those in air pollution

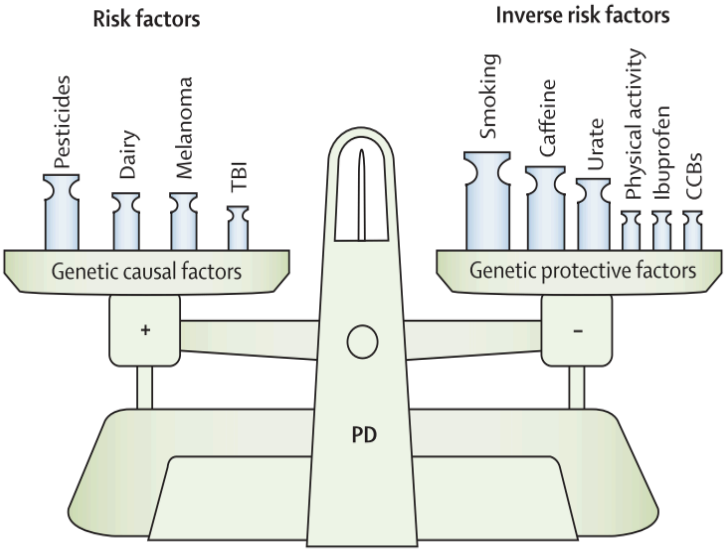

Fig. 3. Graphic representation of genetic and external factors for developing PD. *The weight's sizes depend on the robustness of the epidemiological evidences published about the factor they represent and they are figurative. TBI=traumatic brain injury. PD=Parkinson's disease. CCBs=calcium channel blockers. Taken from Ascherio et al 2016.*

(Goldman, 2014), methamphetamine use, having medical conditions such as certain types of cancer, traumatic brain injury or type 2 diabetes, alcohol abuse, use of postmenopausal drugs or polyunsaturated fat intake (Ascherio & Schwarzschild, 2016; Eriksson et al., 2013). Conversely, several protective factors have also been identified. Smoking tobacco, caffeine consumption, regular moderate or vigorous physical activity and high blood urate levels have been associated with lower risk of suffering PD (Ascherio & Schwarzschild, 2016)). However, caution is advised when interpreting these findings since they are dependent on the patient cohorts analyzed as well as dependent on other risk factors, including genetic risk (**Fig. 3**). Examples or this interdependent relationship include lower PD risk associated with polyunsaturated fat intake only in people who never smoked regularly (Abbott et al.,

2003), a threefold greater PD risk for patients who previously suffered from brain injury compared to those that did not when exposed to paraquat (P. C. Lee et al., 2012), and higher PD risk for patients suffering from head trauma only in individuals with increased α-syn expression (Goldman et al., 2012). Nevertheless, these findings are consistent with the idea of a multiple-hit hypothesis for PD, where genetics, environment and age all contribute to disease in a complex fashion.

Familial or genetic PD is due to autosomal-dominant or autosomal-recessive mutations in a set of genes also called *PARK* genes. *SNCA (PARK1/4)*, the gene encoding for α-syn was the first gene to be directly related with familial PD development in 1997 (Polymeropoulos et al., 1997). It was found that different *SNCA* mutations result in a higher predisposition of mutant α-syn to form LB-like aggregates. Since then, more than 20 monogenic forms of PD have been discovered (**Table 1**, (Del Rey et al., 2018). In addition to *SNCA*, autosomal-dominant forms of PD are due to mutations in genes such as vacuolar protein sorting 35 (*VPS35*; (Vilariño-Güell et al., 2011; Zimprich et al., 2011), eukaryotic translation initiation factor 4 gamma 1 (*EIF4G1*; (Chartier-Harlin et al., 2011), DNAJ subfamily C member 6 (*DNAJC6*; (Vilariño-Güell et al., 2014), coiled-coil-helix-coiled-coil-helix domain 2 (*CHCHD2*; (Funayama et al., 2015) and leucine-rich repeat kinase 2 (*LRRK2*; (Paisán-Ruíz et al., 2004). The autosomal-recessive forms of PD include those caused by mutations in parkin RBR E3 ubiquitin protein ligase (*PARKIN*; (Kitada et al., 1998), PTEN induced kinase (*PINK1*, (Valente et al., 2004) and parkinsonism-associated deglycase (*DJ-1*; (Bonifati et al., 2003) genes. The currently known functions of all of these genes are gathered in **Table 1**, and are listed together with other genes that have also been proposed to be linked to PD, even though further studies are necessary to confirm such associations (Tran et al., 2020).

Advances in genetic analysis techniques, especially the use of genome wide association studies (GWAS), have allowed for the identification of multiple common genetic risk variants (Fernández-Santiago & Sharma, 2022). These include variants in genes previously identified as monogenic PD-related genes such as LRRK2. Importantly, many of the identified risk variants linked to PD are involved in vesicular trafficking steps (Bandres-Ciga et al., 2019). Among them are genes encoding cyclin G associated kinase (GAK) implicated in clathrin-mediated endocytosis (Cunningham & Moore, 2020) transmembrane protein 175 (TMEM175) a potassium or proton leak channel in endosomes and lysosomes, or the golgi brefeldin A-resistant guanine nucleotide exchange factor 1 (GBF1), which selectively modulates ER-Golgi trafficking (Lopes-da-Silva et al., 2019). Among the greatest

genetic risk variants for PD are those affecting *GBA* which encodes ß-glucocerebrosidase (GCase), a lysosomal enzyme which converts glucocerebroside into ceramide and glucose, and loss of which results in the accumulation of glucocerebroside causing a lysosomal storage disorder called Gaucher disease (Smith et al., 2022). *GBA* heterozygous carriers notably have an increased risk for PD, implicating lysosomal dysfunction in the PD disease process (Smith et al., 2022). In addition, variants in the microtubule-associated protein Tau encoding gene (*MAPT*) have been related to a higher risk of suffering PD (Nalls et al., 2014). Tau protein has an essential role in microtubule assembly and stabilization (Y. Wang & Mandelkow, 2016) especially in axons (Conde & Cáceres, 2009) and has been shown to play a role in PD pathology (Lei et al., 2010) which is consistent with the importance of microtubule organization and vesicular trafficking mechanisms in neurodegeneration in PD (Bandres-Ciga et al., 2019; Brunden et al., 2014; Esteves & Cardoso, 2016; Pellegrini et al., 2017).

Interestingly, various *RAB29* variants have also been related to variable risk of PD in different populations (Chung et al., 2013; Guo et al., 2014; Z. hua Liu et al., 2015; T. W. Liu et al., 2020; Nalls et al., 2014). The function of RAB29 is poorly understood, but it is a RAB GTPase possibly implicated in lysosome integrity (Kuwahara et al., 2016), retromer-mediated protein recycling and trans-Golgi network integrity (S. Wang et al., 2014) where it has been shown to recruit and directly interact with LRRK2 (Z. Liu et al., 2018; Purlyte et al., 2018). Apart from risk variants, a significant proportion of monogenic gene products are also implicated in vesicular trafficking steps including *VPS35*, *DNAJC6*, *ATP13A2*, *SYNJ1* and *LRRK2* (Bandres-Ciga et al., 2019). Therefore, the etiology of both familial and sporadic PD seems to be intimately linked to deficits in various vesicular trafficking steps, which will be further discussed below.

Locus	Location	Gene	Gene product	Function	Inheritance
PARK1/4	(4q21)	SNCA	α-synuclein	Neurotransmitter release and regulation of synapse	AD
PARK2	(6q25.2-q27)	PRKN	Parkin	Mitochondrial quality control, mitophagy	AR
PARK6	(1p35-p36)	PINK1	PTEN-induced kinase 1	Mitochondrial quality control, mitophagy	AR
PARK7	(1p36)	DJ-1	DJ-1	Protection against oxidative stress	AR
PARK8	(12q12)	LRRK2	Leucine rich repeat kinase 2	Membrane trafficking	AD
PARK9	(1p36)	ATP13A2	Cation-transporting ATPase 13A2	Cation homeostasis, lysosomal function	AR
PARK17	(16q11.2)	VPS35	Vacuolar protein sorting 35	Recycling of membrane proteins between endosomes and the trans-Golgi network	AD
PARK19	(1p31.3)	DNAJC6	DNAJ subfamily C member 6	Clathrin mediated endocytosis	AR
PARK15	(22q12–q13)	FBXO7	F-box protein 7	Phosphorylation-dependent ubiquitination	AR
PARK20	(21q22.11)	SYNJ1	Synaptojanin-1	Regulation of synaptic vesicle endocytosis	AR
PARK14	(22q12–q13)	PLA2G6	Phospholipase A2, group 6	Phospholipid remodeling, mitochondrial function	AR
PARK22	(7p11.2)	CHCHD2	Coiled-coil-helix-coiled-coil- helix domain 2	Regulation of mitochondrial metabolism under oxidative stress	AD
PARK11	(2q36–q37)	GIGYF2	GRB10 interacting GYF protein 2	Negative regulation of cell growth	AD
PARK13	(2p13)	Omi/HTRA2	High-temperature requirement A2	Neuroprotection	AR
PARK23	(15q22.2)	VPS13C	Vacuolar protein sorting 13C	Maintenance of mitochondrial function	AR
PARK18	(3q27.1)	EIF4G1	Eukaryotic translation initiation factor 4 ɣ 1	Regulation of mRNAs translation	AD
PARK5	(4p13)	UCHL1	Ubiquitin C-terminal hydrolase L1	Ubiquitin–proteasome system and neuronal survival	AD
-	(1q21)	GBA	Glucocerebrosidase	Lysosomal function	RF
-	(17q21.31)	MAPT	Microtubule-associated protein Tau	Modulates the stability of (axonal) microtubules	RF
AD autosomal dominant; AR autosomal recessive; RF risk factor; del deletion.					

Table 1. Summary of Parkinson's genes first identified in familial and early-onset cases of PD and most important risk factors. Modified from (Tran et al., 2020)

1.4. Molecular Mechanisms

α-syn aggregation is one of the main pathological hallmarks of PD. α-syn is the major component of LBs and it can exist in different conformational states including monomeric, oligomeric and fibrillar forms. Although its native form is unfolded, some factors such as mutations in the SNCA gene or different post-translational modifications lead to α-syn phosphorylation favoring its misfolding and thus, aggregation and LB formation (Soni & Shah, 2022). When mutated or forming oligomers, α-syn can still bind to the LAMP-2A receptor on the lysosome but it is not degraded by chaperone-mediated autophagy as if it were in its native form (Lynch-Day et al., 2012). α-syn may propagate then through the nervous system in a prion-like manner through different mechanisms (Quek & Hill, 2017). There is extensive evidence suggesting that α-syn aggregation may contribute to neurodegeneration (Bengoa-Vergniory et al., 2017; Blesa & Przedborski, 2014; Bougea, 2021), although this does not necessarily occur for all PD cases (Førland et al., 2018; Mollenhauer et al., 2019; Stewart et al., 2014). For instance, and as mentioned before, LBs can be absent in postmortem brains, especially in patients suffering from monogenic forms of the disease. In addition, LBs may be neuroprotective, perhaps dependent on the neural cell type (Breydo et al., 2012; Chartier & Duyckaerts, 2018). In either case, the relationship between α-syn, PD pathology and neurodegeneration is complex and not fully understood.

Another important pathological feature of PD is mitochondrial dysfunction. As previously mentioned, some of the mutations related to monogenic forms of PD affect genes encoding essential components of the mitophagy process, in particular *PINK1* and *PARKIN,* which codify proteins in charge of marking depolarized mitochondria for degradation (Vives-Bauza et al., 2010). Furthermore, exposure to MPTP or other substances like rotenone or paraquat, known disruptors of mitochondrial function, are directly related to PD development (Kouli et al., 2018; Mullin & Schapira, 2015). MPTP and rotenone cause mitochondrial dysfunction by directly binding to complex I and causing its inhibition (Pravdic et al., 2012; Ramsay et al., 1991) whereas paraquat interferes with the NADPH-cytochrome P450 reductase, accepting electrons and indirectly inhibiting complex I activity as a result (Chung Zhi See et al., 2022). The inhibition of complex I leads to a decrease in ATP and an increase in reactive oxygen species (ROS) levels in SNpc neurons resulting in altered expression of mitochondrial genome-encoded genes and ultimately causes mitochondrial fragmentation and ROS-mediated cell death (Goiran et al., 2022). The toxicity of MPTP is dependent on neuroglia, since it needs to be previously converted to a cation (MPP+) by either astrocytes or microglia, respectively, in order to be efficiently taken up by

dopaminergic cells through the dopamine transporter (DAT; (Machado et al., 2016; Rappold et al., 2011; Singer et al., 1986).

Apart from their involvement in toxin-related pathological mechanisms, neuroglia are also implicated in the process of PD via neuroinflammation. There are several studies that have corroborated the presence of reactive microglia (a type of CNS resident macrophage) in the midbrain of PD patients (Gerhard et al., 2006; McGeer et al., 1988; Ouchi et al., 2009; S. Pradhan & Andreasson, 2013), even in prodromal stages (Stokholm et al., 2017), presenting morphologies of activation and expressing inflammatory markers (Williams et al., 2022). As a consequence of microglia secretion of pro-inflammatory factors (Il-1α, TNFα, and C1q), astrocytes switch to a reactive and neurotoxic state (Liddelow et al., 2017). Furthermore, macrophages, dendritic cells and T lymphocytes are recruited, amplifying the inflammatory response (Engelhardt et al., 2017). There is evidence of neuroinflammation originating in the midbrain and extending through other brain regions (Terada et al., 2016). However, it is not fully understood if this process takes place as a reaction against dopaminergic neuronal death triggered by cell-autonomous mechanisms or if, on the contrary, it initiates as a result of other factors (i.e. genetic risk variants (Williams et al., 2022), α-syn accumulation (Scheiblich et al., 2021) or exposure to neurotoxins, thereby contributing to neurodegeneration (Zhu et al., 2022).

As mentioned before, various genes, mutations in which are associated with PD encode for gene products which have important roles in membrane trafficking (**Table 1**), with VPS35, synaptojanin-1 and LRRK2 among the most significant ones. The biology of LRRK2 and how it may be implicated in vesicular trafficking steps will be thoroughly addressed below.

2. LRRK2

The locus containing the gene encoding for LRRK2, *PARK8*, was first linked to PD in 2002 by Funayama and colleagues (Funayama et al., 2002). Two years later, 2 independent laboratories identified *LRRK2* as the causative gene (Paisán-Ruíz et al., 2004; Zimprich et al., 2004), including Paisán-Ruiz and colleagues at the Basque country in Spain, who alternatively named LRRK2 as *dardarin.* This name comes from the Basque word *dardara* which means tremor in English. LRRK2 mutations are the most common cause of genetic PD, causing around 5-6% of total cases, and they are also implicated in 1-2% of idiopathic PD cases, this prevalence being higher in certain ethnic groups (Helton et al., 2021). LRRK2-related PD and sporadic PD are greatly similar in clinical features and treatment response, which makes studying LRRK2 especially valuable for both kinds of PD (Kett & Dauer, 2012; Kluss et al., 2019; Tolosa et al., 2020).

2.1. Structure and Mutations

LRRK2 is a large protein, formed by 2527 amino acids (286 kDa) and belonging to the ROCO/RIPK (Receptor-interacting serine/threonine-protein kinase 1) families of proteins (Leandrou et al., 2019). The ROCO proteins received their name and are characterized by the presence of two domains, a Ras-like GTPase domain called Ras of complex protein (Roc) and a unique domain called C-terminal of Roc (COR) (Park et al., 2022). The RIPKs, in turn, are a small family of seven Ser/Thr and TKL (Tyr-kinase-like) kinases that share a significantly homologous kinase domain (Cuny & Degterev, 2021). The kinase domain (Kin) together with the Roc/COR domains situated upstream comprise the catalytic core of the protein, which is surrounded by different protein-protein interaction domains: armadillo (ARM), ankyrin (ANK) and Leucine-rich repeat (LRR) domains upstream and WD40 domains downstream (**Fig. 4**).

All 8 known LRRK2 mutations that cause autosomal-dominant PD are clustered in the catalytic core of the protein (Mata et al., 2016; Usmani et al., 2021). The most frequent LRRK2 mutation worldwide is G2019S, located in the kinase domain (Guedes et al., 2010). I2020T is also located at the kinase domain, N1437H, R1441C/G/H/S are located at the Roc domain and Y1699C at the COR domain (Smith et al., 2022). All of these mutations have been reported to cause an increase in the kinase activity (Martin Steger et al., 2016; Z. Liu et al., 2018). This kinase hyperactivity is the main pathological characteristic of PD-associated LRRK2 variants and is mediated by multiple mechanisms that will be further discussed. Apart from these pathogenic mutants there are also many identified LRRK2 polymorphisms which enhance risk for PD in specific populations, such as G2385R, R1628P and Y2189C (Ahmadi Rastegar & Dzamko, 2020;

Smith et al., 2022). T1410M is a variant with an unclear pathogenicity that may show a defective tertiary structure that hampers GTP hydrolysis (Deng et al., 2008).

In physiological conditions, LRRK2 activity has been shown to be regulated by complex intra- and intermolecular dynamics. LRRK2 has been repeatedly shown to form dimers, even though the specific mechanism regulating the balance between these two states remains unknown (Plotegher & Civiero, 2012). Dimer formation seems to be closely related to LRRK2 kinase activity, given that the inhibition of dimerization leads to a decrease in LRRK2 substrate phosphorylation (Helton et al., 2021). A model has been proposed in which LRRK2 is found mostly in a monomeric and inactive state in the cytoplasm and in dimers and kinase-active when attached to cellular membranes (Helton et al., 2021). This model is consistent with the Roc/COR domain being recently reported to be responsible for LRRK2 dimerization, and Roc/COR pathogenic mutations are concentrated at the protein-protein interaction interface (Myasnikov et al., 2021) and alter LRRK2 subcellular localization (Beilina et al., 2014; Madero-Pérez, Fdez, et al., 2018; Madero-Pérez, Fernández, et al., 2018). Roc/COR and kinase domains are in close spatial proximity in the

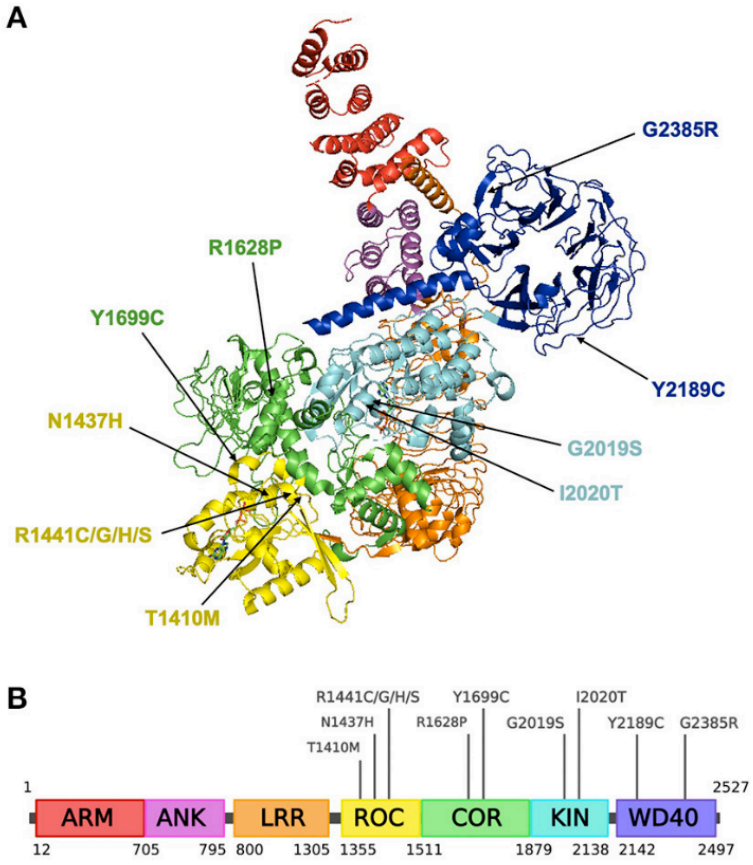

Fig. 4. Overall domain structure of LRRK2 and location of the main PD related mutations and some identified variants. *(A) Ribbon structural model of LRRK2 monomer. (B) Schematic representation of LRRK2 domains. (Smith et al., 2022)*

quaternary structure of LRRK2 (Myasnikov et al., 2021; Watanabe et al., 2020). Interestingly,

Roc/COR mutations have been reported to cause a higher increase in LRRK2 kinase activity as compared to the G2019S mutation when determined *in vivo* (Steger et al., 2016). All these data highlight the previously reported crosstalk between the Roc and kinase domains (Terheyden et al., 2016).

A recent structural study proposed a model for LRRK2 monomeric and dimeric forms that supports previously described interactions between LRRK2 and certain RAB proteins. Some members of the RAB GTPases family have been reported to recruit and activate LRRK2. Specifically the RAB32 subfamily which includes RAB29, RAB32 and RAB38, has been shown to interact with LRRK2 through its ARM domain in a GTPase dependent manner (McGrath et al., 2021). This interaction led to a subsequently published model of LRRK2-dimer RAB29 recruitment to membranes which is shown in **Fig. 5** (Myasnikov et al., 2021).

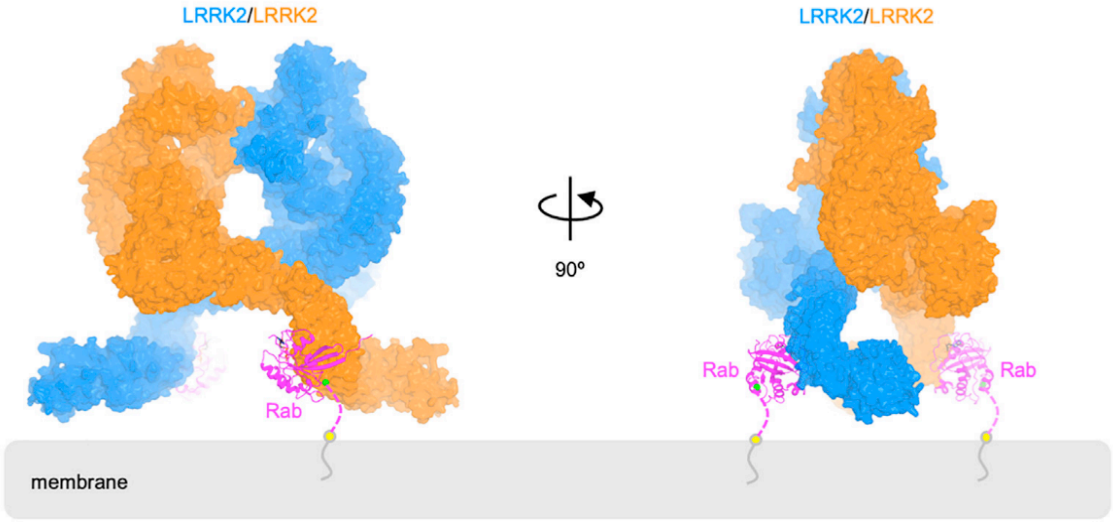

Fig. 5. Proposed model of LRRK2-RAB complex at the PM*. One unit of LRRK2 is represented in orange, the other one in blue. RAB is represented in magenta. Taken from (Myasnikov et al., 2021).*

Given the crucial role of LRRK2 kinase activity in PD pathological processes, specific LRRK2 kinase inhibitors have therefore emerged as the most promising clinical approach against LRRK2 PD. There are two types of inhibitors, type I, which are ATP competitive and type II, which are allosteric (Tasegian et al., 2021). Important LRRK2 specific kinase inhibitors widely used in both *in vitro* and *in vivo* studies such as Inhibitor-1, GSK2578215A and MLi-2 belong to type I kinase inhibitors. Other specific type I inhibitors include DNL151 and DNL201, developed by Denali Therapeutics, which have successfully completed clinical trial phase I and Ib, and DNL151 (also called BIIB122), which recently moved to phase II clinical trials (https://clinicaltrials.gov/study/NCT05348785, Azeggagh & Berwick, 2022; Jennings et al., 2022). However, it has been published recently that type I inhibitors enhance LRRK2 association

with microtubules, hampering kinesin and dynein motility (Deniston et al., 2020) which is consistent with previous studies (Blanca Ramírez et al., 2017; X. Deng et al., 2011) and could potentially affect vesicular trafficking events crucial for normal neuronal function (Leschziner & Reck-Peterson, 2021). On the other hand, currently available type II inhibitors which do not cause microtubule association of LRRK2 do not show enough specificity for LRRK2 (Tasegian et al., 2021). Given the reported side effects of type I inhibitors in peripheral tissues, other LRRK2 targeting strategies including the development of specific type II inhibitors or antisense oligonucleotides to decrease LRRK2 protein levels are currently being pursued.

2.2. Expression

LRRK2 is a widely expressed protein along human tissues. Its highest expression levels can be found in kidney and lung, which is consistent with the side effects found in those tissues in specific kinase inhibitor studies or LRRK2 knockout (KO) studies in different animal model systems (Baptista et al., 2013; Fuji et al., 2015; Herzig et al., 2011). These data also suggest a relevant physiological role for LRRK2 in those tissues which remains largely unknown. LRRK2 is also highly expressed in the immune system and moderately expressed in other tissues such as the enteric system, the bone marrow and the brain (Biskup et al., 2007; Wallings & Tansey, 2019, https://www.proteinatlas.org/ENSG00000188906-LRRK2/tissue). LRRK2 is detectable in the CNS, nonetheless, there is no current consensus about its expression levels in specific brain regions and cell types given the variety of published data (Dzamko et al., 2017; Galter et al., 2006; Sharma et al., 2011; Trabzuni et al., 2013; Zimprich et al., 2004). These discrepancies could be due in part to the technical difficulties in relation to reproducibility and specificity of the various LRRK2 antibodies employed in brain tissue preparations (Davies et al., 2013; Ferraro et al., 2022).

In the immune system, LRRK2 presents high levels of expression in peripheral blood cells such as monocytes, macrophages, neutrophils and dendritic cells (Fan et al., 2018; Hakimi et al., 2011; M. Zhang et al., 2022). Furthermore, LRRK2 expression levels are related to various immunological mechanisms and diseases as will be discussed in the next section.

2.3. Molecular mechanisms

Peripheral immune system

There is a bidirectional link between neurodegeneration and the inflammatory response, since conditions affecting the brain such as ageing, traumatic brain injury or chronic diseases can lead to chronic inflammation and reciprocally, peripheral infections and the associated inflammatory responses may contribute to neurodegeneration. LRRK2 has been linked to the immune system through GWAS studies as well as expression studies showing high LRRK2 expression in multiple peripheral and brain immune cells (Kung et al., 2022; Russo et al., 2022). In line with this, various LRRK2 genetic variants have been linked to an increased risk of inflammatory bowel disease, especially Crohn's disease (Barrett et al., 2008; J. Z. Liu et al., 2015; Witoelar et al., 2017). One of the identified variants has been reported to increase LRRK2 kinase activity (Hui et al., 2018), supporting the notion that LRRK2 activity may play a role in chronic inflammation which contributes to neuroinflammation thus leading to PD development.

LRRK2 expression levels increase in peripheral immune cells in human monocytes, macrophages, leukocytes and B and T cells in response to microbial infections, which is anticipated to also increase overall LRRK2 kinase activity (Ahmadi Rastegar & Dzamko, 2020; Shutinoski et al., 2019; Wallings et al., 2020). Even in the absence of immunological threats, LRRK2 expression levels are upregulated in these same cell types and in neutrophils in sporadic PD patients, causing an increased proinflammatory cytokine release from monocytes and T cells (Ahmadi Rastegar & Dzamko, 2020; Atashrazm et al., 2019; Cook et al., 2017). Also, higher levels of proinflammatory cytokines were found in blood, cerebrospinal fluid and brain of PD patients compared to healthy ones (Marogianni et al., 2020). These data taken together support LRRK2 as a regulator of the inflammatory response and suggest an early role of peripheral inflammation in PD. Additionally, upon immune stimulation, increases in LRRK2 kinase activity have been reported in neutrophils from PD patients (Atashrazm et al., 2019) and in healthy human peripheral blood mononuclear cells (Thirstrup et al., 2017), which points to the relevance of LRRK2 kinase activity in the immune system.

LRRK2 has also been recently linked to other immune cell functions, i.e. migration to infection or injury locations. Mild defects have been described in human monocytes derived from induced pluripotent stem cells (iPSCs) from G2019S LRRK2 PD patients (Speidel et al., 2016). Conversely, G2019S LRRK2 is associated with an increased chemotactic motility of peripheral immune cells in mice which is reversed by specific kinase inhibitors (Moehle et al., 2015).

Therefore, LRRK2 may be involved in immune cell migration, acting in a cell-type-dependent manner.

There is also evidence of detrimental mitochondrial function in immune cells derived from PD patients carrying the G2019S LRRK2 mutation. Mitochondrial DNA damage was observed in PD patient-derived immune cells as compared to healthy age-matched controls (Howlett et al., 2017). As this defect is reverted upon the specific LRRK2 kinase inhibitor GNE-7915, Gonzalez-Hunt and colleagues propose mitochondrial DNA damage as a potential biomarker of LRRK2 kinase activity in LRRK2-PD and sporadic PD patients (Gonzalez-Hunt et al., 2020), further confirmed by Qi and colleagues (Qi et al., 2023).

Autophagy has extensively been linked to LRRK2 function, and this link was first described precisely in immune cells including RAW264.7 murine macrophages (Schapansky et al., 2014). The study showed that there is an increase in LRRK2 translocation to the phagosome after an immune response in these cells, which was not observed upon LRRK2 knockdown (KD) or specific LRRK2 kinase inhibition. Recently, a pathway was described which further links LRRK2 to the immune system. LRRK2 has been shown to regulate the activation of the Transcription Factor EB (TFEB), a master regulator of both the autophagy and lysosomal machinery. LRRK2 KO macrophages show defects in TFEB activation while G2019S LRRK2 carrying macrophages present overactivated TFEB, which is also responsible for the transcriptional regulation of chemokines and cytokines that causes macrophage activation (Nabar et al., 2022). This could contribute to the defective immune response observed in LRRK2 deficient mice following an infection (Liu et al., 2017). However, opposing roles for LRRK2 in TFEB activation have also recently been reported (Yadavalli & Ferguson, 2023), and the precise link between LRRK2 and TFEB activation remains to be determined. Moreover, R1441C LRRK2 bone marrow derived macrophages have a reduced expression of the LC3-II autophagy marker, suggesting impaired autophagy (Hakimi et al., 2011), but the specific mechanisms implicated in the LRRK2-autophagy link in immune cells remains still unclear.

Lastly, LRRK2 also plays a role in phagocytosis, even though the precise mechanisms remain unclear. There is evidence for a positive correlation between LRRK2 kinase activity and phagocytic activity. Macrophages derived from G2019S LRRK2 human and mice show increased phagocytosis mediated by LRRK2 phosphorylation of an actin-cytoskeletal regulator, while LRRK2 KO macrophages and specific kinase inhibition treatment cause the opposite effect (Kim et al., 2018). Other studies show that LRRK2 KO and kinase inhibition do not impact macrophage phagocytic function *per se* but rather affect phagosome formation, maturation and function (Härtlova et al., 2018). Specifically, LRRK2 may mediate phagosome formation and function

through interaction with RAB5 and recruitment of RAB8 and RAB10 proteins (K. S. Kim et al., 2018; H. Lee et al., 2020). In this context, a stimulus-dependent mechanism has been proposed through which specific stimuli would trigger different phagocytosis pathways, implying LRRK2 playing distinct roles according to the given conditions (Russo et al., 2022).Central Nervous System

Microglia

Microglia are the resident and main immune cells of the brain, being essential for cerebral homeostasis. During embryonic development, these cells arise from monocytic precursors, in the same way as dendritic cells or macrophages (Zhang et al., 2022). In fact, microglia perform similar functions to those of macrophages, such as detecting changes in their close milieu or removing cellular debris (Colonna & Butovsky, 2017). Given these similarities, there are various shared cellular mechanisms affected by LRRK2 between these two cell types, including cell migration, autophagy, phagocytosis and mitochondrial function.

G2019S LRRK2 mouse microglia show defective motility while LRRK2 KO mouse microglia have enhanced motility. The mechanism behind these effects is thought to involve LRRK2 phosphorylation of the Focal Adhesion Kinase and the consequent attenuation of its function (Choi et al., 2015). As previously mentioned, LRRK2 was first identified as a regulator of autophagy in RAW264.7 murine macrophages as well as in BV2 murine microglia (Schapansky et al., 2014). Microglia derived from G2019S LRRK2 patients show enhanced phagocytosis (Panagiotakopoulou et al., 2020), perhaps mediated by distinct mechanism including those related to phagosome maturation or lysosomal health. Unexpectedly, microglia from LRRK2 KO mice also show enhanced phagocytosis, which is associated with an increase of RAB5 positive endosome numbers (Maekawa et al., 2016). Whilst these results are contradictory, they point to a regulatory role for LRRK2 in microglial phagocytosis.

Interestingly, BV2 cells and primary murine microglia expressing G2019S LRKK2 present a decrease in mitochondrial area, mediated by an increase of the mitochondrial fission protein Drp1 in a LRRK2 kinase-dependent manner, which correlates with a stimulation of pro-inflammatory responses (Ho et al., 2018). In addition, recent studies in mice suggest that microglia establish a network of nanotubes through which they redistribute healthy mitochondria, dampening cytotoxicity and avoiding cell death associated with α-syn accumulation, and this process is disturbed in G2019S LRRK2 microglia (Scheiblich et al., 2021).

The most studied functions for microglia in the context of LRRK2-PD are undoubtedly related to α-syn clearance and its role in neuroinflammation, and a complex relationship between LRRK2, α-syn and neuroinflammation has been described. Under normal physiological conditions, microglia are considered to be in a resting state. They become activated or reactive upon any variation in brain homeostasis, including pathological events or changes in the extracellular matrix resulting from cell death, to create a neuroinflammatory environment (Jang et al., 2009; C. Kim et al., 2013; Sadasivan et al., 2017; Sanchez-Guajardo et al., 2013; Smeyne et al., 2016). An exacerbated or chronic activation of microglia can then induce neuronal death (Dahlke et al., 2015). There is published evidence that points towards the regulatory role of LRRK2 in α-syn-mediated microglial immune responses, since microglia from LRRK2-KO mice show alterations in inflammatory and oxidative-stress pathways upon treatment with α-syn pre-formed fibrils (Russo et al., 2019). Recent studies suggest that this involves LRRK2-specific phosphorylation and nuclear translocation of the Nuclear Factor of Activated T cells, cytoplasmic 2 (NFATc2), as detected in PD/DLB patients (C. Kim et al., 2020). Accordingly, conditioned media from G2019S LRRK2 microglia creates an inflammatory milieu that causes a detrimental neurite elongation in DA neurons derived from iPSC-derived neural precursor cells from LRRK2 PD patients in vitro (Panagiotakopoulou et al., 2020).

How exactly LRRK2 regulates peripheral and neural inflammatory responses and the specific role for microglia in this process remains unclear. Despite previous studies reporting LRRK2 expression in microglia from healthy human brain and in murine BV2 cells, and increased LRRK2 protein levels in primary rat microglia upon immunological stimulation (B. Kim et al., 2012; Miklossy et al., 2006; Moehle et al., 2012; Russo et al., 2015), there is evidence for the absence of LRRK2 protein expression in human microglia (Kozina et al., 2018). In line with these studies, it has been proposed that microglia do not initiate neuroinflammation, but that neuroinflammation occurs secondary to an altered peripheral immune response caused by pathogenic LRRK2. This in turn triggers LRRK2 upregulation in microglia and creates a "self-perpetuating cycle of inflammation and neurodegeneration" (Kozina et al., 2018).

Two mechanisms for such cycle have been proposed: the actual recruitment of peripheral immune cells or an increase in circulating pro-inflammatory cytokines. Regarding the first mechanism, recent studies show that pathogenic α-syn recruits infiltrating monocytes through increased LRRK2 expression and subsequent RAB10 substrate phosphorylation (Xu et al., 2022). The second mechanism is backed by a study in LRRK2 mutant mice, where lipopolysaccharide induced SNpc neurodegeneration is associated with increased pro-inflammatory cytokine release by peripheral leukocytes but not by microglia or infiltrated cells (Kozina et al., 2018, 2022). The

latter model is also supported by the high expression levels of LRRK2 in peripheral immune cells, as previously mentioned. Finally, G2019S LRRK2 mutant-altered immune responses may require multiple contacts with inflammatory triggers along the lifespan of patients in order to cause dopaminergic neuronal cell death (Schildt et al., 2019). In either case, current work suggests that pathogenic LRRK2 affects microglial function in various ways and via various downstream targets, leading to deficits in microglial motility, alterations in the phagocytic/lysosomal degradation of

protein aggregates/cell debris, and transcriptional alterations which impact upon the production of proinflammatory cytokines (**Fig. 6**).

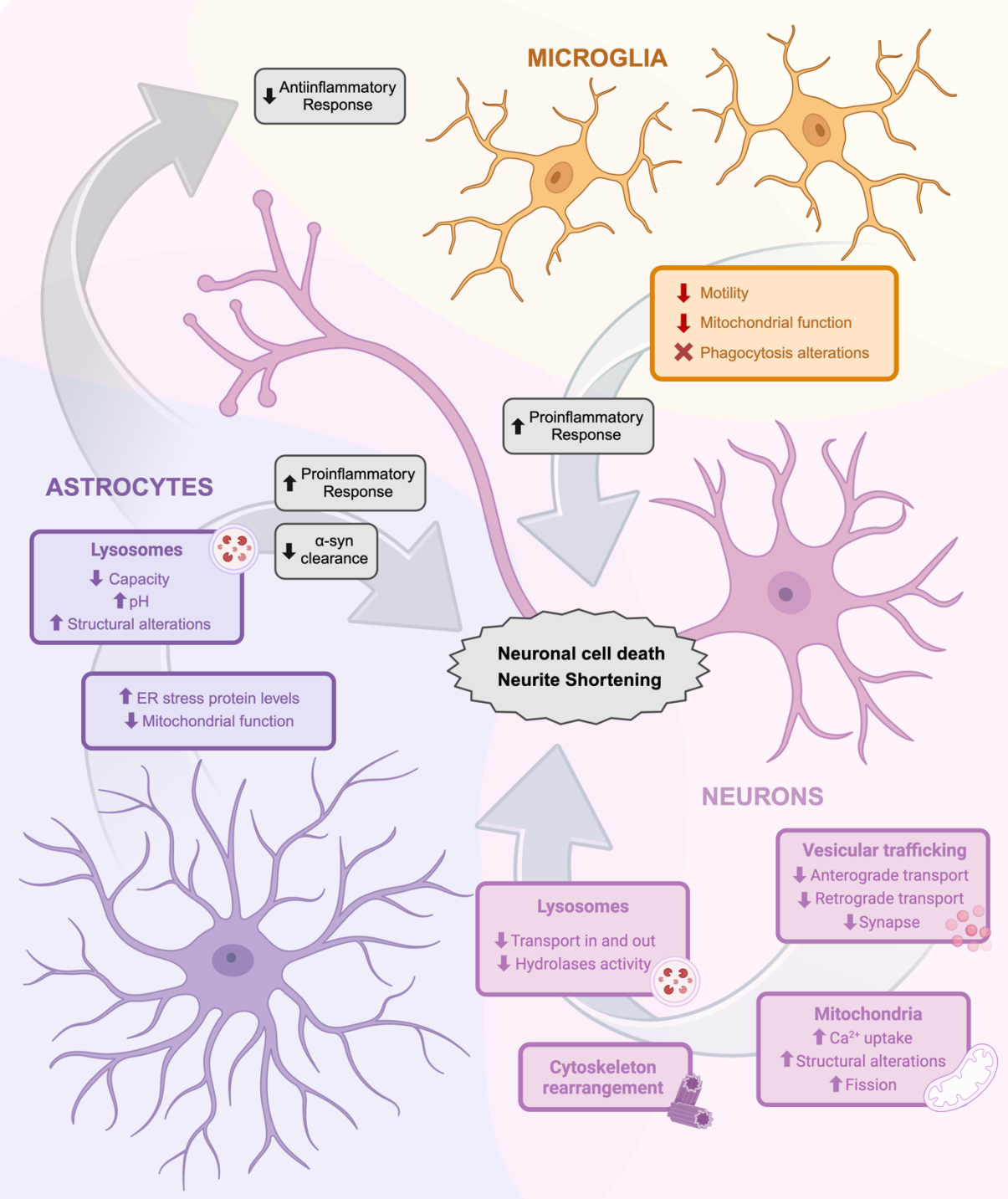

*Fig. **6**. Main cellular alterations in a pathogenic LRRK2 context in microglia, astrocytes and neurons. Created with BioRender.com.*

Astrocytes

Astrocytes are the major and most numerous cell type of the CNS, outnumbering neurons by over five-fold. This is due to their essential roles in maintaining CNS homeostasis and providing an optimal environment for neurons. Astrocyte functions include metabolic exchange with neurons, regulation of neurotransmitter levels in the synaptic cleft and blood-brain barrier maintenance (Iseki et al., 2023). LRRK2 is especially active in primary mouse astrocytes, where high levels of pT73 phosphorylated RAB10 substrate are found. In fact, astrocytes derived from G2019S LRRK2 knock-in (KI) mice show nearly twofold increased levels of pT73 RAB10, suggesting that endogenous G2019S LRRK2 displays a robust increase in kinase activity as compared to wildtype LRRK2 in this cell type (Wang et al., 2021).

The LRRK2 kinase activity has been linked to a variety of astrocytic functions. Similarly to microglia, astrocytes can reach a reactive state when exposed to pathological elements or stressful environments, which includes normal ageing (Clarke et al., 2018) and also stimulation via activated microglia (Liddelow et al., 2017). This reactive state has been found in PD astrocytes and implies multiple gene expression modifications which lead to a change in the astrocytic phenotype (Zamanian et al., 2012). Astrocytes derived from G2019S LRRK2 patient iPSCs show significantly increased secretion of proinflammatory cytokines upon inflammatory stimulation compared to control astrocytes (Sonninen et al., 2020). Interestingly, astrocytes derived from iPSCs from G2019S LRRK2 PD patients show downregulation of transforming growth factor beta 1, an anti-inflammatory cytokine which normally inhibits microglia inflammatory responses (Paglinawan et al., 2003; Booth et al., 2019). This data suggests that pathogenic G2019S LRRK2 has an increased activity in astrocytes which is implicated in inflammatory pathways within this cell type, ultimately also leading to an exacerbated inflammatory response which involves microglia.

In relation to α-syn accumulation, co-cultures of iPSC-derived neurons and astrocytes from healthy control and G2019S LRRK2 PD patients suggests a clear non-cell-autonomous implication of astrocytes in the disease process. Control neurons grown on top of PD astrocytes showed signs of neurodegeneration and α-syn accumulation, while co-culture of control astrocytes with PD neurons reverted the PD phenotypes in the neurons (di Domenico et al., 2019). Astrocytes internalize and secrete a high fraction of α-syn aggregates in both mono- and co-cultures, with microglia additionally captivating and clearing some of the aggregates in co-cultures (Rostami et al., 2021). Given the previously described defects in microglia expressing pathogenic LRRK2, α-syn accumulation and pathology seems to be a result of various mechanistic and cell-type dependent alterations mediated by altered LRRK2 function.

LRRK2 has also been related to ER Ca^{2+} homeostasis in primary astrocytes from G2019S LRRK2 KI mice. Treatment with α-syn resulted in increased expression of ER stress proteins and consequent cell death of G2019S LRRK2 astrocytes. Moreover, G2019S LRRK2 can localize to the ER membrane, interacting with and inhibiting the calcium pump or sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA), resulting in the formation of mitochondria-ER contact sites, mitochondrial Ca^{2+} overload and mitochondrial dysfunction (Lee et al., 2019). Interestingly, G2019S LRRK2 does not seem to have an effect on ER stress in neurons, but when neurons are co-cultured with G2019S LRRK2 astrocytes, neuronal death increases and neurite length shortens upon α-syn treatment as compared to co-cultures with wildtype astrocytes. Mitochondrial dysfunction has also been observed in G2019S LRRK2 astrocytes derived from patient iPSCs, which show altered mitochondrial morphology, reduced mitochondrial activity and increased generation of ROS, which hinders neuronal homeostasis maintenance (Ramos-Gonzalez et al., 2021). Therefore, pathogenic LRRK2 may play a unique role in astrocytes related to abnormal ER Ca^{2+} homeostasis, with downstream effects on mitochondrial function which ultimately affects proper neuronal function.

Pathogenic LRRK2 also causes various discrete vesicular trafficking deficits in astrocytes. Mutant G2019S, R1441C and Y1699C LRRK2 expression has been shown to cause lysosomal enlargement and a reduction in lysosomal capacity in mouse astrocytes (Henry et al., 2015). G2019S LRRK2 expression additionally reduces optimal lysosomal pH and increases the expression of the lysosomal ATPase ATP13A2, mutations in which cause monogenic forms of PD (**Table 1**; (Henry et al., 2015). Whilst the reason for this increase remains unknown, it could be part of a compensatory mechanism for the lysosomal deficits (Wang et al., 2021). The lysosomal structural alterations are rescued with a specific LRRK2 kinase inhibitor, indicating that they depend on the LRRK2 kinase activity (Henry et al., 2015). Specific LRRK2 kinase inhibitors also significantly rescue functional lysosomal disturbances such as pH alkalinization and a decrease in cathepsin B activity in mouse astrocytes caused by a PD-related mutation in GBA1, the gene encoding for GCase, suggesting possible crosstalk between LRRK2 and GCase (Sanyal et al., 2020).

Importantly, recent data suggest that LRRK2 is recruited to damaged lysosomes, followed by the formation of lysosomal tubules which release lysosomal membranous vesicular content (Bonet-Ponce et al., 2020), a process termed Lysosomal Tubulation/sorting mediated by LRRK2 (LYTL, Bonet-Ponce & Cookson, 2022). Mechanistically, LRRK2 at the damaged lysosome can phosphorylate RAB10 and RAB35, which is followed by recruitment of the microtubule motor JIP4 and lysosomal tubule formation. Hence, the normal function of LRRK2 may be related to maintaining proper lysosomal homeostasis, which may be deficient in the context of pathogenic

LRRK2. Interestingly, RAB10 phosphorylation by LRRK2 at lysosomes seems uniquely dependent on the subcellular positioning of lysosomes, mainly occurring in perinuclear lysosomes (Kluss et al., 2022). This may be at least in part mediated by the localization and/or regulation of the phosphatase PPM1H, which specifically dephosphorylates the LRRK2 kinase substrates RAB8 and RAB10 in vivo (Berndsen et al., 2019). Further studies will be required to determine whether LYTL is also active in neurons, or an astrocyte-specific function of LRRK2. Altogether, current work indicates that pathogenic LRRK2 causes lysosomal and mitochondrial deficits in astrocytes which is associated with impaired α-syn clearance and pro-inflammatory cytokine release (**Fig. 6**).

Neurons

In addition to the varied non-cell-autonomous mechanisms described for LRRK2-mediated neuronal pathology, numerous studies have also found LRRK2-mediated alterations in neuronal cell models. For instance, several different studies have found an increase in α-syn expression in iPSC-derived DA neurons from G2019S LRRK2 PD patients (Sison et al., 2018). In addition, the process of α-syn aggregation and the emergence of associated pathological phenotypes is accelerated in primary neurons from G2019S LRRK2 transgenic mice and in iPSC-derived neurons from G2019S LRRK2 patients (Bieri et al., 2019). By contrast, iPSC-derived LRRK2 KO neurons or primary neurons from G2019S LRRK2 mice upon LRRK2 kinase inhibitor treatment showed decreased α-syn aggregation. However, various other studies indicate that LRRK2 kinase inhibitors are unable to revert α-syn pathology, which is in line with the fact that a significant subset of LRRK2 PD cases lack LB pathology (Henderson et al., 2018; Kalia & Lang, 2015). Therefore, whilst a link between α-syn aggregation and pathogenic LRRK2 is evident in cultured cells, mechanisms unrelated to α-syn pathology may drive cell death in LRRK2-related PD in the intact brain.

Mitochondrial disturbances have also been described in G2019S LRRK2 iPSC-derived neurons, such as decreased total mitochondrial content, increased mitochondrial fission, and deficits in mitochondrial function, as evidenced by low levels of ATP and increased mitochondrial ROS (Schwab et al., 2017; Su & Qi, 2013). It is worth mentioning that these alterations were specifically observed in cultured DA neurons but not in glutamatergic or sensory neurons (Schwab et al., 2017). Other studies have reported an increase in mitochondrial calcium transporters leading to increased mitochondrial calcium uptake in primary cortical neurons expressing G2019S and R1441C LRRK2 mutations. This upregulation seems to be mediated by the ERK1/2 (MAPK3/1) pathway and ultimately results in dendrite and neurite shortening, and both inhibition of calcium uptake and potentiation of calcium export were neuroprotective and

attenuated this phenotype (Verma et al., 2017). Other studies indicate that G2019S LRRK2 may interact with and phosphorylate the dynamin like protein 1 (Drp1) to cause augmented mitochondrial fission and neurite shortening in iPSC-derived DA neurons from G2019S LRRK2 patients (Su & Qi, 2013). Finally, structural mitochondrial alterations are also evident in the brains of various pathogenic LRRK2 KI mice (H. Liu et al., 2021; Yue et al., 2015), consistent with an important role for LRRK2 in mitochondrial function. However, it remains unclear whether the mitochondrial deficits are a direct consequence of pathogenic LRRK2 function, or whether they are indirect reflections of deficits in LRRK2-mediated mitophagy and lysosome-mediated mitochondrial clearance mechanisms.

Interactions of LRRK2 with the microtubule cytoskeleton have long been known (Calogero et al., 2019). In fact, around 40% of all known LRRK2 interactors are implicated in cytoskeleton-related functions. There is a well-documented link between LRRK2 and both filamentous actin and microtubules, which are the major components of the cytoskeleton and therefore implicated in essential cellular processes such as cell division and motility, intracellular transport, and cell shape maintenance (Civiero et al., 2018). Neurons from G2019S LRRK2 mice show rearrangement of the actin cytoskeleton which may partially cause the reduced neurite outgrowth/neurite shortening (Jaleel et al., 2007; Parisiadou et al., 2009) extensively related to mutant LRRK2 in neuronal cell models (Civiero et al., 2018). PAK6, a member of the PAKs (p21-activated kinases), serine/threonine-protein kinases which play essential roles in signal transduction, has been shown to rescue the neurite shortening phenotype in primary cortical neurons from G2019S LRRK2 BAC transgenic mice through 14-3-3ɣ phosphorylation (Civiero et al., 2017). This phosphorylation results in the inability of 14-3-3ɣ to bind LRRK2, and at the same time PAK6 interacts with the Roc domain of LRRK2 and impairs its kinase activity (Blanca Ramírez et al., 2017; Civiero et al., 2017; Cogo et al., 2022).

In striatal cholinergic neurons in the intact mouse brain, pathogenic mutant LRRK2 interferes with the formation of the primary cilia, a ubiquitous microtubule-based cellular structure essential for detecting and responding to different extracellular signals. Pathogenic G2019S and R1441C LRRK2 seem to hinder cilia formation through a mechanism that involves RAB10 phosphorylation and its interaction with RILPL1 (RAB interacting lysosomal protein-like 1), a protein localized to the base of cilia (Dhekne et al., 2018). Whilst a neuroprotective pathway between striatal cholinergic neurons and dopaminergic neurons may be important for dopaminergic cell health (Hussein et al., 2021; Malave et al., 2021), subsequent studies did not reveal differences in this pathway in non-ciliated cholinergic neurons in pathogenic mutant LRRK2 mice (Khan, 2021), and the role for this ciliary phenotype in the context of PD remains unknown.

In neurons, LRRK2 has also been functionally linked to various intracellular trafficking steps. For example, in iPSC-derived DA neurons from G2019S, R1441C and R1441G LRRK2 PD patients, the activity of the lysosomal hydrolase GCase is significantly reduced in a RAB10-phosphorylation-mediated manner, which correlates with an accumulation of oxidized dopamine and α-syn. Treatment with a LRRK2 kinase inhibitor was sufficient to rescue these phenotypes (Ysselstein et al., 2019), again highlighting an important role for the LRRK2 kinase activity at the lysosome. Macleod and colleagues reported that pathogenic mutations in LRRK2 and VPS35 impair retromer-mediated trafficking through a RAB29-mediated process in primary neurons from both rat and mice (MacLeod et al., 2013). The retromer complex is responsible for retrograde transport between lysosomes and the Golgi apparatus, suggesting that pathogenic LRRK2 may impact upon proper trafficking to and from the lysosome.

Deficits in vesicle trafficking at the synapse have also been described in LRRK2-PD models. LRRK2 can be located in the synaptic compartment where it may interact with or phosphorylate proteins which regulate synaptic vesicle endocytosis. At the Drosophila neuromuscular junction, LRRK2 has been shown to phosphorylate EndophilinA, thereby impairing synaptic vesicle endocytosis (Matta et al., 2012). A decrease in synaptic vesicle endocytosis is observed in DA neurons from G2019S LRRK2 mice (Pan et al., 2017). Consistently, in G2019S, R1441C and R1441G LRRK2 PD patient-derived DA neurons, LRRK2 has been shown to phosphorylate and inhibit auxilin, which is necessary for uncoating of clathrin-coated vesicles. This has been suggested to cause the accumulation of oxidized dopamine in DA neurons (Nguyen & Krainc, 2018), even though the putative underlying mechanism for this remains unknown.

Interestingly, LRRK2 also plays a role in anterograde transport through anchoring Sec16A at the rough endoplasmic reticulum (ER) exit site, with either LRRK2 depletion or mutant R1441C LRRK2 expression causing Sec16A misplacing. This causes impairment of ER-Golgi protein transport, impeding proper trafficking of glutamate receptors to the synaptic surface (Cho et al., 2014). Conversely, G2019S LRRK2 has also been reported to cause defective internalization of the dopamine receptor D1 and delayed transport of the D2 receptor from the Golgi apparatus to the cell membrane, resulting in alterations in dopaminergic signal transduction (Rassu et al., 2017). In summary, LRRK2 seems to regulate various anterograde as well as retrograde vesicular trafficking events as relevant for proper neuronal and synaptic function itself (Boecker et al., 2021).

A schematic representation of the main mechanisms affected by pathogenic LRRK2 in different CNS cell types is showed in **Fig. 6.**

2.4. Interactors and regulation

Apart from cytoskeletal components as outlined above, LRRK2 has been reported to interact with a vast variety of proteins. A computational analysis of published literature about the LRRK2 interactome suggests that LRRK2 itself is the most robust LRRK2 interactor, as it can form dimers and oligomers via interdomain interactions as mentioned above (Myasnikov et al., 2021; Tokars et al., 2022).

Over 400 proteins are considered to be possible LRRK2 interactors and have been classified into families, which include cytoskeletal proteins, ribosomal proteins, protein kinases, GTPases including the RAB proteins, chaperones as well as mitochondrial proteins (Zhao et al., 2023). The most consistent and independently validated LRRK2 interactors include the 14-3-3 chaperone proteins and the RAB proteins, and the latter also serve as substrates for the LRRK2 kinase activity. Therefore, this section is focused on these two families of proteins.

14-3-3 proteins comprise a highly conserved family of chaperone-type proteins that regulate many diverse cellular functions such as cell cycle, mitosis, intracellular signaling and exocytosis (review in (Giusto et al., 2021)). Humans express 7 different isoforms of 14-3-3, 6 of which have been reported to directly interact with LRRK2 with different affinities (Manschwetus et al., 2020). 14-3-3 proteins bind to their interacting partners when they are in a phosphorylated state, and this is also observed with LRRK2, where phosphorylation sites in the N-terminus of LRRK2 (such as S935) allow for 14-3-3 binding. Certain PD associated mutations in LRRK2, such as R1441C/G/H and Y1699C show diminished N-terminal phosphorylation and thus, an impaired 14-3-3 binding (Li et al., 2011). The pathological decrease in 14-3-3 binding to LRRK2 can cause altered kinase activity as well as altered subcellular localization, and may in this manner contribute to disease-relevant phenotypes (Lavalley et al., 2016; Muda et al., 2014; Nichols et al., 2010).

Phosphorylation of LRRK2 at the N-terminus may involve cAMP dependent protein kinase (PKA; (Muda et al., 2014), but other kinases have been reported to phosphorylate LRRK2 at those sites as well, including protein kinase C zeta (Zach et al., 2010), or casein kinase 1-alpha (Chia et al., 2014).

Importantly, LRRK2 also autophosphorylates itself, and autophosphorylation can be employed as a readout for kinase activity (Greggio et al., 2008; Sheng et al., 2012). Multiple autophosphorylation sites have been proposed but Ser1292, situated in the LRR domain is a clear and independently validated autophosphorylation site, and is currently used as an indicator for LRRK2 activity *in vivo*. Its phosphorylation is increased by different pathological LRRK2 mutations

including G20219S and to a lesser degree R1441C/G (Sheng et al., 2012; Kluss et al., 2018; Iannotta et al., 2020).

Kinase substrates

As mentioned above, various proteins have been proposed to be substrates for the LRRK2 kinase activity. These include Endophilin A, involved in synaptic vesicle endocytosis, the prototype tumor suppressor p53, different mitogen-activated protein kinases, the ribosomal protein s15, Akt1, which is implicated in growth factor and hormone signal transduction, the microtubule affinity-regulating kinase 1 and the ERM proteins, in charge of linking the actin cytoskeleton to different membranes (Matta et al., 2012; Arranz et al., 2015; Ho et al., 2015; Ohta et al., 2011; Gloeckner et al., 2009; C.-Y. Chen et al., 2012; Martin et al., 2014; Krumova et al., 2015; Parisiadou et al., 2009). However, none of these substrates have been independently validated by multiple independent groups, and there is no evidence that they are phosphorylated by endogenous LRRK2 in vivo, as they have been mainly studied under conditions of LRRK2 overexpression (Martin Steger et al., 2016).

In cells expressing endogenous LRRK2, RAB proteins were first described as in vivo LRRK2 kinase substrates by Steger and colleagues in a dual phosphoproteomic screen (Steger et al., 2016) which opened a whole new area of research in the LRRK2-PD field. Interestingly, only the positive control pS935-LRRK2 and pT73-RAB10 passed the filtering criteria of this screen. They confirmed direct phosphorylation of T73-RAB10 by LRRK2 in vitro using a kinase assay. Given that T73 is a highly conserved residue situated in the switch II domain in over 40 RAB-family members (**Fig. 7**), they proceeded to investigate whether other RAB GTPases were LRRK2 substrates and thereby confirmed that RAB3A, RAB8A and RAB12 could also serve as LRRK2 kinase substrates.

In a follow-up study, they systematically analyzed 50 different RAB proteins and found 14 of them (RAB3A/B/C/D, RAB5A/B/C, RAB8A/B, RAB10, RAB12, RAB29, RAB35 and RAB43) to be phosphorylated by LRRK2 when overexpressed (Steger et al., 2017). Importantly, they provided evidence for phosphorylation of 10 endogenous RAB proteins by endogenous LRRK2, including RAB3A/B/C/D, RAB8A/B, RAB10, RAB12, RAB35 and RAB43. The generation of phosphostate-specific antibodies against pRAB8, pRAB10 and pRAB12 allowed for the determination that these RAB proteins are endogenously phosphorylated by endogenous LRRK2, and therefore serve as authentic substrates for the LRRK2 kinase activity (Lis et al., 2018). Several posterior studies independently confirmed these previously identified RAB proteins as LRRK2 substrates and highlighted the possibility of additional ones such as RAB1 (Jeong et al., 2018; Nirujogi et al., 2021). In either case, a different subset of RAB proteins may be preferentially

phosphorylated by LRRK2 in a cell type-specific and tissue-specific manner (Lara Ordóñez et al., 2021; Nirujogi et al., 2021; Steger et al., 2017). The LRRK2 preference for different RAB substrates also is likely conditioned by their relative abundance and by counter-active phosphatases as well as their specificity (S. R. Pfeffer, 2023) as evidenced by the phosphatase PPM1H, which dephosphorylates pRAB8, pRAB10 and pRAB35, but not pRAB12 (Berndsen et al., 2019).

Below, a general introduction to the function and regulation of the RAB protein cycle will be presented, along with a description of the RAB proteins most relevant to LRRK2-related PD, namely RAB8, RAB10 and RAB29.

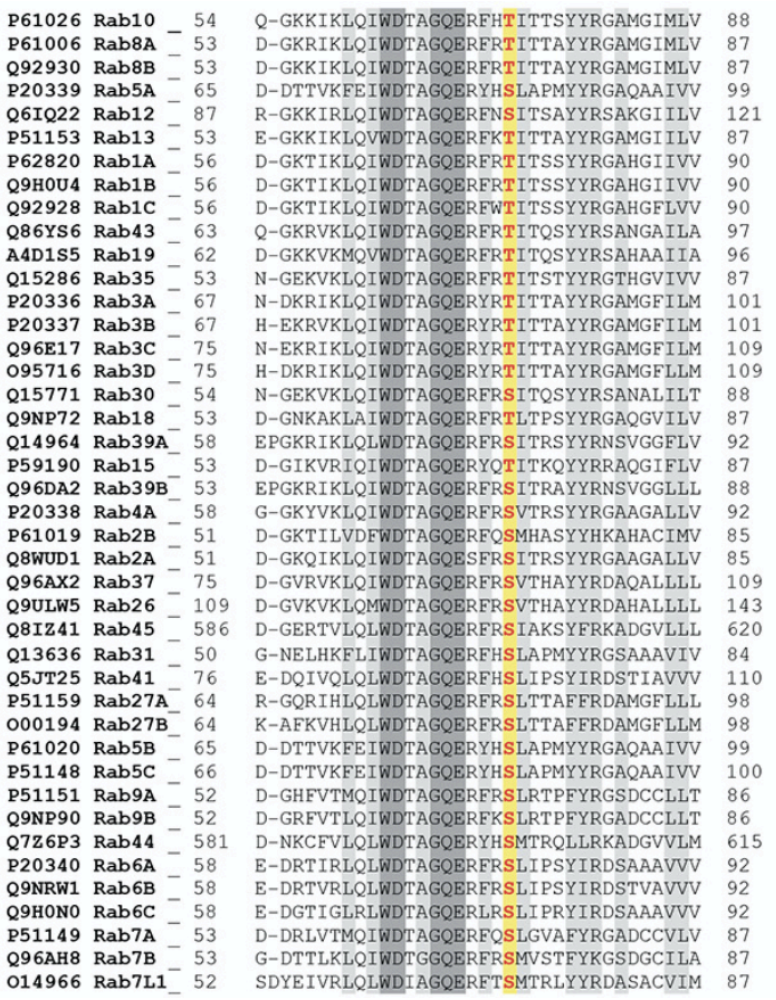

Fig. 7. Sequence alignment for several human RAB GTPases, including LRRK2 substrates. *Color legend: dark gray: 100% similarity, light gray: 80-100% similarity, yellow: highly conserved T73-RAB10 residue and equivalent sites for different RAB proteins. Taken from (Martin Steger et al., 2016).*

3. RAB PROTEINS

3.1. Cycle and regulation

RAB proteins comprise the largest family (more than 60 members in humans) of small GTPases which are part of the superfamily of Ras proteins (Elias et al., 2012). They are well known regulators of intracellular vesicular trafficking events, conferring functional identity to numerous membrane compartments and thereby assuring adequate intracellular transport of cargoes to their destinations (Pfeffer, 2013). The quantity and variety of specific processes regulated by RAB GTPases is truly ample, ranging from vesicle maturation to trafficking between organelles to vesicle tethering processes (reviewed in (Wandinger-Ness & Zerial, 2014)). **Table 2** lists a set of RAB proteins whose functions are well understood, as well as the RAB proteins which are endogenous LRRK2 kinase substrates, along with their reported localization and functions.

RAB GTPases can exist in either a cytosolic or membrane-bound form (**Fig 8**). Upon synthesis, GDP-bound RABs are recognized and recruited by RAB escort proteins (REPs) and then presented to RAB geranylgeranyl transferase (RABGGTB, also GGTase II) which transfers one or two lipophilic geranylgeranyl groups to C-terminal cysteine residues (Müller & Goody, 2017; Nagai-Ito et al., 2022). The prenylated RAB protein is then escorted by REP to its target membrane and anchored via its geranylgeranyl groups (Wandinger-Ness & Zerial, 2014). Situated at their C-terminal, a domain called the hypervariable region seems to be essential and sufficient to determine initial specific membrane targeting for some RABs (Chavrier et al., 1991). For some other RABs however, initial membrane targeting appears to depend on the specific localization of effectors and/or guanine-nucleotide-exchange factors (GEFs) and their interaction with RAB proteins (Banworth et al., 2022; F. Li et al., 2014). In addition, RAB domains other than the hypervariable region have also been suggested to be implicated in specific membrane targeting of newly synthesized RAB proteins (Ali et al., 2004).

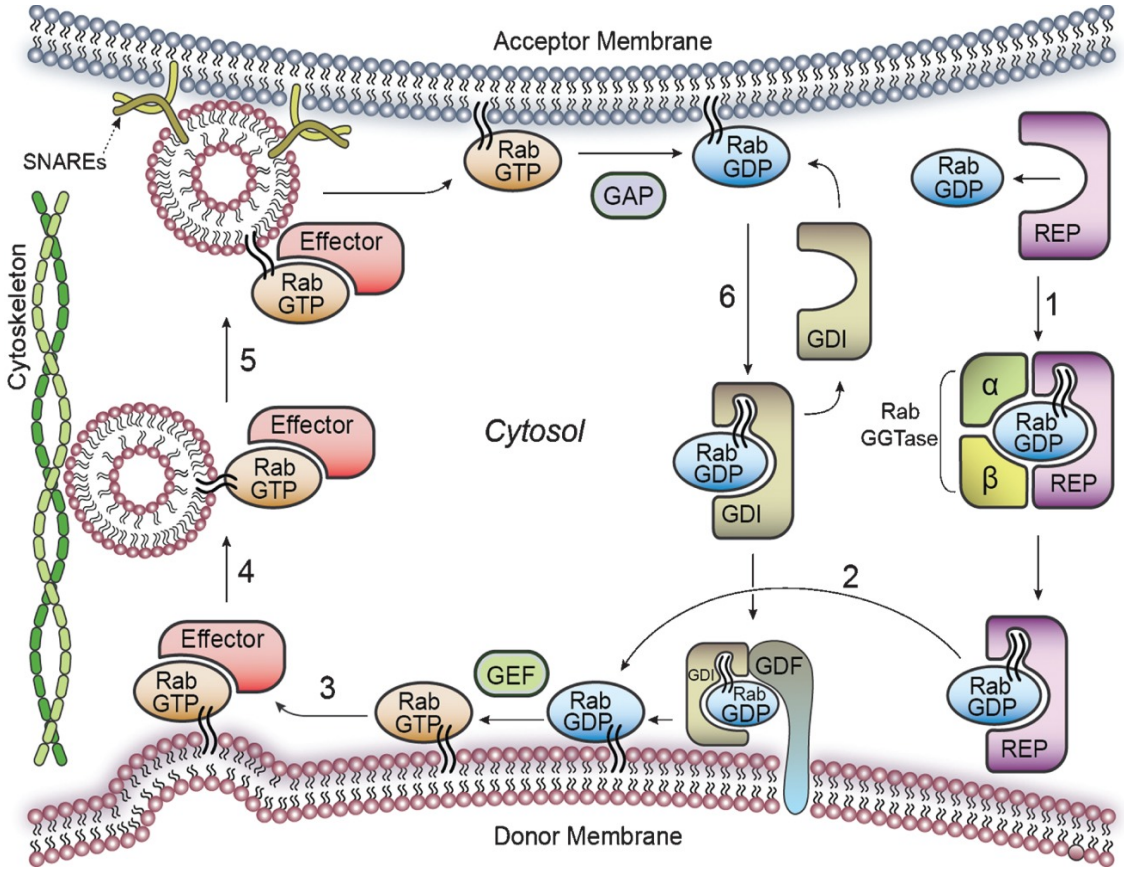

Fig. 8. RAB GTPase generic cycle. *Newly synthesized inactive RAB proteins are recruited by REPs which presents RABs to RAB GGTase II (1), that prenylates them at their C-terminal. RABs are then escorted by REP to its target membrane and anchored via its prenylated groups (2). GEFs recognized inactive RAB and cause nucleotide exchange, allowing RAB to recruit and interact with its effectors, which mediate vesicle budding (3), transport (4) and tethering at the target membrane (5). After fulfilling their functions GTP-bound RABs are recognized and inactivated by GAPs. Membrane-bound inactive RAB GTPases are then specifically recognized by GDIs (6), which can form a repository of cytoplasmic RABs ready for their next cycle. GDIs also help insert RABs back into their correct membranes. Modified from (Seixas et al., 2013).*

RAB	LRRK2 substrate	Cellular location	Function	References
RAB1	No	Golgi	Golgi integrity	Haas 2007
		ERGIC (or IC) and ERES	ER-Golgi traffic (anterograde) ("secretory machinery")	Segev 1988
				Stenmark 2009
				Westrate 2020
RAB2	No	Cis-Golgi, phagophore	Retrograde trafficking	Chavrier 1990
			Autophagosome and autolysosome formation	ding 2019
			Autophagosome-lysosome fusion	zhao 2021
RAB3	Yes	Synaptic vesicles	Synaptic vesicle fusion with the PM	Pavlos 2010, Xiachun Wang 2023
RAB4	No	EEs/LEs/REs	Fast recycling pathway	Van der Sluijs 1996,
				McEwen 2007
RAB5	No	EEs	Endosome maturation, motility, size and membrane identity	Naslavsky 2023
			Endocytosis	Tremel 2021
			Retromer recruiting	Rojas 2008
RAB6	No	Golgi	Intragolgi retrograde transport	Martinez 1994,
			Golgi to ER retrograde transport	Hefferman 2022,
				Dornan 2023
		Exocytic vesicles	Golgi to PM transport (cell specific)	Grigoriev 2007
RAB7	No	EEs and LEs	Transport from early to late endosomes and lysosomes	Feng 1995
			Endosome maturation	Casanova 2017
			Retromer recruiting	Rojas 2008
			Late endosome-lysosome fusion	Yasuda 2016
		Lysosomes	Lysosome biogenesis	Bucci 2000
			Lysosome pH and positioning	Johnson 2016
		Autophagosome	Autophagy regulation	Hyttinen 2013
				Zhan 2017
RAB8	Yes	ERC	Endocytic recycling	Roland 2009
		Recycling Endosomes		Kobayashi 2014
		TGN	Golgi to PM transport in polarized cells	Huber 1993
				Sato 2014
		Exocytic vesicles		Shibata 2016
		Primary cilia	Ciliogenesis	Nachury 2007
				B.Zhang 2015
RAB10	Yes	TGN	Golgi to PM transport in polarized cells	Schuck 2007
		ERC	Endocytic recycling	Roland 2009
		Recycling endosomes		Etoh 2019
		Primary cilia	Ciliogenesis	Sano 2011
				Sato 2014
RAB11	No	REs, ERC, TGN	Slow endocytic recycling,	Ullrich 1996
				Maxfield 2004
RAB12	Yes	ERC	Atypical degradation pathway	Mitsui 2011
		Autophagosomes	Autophagosome trafficking in starvation-induced autophagy	Xu 2015
		TGN	Post Golgi trafficking to PM of EGFR	Jinhui Wang 2023
RAB22A	No	REs	Endosomal cargo recycling	Naslavsky 2023
				Kong 2023 and refs within
			Biogenesis of recycling tubules	
RAB29	No	TGN	TGN integrity and funtionality	Aoki 2017
				Wang 2014
			M6PR retromer-mediated recycling	
		Phagosome	Phagosomal maturation	Pradhan 2018
			Phagosome-lysosome fusion	Shrivastava 2022
RAB35	Yes	PM and REs	Endocytic recycling	Sato 2008
				Remsburg 2021
RAB43	Yes	Golgi	Biogenesis and maintenance of Golgi	Haas 2007
			Transport through medial Golgi	John V Cox 2016
			ER-Golgi transport	Chunman Li 2017

Table 2. Location and main functions of some of the best characterized RAB proteins including all LRRK2 substrates.

Once the RAB protein is inserted into its target membrane, it undergoes what is also called the GTPase cycle. RAB proteins act as a "molecular switch", whereby they are in an active "on" state when GTP bound, and in an inactive "off" state when GDP bound (Zhen & Stenmark, 2015). This nucleotide cycle is finely regulated by GEFs and GTPase-activating proteins (GAPs), specific for either single RAB GTPases or RAB subfamilies (F. Barr & Lambright, 2010). RAB proteins bind to GDP with very high affinity, and thus need accessory proteins for nucleotide exchange. GEFs recognize and bind to inactive GDP-bound RAB GTPases (**Fig. 8**) provoking conformational changes mainly in the switch I and switch II domains. This results in the opening of the nucleotide binding site, allowing for nucleotide exchange. Given that the cytosolic concentration of GTP is ten times higher than that of GDP (Traut, 1994), GTP then binds to RAB, switching it to an active, GTP-bound conformation which causes GEF to dissociate from RAB (Barr, 2013). In such GTP-bound state, the RAB is active and stabilized at the membrane. Directed point mutations in key residues can mimic both GTP and GDP-bound states of RAB proteins, which makes them a valuable tool for functional studies (Zhen & Stenmark, 2015).

In their GTP-bound active form, RAB GTPases are able to recruit their specific set of effector proteins in a spatiotemporally regulated manner especially via interactions of their switch I and switch II domains (**Fig. 9**) (Zhen & Stenmark, 2015). These effector interactions carry out downstream functions of RAB GTPases along all the vesicular trafficking processes which include cargo selection, vesicle formation, motility and tethering at the proper target membranes (Hutagalung & Novick, 2011; Mizuno-Yamasaki et al., 2012; Stenmark, 2009). Some proteins can

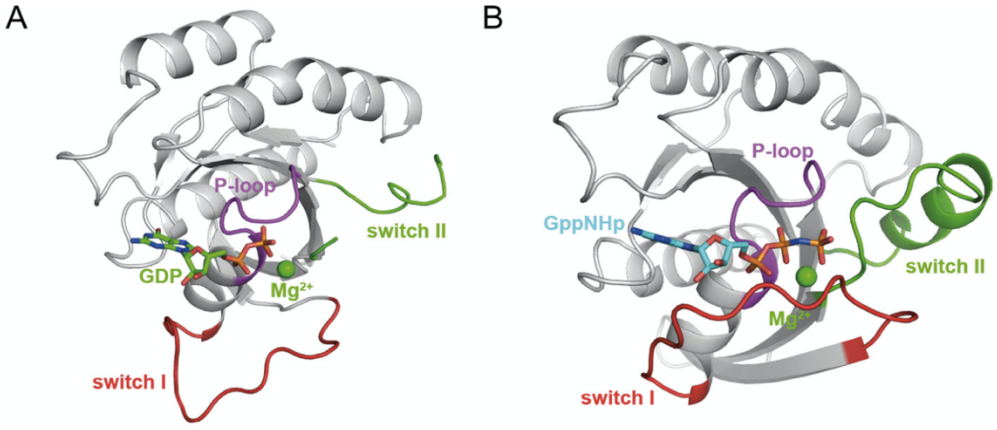

Fig. 9. Comparison of RAB8 structure in its inactive and active forms. *In its inactive form bound to GDP (A), switch domains show unfolded confirmations while in its active conformation bound to GTP (B) switch domains adopt well-folded conformations, and in this conformation can contribute to effector protein binding. Modified from (Guo et al., 2013).*

be effectors for more than one RAB GTPase, and RAB proteins can work in cascades that functionally connect different RABs (Mizuno-Yamasaki et al., 2012; S. R. Pfeffer, 2013).

After fulfilling their respective functions, active GTP-bound RABs are recognized by GAPs which enhance the inherently low RAB GTPase activity to promote hydrolysis of GTP into GDP, thus returning the RAB proteins into their inactive form. Some RAB GTPases show a high intrinsic GTPase activity by themselves and thus are less dependent on GAPs for their inactivation (F. Barr & Lambright, 2010). Membrane-bound inactive RAB GTPases are then specifically recognized by RAB GDP dissociation inhibitors (GDIs). GDIs detach GDP-bound RABs from the membrane and chaperones them in the cytosol, in that manner providing a pool of soluble RABs available for another event of vesicular transport (Pfeffer & Aivazian, 2004). They are also required to deliver RAB proteins to their correct membranes, where they are then activated by the resident GEFs for a new RAB cycle to occur. The full RAB cycle is schematically represented in **Fig. 8.**

All human RAB proteins share a highly conserved switch II domain (**Fig. 7**, Steger et al., 2016). Despite the fact that LRRK2 phosphorylation site is located at this domain, only the few previously mentioned members of the RAB family are confirmed LRRK2 substrates in vivo. To date, it is still not well understood what determines whether a RAB protein is a LRRK2 substrate or not, nevertheless RAB8 and RAB10 are known to be its most prominent substrates, with RAB29 playing a role in recruiting LRRK2 to membranes.

3.2. RAB8

Localization and function

RAB8 exists as two different isoforms, RAB8A and RAB8B, that slightly differ in their tissue distribution, with RAB8A being more abundant in lung and kidney and RAB8B in heart, spleen and brain (H. H. Ward & Wandinger-Ness, 2018). RAB8 was first described to be located at the cell periphery, specifically at ruffling areas in *Saccharomyces cerevisiae* (Y. T. Chen et al., 1993). This localization suggested a role for RAB8 in exocytic functions related to cell morphology and cell polarization. Indeed, in mammalian cells RAB8 has been implicated in axonal transport and neurite outgrowth (Furusawa et al., 2017; Kobayashi et al., 2014), protrusion formation (Hattula et al., 2006), cytoskeletal reorganization (Bravo-Cordero et al., 2016; Per et al., 1996) and maintenance of cell polarity (Devergne et al., 2017; Vidal-Quadras et al., 2017). RAB8 also localizes to primary cilia and has been associated with cilia formation in a mechanism along with several other proteins (S. Feng et al., 2012; Nachury et al., 2007; Yoshimura et al., 2007). Primary cilia dysfunction has been implicated in various diseases (Yoshimura et al., 2007) including in several PD models (Dhekne, Yanatori, Gomez, Tonelli, Diez, et al., 2018; Khan et al., 2021; Schmidt et al., 2022).

In agreement with those above-mentioned functions, RAB8 has been localized to the Golgi apparatus and it has been implicated in a myriad of post-Golgi trafficking events. These include polarized trafficking (Nakajo et al., 2016; Sato et al., 2007), cilia-directed trafficking (Mahajan et al., 2023; J. Wang et al., 2012) and exocytosis (Hampson et al., 2013; M. J. Kim et al., 2001).

However, and apart from such exocytic membrane trafficking events (Banton et al., 2014; Huber et al., 1995), RAB8 has also been implicated in autophagy-related transport, retromer-mediated trafficking (Kim et al., 2001) and importantly in endocytic recycling steps (Esseltine et al., 2012; Finetti et al., 2015). This is consistent with other reports about its localization to a tubular early recycling compartment (ERC) (Etoh & Fukuda, 2019).

RAB8 and LRRK2

RAB8 is one of the major LRRK2 kinase substrates. Pathological LRRK2 mutations increase RAB8 phosphorylation levels, reducing its ability to interact with its GEF, Rabin8 (Steger et al., 2017). Importantly, studies with phosphomimetic mutant variants of RAB8 also show a reduced level of interaction with GDI1/2 and the absence of interaction with GAP and various effector proteins, suggesting that phosphorylated RABs may accumulate in membranes unable to interact with neither their regulatory or effector proteins, as represented in **Fig. 10**. Whilst

phosphomimetic mutant versions of RAB proteins were subsequently shown not to be functional or properly localized (Dhekne, Yanatori, Gomez, Tonelli, Diez, et al., 2018), phosphor-state-specific antibody staining of cells expressing pathogenic LRRK2 has revealed that the endogenously phosphorylated RAB proteins indeed accumulate in membranes. This is consistent with the idea that they are trapped in membranes yet unable to perform their functions for membrane trafficking, as unable to bind to their effector proteins, hence causing a loss of function phenotype.

Similar to phospho-RAB10, inducing lysosomal membrane damage also causes accumulation of phospho-RAB8 in damaged lysosomal membranes (Eguchi et al., 2018). In contrast, and in the absence of lysosomal damage, phospho-RAB8 has been localized to a pericentrosomal ERC (Lara Ordónez et al., 2019; Madero-Pérez, Fdez, et al., 2018), consistent with its reported normal endogenous localization. Interestingly, RAB8 phosphorylation by LRRK2 not only interferes with its interaction with GEFs, GAPs and effector proteins, but uniquely allows for its interaction with RILPL1 and RILPL2 (Fdez et al., 2022; Waschbüsch et al., 2021). These two proteins are centrosome-localized, and thus able to bind to phospho-RAB8 localized to the pericentrosomal ERC. Increased RAB8 phosphorylation by pathogenic LRRK2 alters centrosome positioning, affecting neurite outgrowth and cellular polarization and migration. In patient-derived cells, it causes defects in centrosome cohesion (Madero-Pérez et al., 2018). This process seems to be caused by the centrosomal accumulation of pRAB8 and pRAB10 and the displacement of a centrosome cohesion protein, CDK5RAP2 in a RILPL1-dependent mechanism, involving also downstream and upstream LRRK2 regulators (Fdez et al., 2022; Lara Ordónez et al., 2019; Lara Ordóñez et al., 2022). Therefore, LRRK2-mediated phosphorylation of RAB8 causes unique centrosome-related alterations in a dominant fashion, whilst also interfering with proper RAB8 function for the membrane trafficking steps RAB8 is normally involved in.

3.3 RAB10

Localization and function

RAB10 has been described to localize to a large variety of intracellular membrane compartments including primary cilia, endosomes, trans-Golgi network (TGN) and the ER (Schuck et al., 2007; English & Voeltz, 2013; Y. Liu et al., 2013; Babbey et al., 2010). RAB10 belongs to the RAB8 subfamily, with RAB8 its closest homolog (Stenmark & Olkkonen, 2001). Accordingly, some of RAB10 functions are partially redundant with those of RAB8, such

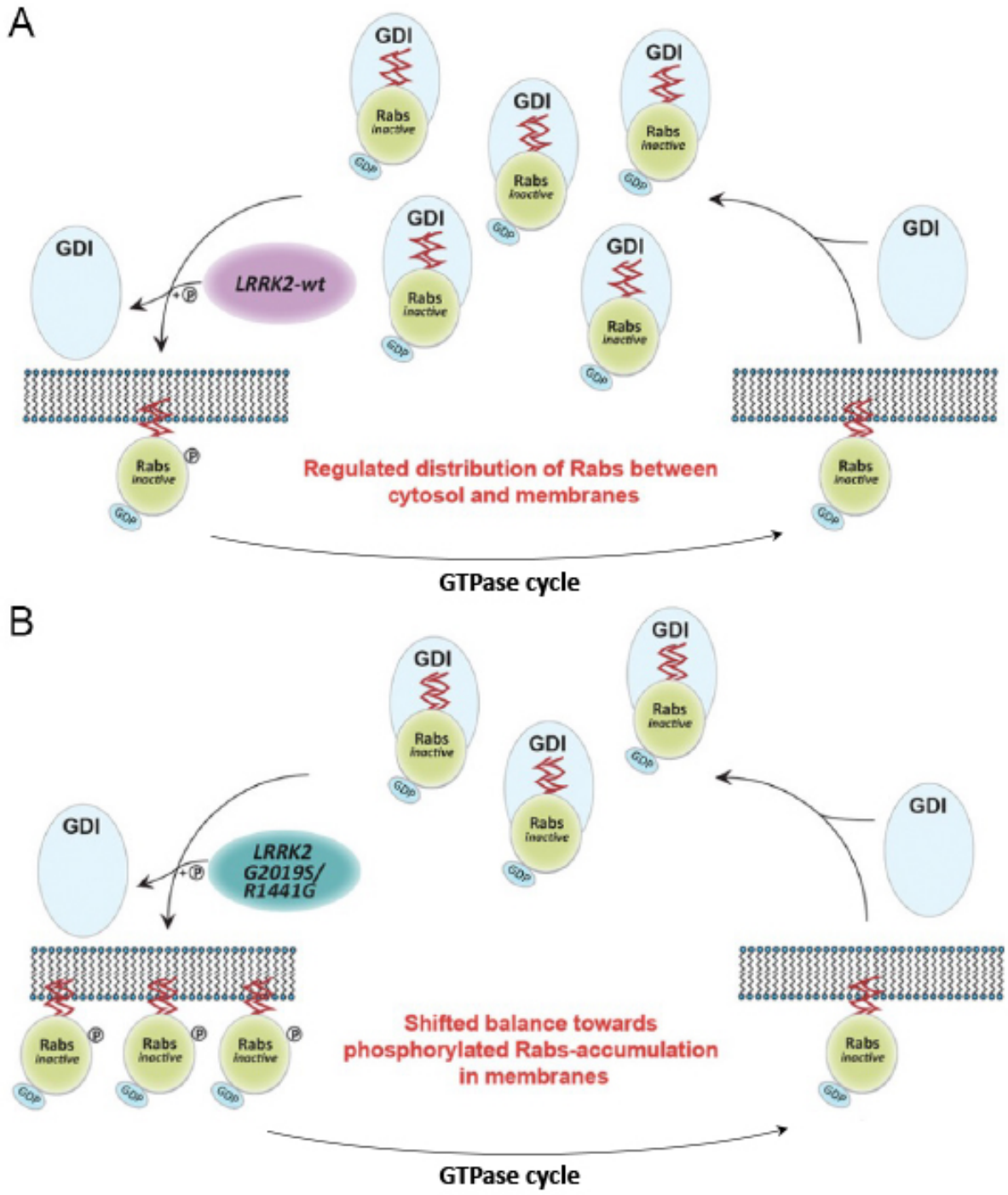

Fig. 10. Model for RAB GTPase phosphorylation by LRRK2 causing accumulation of phospho-RAB proteins in membranes. *(A) In physiological conditions, inactive GDP-bound genaryl-geranyl modified RAB GTPases bound to GDIs are inserted and extracted from their target membranes (B) Under pathogenic conditions, hyperactive LRRK2 causes phosphorylation of RAB GTPases with decreases their affinity for GDIs., such that the phospho-RAB proteins accumulate in membranes unable to be extracted by GDI and also unable to be activated by their GEFs to perform their functions in membrane trafficking. Modified from (Martin Steger et al., 2016).*

as neurite outgrowth and polarized transport (Tao et al., 2019; T. Wang et al., 2011). Other functions may be specific to RAB10 and not shared by RAB8, such as an involvement in ER dynamics and morphology (Chang & Blackstone, 2013; English & Voeltz, 2013), phagocytosis (Z. Li et al., 2016; Palmisano et al., 2017), or the insulin-dependent mobilization and plasma membrane transport of the glucose transporter GLUT4 (Brumfield et al., 2021; Bruno et al., 2016; Sano et al., 2011).

Importantly, RAB10 is also localized to the ERC where it largely colocalizes with RAB8A ((Babbey et al., 2006; Etoh & Fukuda, 2019; Lara Ordónez et al., 2019), and has also been implicated in endocytic recycling events, suggesting partially redundant roles for RAB8 and RAB10 in this membrane trafficking process (Chua & Tang, 2018; Shi et al., 2010).

RAB10 and LRRK2

At least in non-neuronal cells, RAB10 is the most prominent LRRK2 kinase substrate. As previously mentioned, lysosomal damage has been shown to cause accumulation of phospho-RAB10 on damaged lysosomes followed by LYTL to repair lysosomal damage, and such process may be impaired in the context of pathogenic LRRK2 (Bonet-Ponce 2020). In the absence of lysosomal damage, phospho-RAB10 accumulates in the ERC, which is followed by centrosome cohesion and primary ciliogenesis defects and mediated by the interaction of phospho-RAB10 with RILPL1 and RILPL2, identical to that reported for phospho-RAB8 (Dhekne, Yanatori, Gomez, Tonelli, Diez, et al., 2018; Fdez et al., 2022; Lara Ordónez et al., 2019; Lara Ordóñez et al., 2022).

Other locations for the accumulation of RAB10 upon its phosphorylation by LRRK2 have been described as well. In fibroblasts derived from PD patients carrying G2019S and R1441C LRRK2 mutations, phospho-RAB10 can accumulate on depolarized mitochondria impairing depolarization-induced mitophagy and mitochondrial function (Chua & Tang, 2018), even though these findings have not been independently validated. In contrast to RAB8, the consequences of RAB10 phosphorylation regarding its interactions with GEFs, GAPs and GDI have not been biochemically described in detail. However, it is known that RAB10 phosphorylation impedes its interaction with its GAP AS160 (Z. Liu et al., 2018), which points towards a loss of function of RAB10 that would affect its normal role in membrane trafficking.

Intriguingly, a role for LRRK2 phosphorylation of RAB10 has also been described for Alzheimer´s disease. Phospho-T73-RAB10 accumulation was observed in neurofibrillary tangles in hippocampal tissues of patients with AD as compared to control cases (T. Yan et al., 2018). However, further studies are warranted to corroborate these findings.

3.3. RAB29

RAB7L1 (RAB7-like 1, nowadays called RAB29) was first named alluding to its homology with RAB7 (Shimizu et al., 1997). RAB29 is encoded by the homonymous gene that, together with other 4 genes conforms to the PARK16 locus, originally linked to PD by a GWAS analysis in a Japanese population in 2009 (Satake et al., 2009). Since then, plenty of variants have been identified which cause either increased or reduced risk of suffering from PD in distinct populations (Tucci et al., 2010; Y. Yan et al., 2011; Gan-Or et al., 2012; Chung et al., 2013; Guo et al., 2014; Goudarzian et al., 2015; L. Wang et al., 2016; Khaligh et al., 2017).

Location and function

RAB29 is a relatively poorly studied RAB protein which is mainly localized to the TGN (MacLeod et al., 2013; Beilina et al., 2014; Kuwahara et al., 2016; Z. Liu et al., 2018; Purlyte et al., 2018) and has been implicated in diverse vesicular trafficking pathways, in TGN integrity and function (Wang et al., 2014, Aoki et al., 2017) and in ciliogenesis (Madero-Pérez, Fernández, et al., 2018; Onnis et al., 2015). It seems to play a role in the regulation of immune synapse assembly (Onnis et al., 2015), phagosomal maturation (Pradhan et al., 2018), phagosome-lysosome fusion (Shrivastava et al., 2022) and retrograde trafficking of the mannose 6-phosphate receptor (M6PR) (Wang et al., 2014).

RAB29 has been referred to as an atypical RAB GTPase, since it is resistant to being extracted from membranes by GDI and it does not form a complex with GDIs in the cytosol. It also seems to be less efficiently geranylgeranylated (Beilina et al., 2014; Gomez et al., 2019; Nagai-Ito et al., 2022). Until very recently, neither GEFs nor GAPs have been described for RAB29 (Shrivastava et al., 2022).

RAB29 and LRRK2

In regard to its relationship with LRRK2, RAB29 is also different from other RAB proteins such as RAB8 and RAB10. Under endogenous conditions, it does not seem to serve as a kinase substrate for LRRK2. However, RAB29 has been reported to interact with the ANK domain of LRRK2 and recruit LRRK2 to either the TGN or to lysosomes and thereby activate its kinase activity, at least in overexpression experiments (Eguchi et al., 2018; Fujimoto et al., 2017; Z. Liu et al., 2018; Purlyte et al., 2018). This recruitment and activation in turn causes the accumulation of phosphorylated RAB8 and RAB10 in those membrane compartments, which could in this manner contribute to the LRRK2-mediated membrane trafficking phenotypes. Interestingly, it has been shown that RAB29-mediated LRRK2 targeting to lysosomes enlarged by lysosomal

stress reduced the enlargement and contributed to lysosomal secretion. Furthermore, LRRK2-phosphorylated RAB8A and RAB10 accumulated on stressed lysosomes, suppressing the enlargement and promoting lysosomal secretion, respectively (Eguchi et al., 2018).

In the absence of lysosomal stress, endogenous RAB29 does not affect LRRK2 kinase activity (Kalogeropulou et al., 2020). In addition, and at least in the context of overexpression studies, RAB29 expression can rescue the LRRK2-mediated pathological phenotypes (MacLeod et al., 2013). LRRK2 has also been shown to work along RAB29 to regulate axonal trafficking and neurite outgrowth, proper lysosomal functioning, autophagosome transport and function, retrograde trafficking of M6PR, TGN morphology and intraneural protein sorting (MacLeod et al., 2013; Kuwahara et al., 2016; Fujimoto et al., 2017; Eguchi et al., 2018; M. Feng et al., 2018). Thus, under normal physiological conditions, the relationship between RAB29 and LRRK2 may not involve RAB29 phosphorylation or RAB29-mediated LRRK2 activation, but may be due to the proteins impacting upon similar intracellular vesicular trafficking steps, which requires further investigation.

4. EGFR trafficking

4.1 EGFR trafficking

The EGFR belongs to the ErbB receptor family which pertains to the receptor tyrosine kinase superfamily of signalling receptors. The EGFR plays a key role in several important processes including cell homeostasis, proliferation and migration as well as in neural development and tissue repair (Caldieri et al., 2018; Sibilia et al., 2007). Defects in EGFR signalling pathways are associated with different types of cancers, especially lung cancer and autoimmune disease such as psoriasis (Sasaki et al., 2013; Capuani et al., 2015; S. Wang et al., 2019). It has also been suggested that there is a link between EGFR signalling impairment and neurodegenerative diseases including AD and PD (Romano & Bucci, 2020). Most importantly though, the trafficking pathways of the EGFR are understood in great detail, such that it serves as an excellent cell biological model system to understand how select proteins impact upon endolysosomal degradative and vesicular recycling events.

In the absence of ligand, the EGFR undergoes a continuous cycle of internalization and recycling back to the plasma membrane (PM), ensuring its presence at the cell surface at all times. Ligand binding to the EGFR provokes a conformational change of the receptor that allows it to homodimerize through its intracellular domain, causing a trans-autophosphorylation of multiple residues that ultimately leads to signal transduction to various pathways such as RAS-RAF-MAPK or PI3K-Akt pathways (Zhou & Sakurai, 2022). To date, seven different ligands have been described for EGFR, with EGF being the most frequently used in research assays (Henriksen et al., 2013).

Activated EGFR dimers are rapidly internalized and transported to early endosomes (EEs) where they are sorted either back to the PM or to lysosomes for degradation. The EGFR can undergo both clathrin-mediated endocytosis (CME) and clathrin-independent endocytosis (CIE), and its fate seems to be in part determined by the internalization pathway it follows. CME is the main pathway for EGFR endocytosis, preferentially directing the EGFR to the recycling pathway while a small fraction is targeted to lysosomes for degradation, thus guaranteeing sustained EGFR signalling and functions. CIE on the contrary is thought to promote rapid targeting of the EGFR to lysosomal degradation, thus attenuating its signalling (Zhou & Sakurai, 2022).

Ligand concentration seems to be key to determine which pathway the EGFR follows when internalized, although there is still an open debate about the exact mechanism. Physiological concentrations seem to induce the CME/recycling pathway while saturated concentrations lead to CME saturation and CIE activation, such that an excess of EGF-EGFR complex is directed to lysosomal degradation (**Fig. 11**; (Sigismund et al., 2005). This ligand concentration-dependent trafficking is thought to be mediated by EGFR ubiquitination. An increase in EGFR ubiquitination has been described to perfectly correlate with levels of EGFR internalization by CIE. Indeed, receptor ubiquitination levels correlate with increasing concentrations of EGF in such a fashion that EGFR levels are preserved at low ligand concentrations (Sigismund et al., 2013).

When targeted for degradation, the ubiquitinated EGFR traffics from the EE, a compartment positive for RAB5 to the late endosome/multivesicular body (LE/MVB), a compartment positive for RAB7. At the LE, the EGFR is recognized and sequestered by the ESCRT machinery to be packaged into intraluminal vesicles (ILVs) which terminates its signalling, and the EGFR is subsequently degraded in the lysosome. RAB7 is essential at the endolysosomal

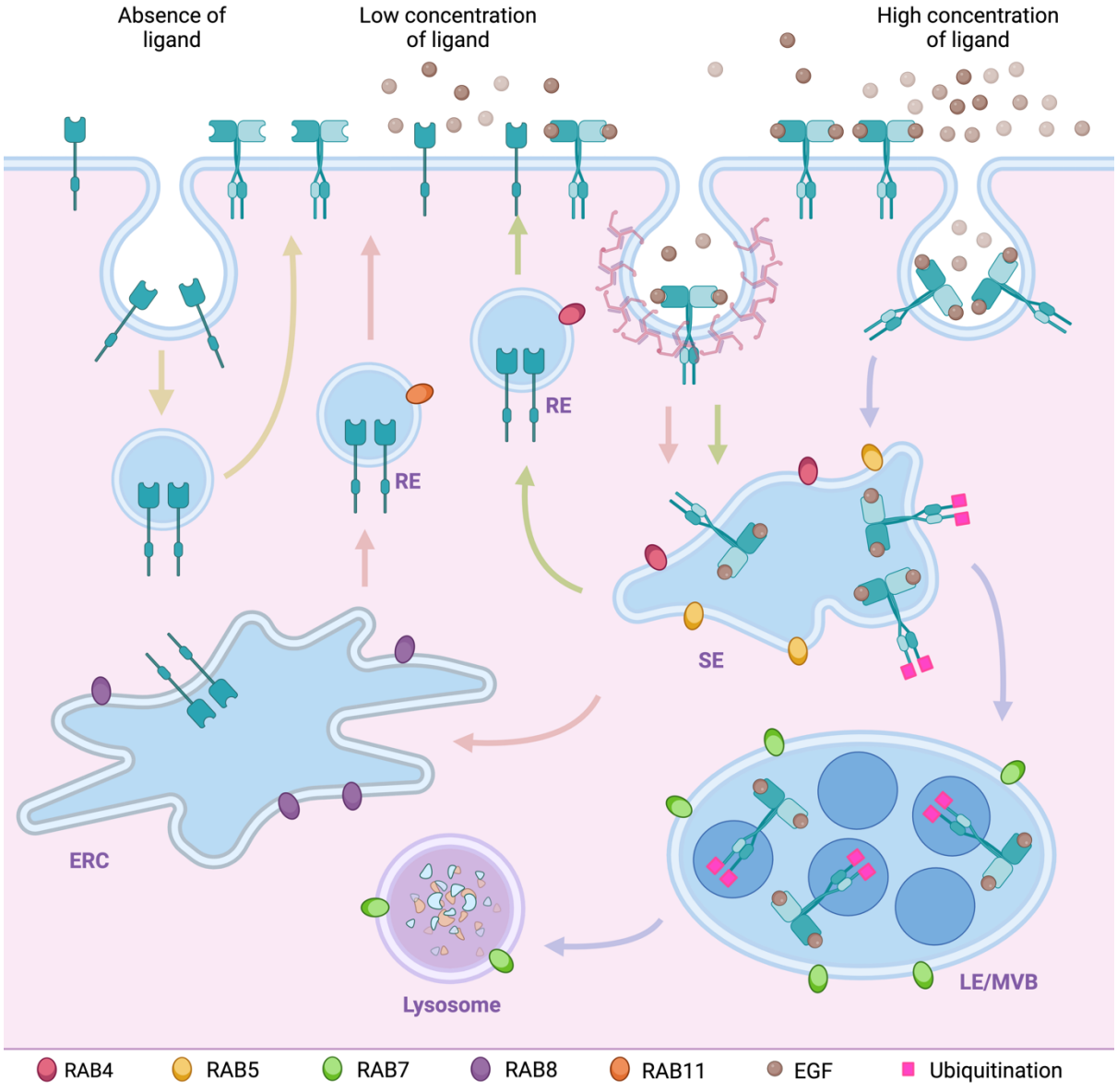

Fig. 11. Main trafficking pathways of the EGFR. *In the absence of ligand, the EGFR follows a basal rate of internalization and recycling back to the PM as a homeostatic mechanism (yellow arrows). Under low concentrations of ligand, the EGFR is mainly endocyted via CME and preferentially destined for fast (green arrow) or slow (orange arrows) recycling back to the PM. Upon higher ligand concentrations, the EGFR is internalized mostly via CIE, ubiquitinated and destined for endolysosomal degradation (purple arrows). RAB proteins which mediate membrane identity and the respective vesicular transport along the different pathways are indicated. ERC: Endocytic Recycling Compartment, RE: Recycling Endosome, SE: Sorting or early Endosome, LE/MVB: Late endosome/Multivesicular body. Created with BioRender.com.*

degradation pathway since it confers membrane identity to the maturing endolysosome. RAB7 also mediates the recruitment of microtubule motor proteins to transport the LE/MVB to the perinuclear region where it is able to fuse with lysosomes and fulfil its degradative function (reviewed in (Bakker et al., 2017)).

The EGFR can also be recycled from the RAB5-positive early EE back to the PM through the two main pathways broadly named as fast and slow recycling pathways (Chi et al., 2011; Goueli et al., 2012). Fast recycling consists of the targeting of the cargo from the EE directly back to the PM, while slow recycling requires the transport of the cargo from the EE to the ERC, localized adjacent to the microtubule-organizing center (MTOC), before being recycled back to the PM (Naslavsky & Caplan, 2018). Fast recycling requires the switching of the cargo from a RAB5 domain to a RAB4 positive domain (Sönnichsen et al., 2000; Zerial & McBride, 2001). Expression of mutant RAB4 and RAB4 KD have been related with impaired recycling and alterations in endosome morphology, which highlights the important role of RAB4 in endosomal trafficking (McCaffrey et al., 2001; Tubbesing et al., 2020). RAB35 has been also related to fast endocytic recycling, specifically in pathways related to cell adhesion, migration and cytokinesis (Kouranti et al., 2006; Allaire et al., 2013) The slow recycling pathway requires the budding of endosomal cargo-containing tubular domains which then fuse with the ERC. RAB11 plays a crucial role in this process, and RAB8 and RAB10 have also been implicated in the slow recycling pathway (Grant & Donaldson, 2009; Magadán et al., 2006; Tubbesing et al., 2020). Hence, the EGFR can undergo recycling through either a fast RAB4-positive compartment or a slower RAB8/RAB10-positive ERC compartment (**Fig. 11**), the latter of which is expected to be impaired upon RAB8 or RAB10 inactivation.

4.2 EGFR Trafficking and LRRK2

LRRK2 was first shown to impair various EGFR trafficking steps by previous work from our lab (Gómez-Suaga et al., 2014a; Rivero-Ríos et al., 2019). In 2014, we determined that overexpression of pathogenic G2019S and R1441C LRRK2 caused an initial reduction in EGF binding to surface receptors as well as a delay in EGFR degradation (Gómez-Suaga et al., 2014a). These effects were less pronounced with active wildtype LRRK2, and not observed with the LRRK2 kinase-inactive variant K1906M. In addition, the effects of pathogenic LRRK2 were reverted with two structurally distinct LRRK2 kinase inhibitors, indicating that they were mediated by the LRRK2 kinase activity. In cells expressing pathogenic LRRK2, internalized fluorescent EGF showed a decrease in colocalization with RAB7 and a decrease in intraluminal vesicle targeting, suggesting a deficit in EGFR transport from EE to LE.

Time-lapse microscopy further revealed an altered LE morphology with tubular structures in cells expressing pathogenic LRRK2 but not kinase-inactive LRRK2. These effects were was also evident in primary dermal fibroblasts from PD patients carrying the G2019S mutation as compared to healthy controls, and were reverted upon treatment with 2 different specific LRRK2 kinase inhibitors. Finally, it was determined by fluorescence recovery after photo-bleaching (FRAP) and pulldown assays that G2019S and R1441C LRRK2 overexpression as well as fibroblasts from G2019S LRRK2 PD patients presented with significantly lower levels of GTP-bound active RAB7. Interestingly, RAB7 GAP TBC1D15 expression was shown to mimic the described impairments as well as the decrease in active RAB7 levels. These data led to a model whereby pathogenic LRRK2 delays endolysosomal trafficking by decreasing the activity of RAB7.

Since RABA is a prominent LRRK2 kinase substrate, further work was performed to determine whether RAB8A was implicated in the above-described phenotypes mediated by pathogenic LRRK2 kinase activity (Rivero-Ríos et al., 2019). RAB8A was independently confirmed to serve as a LRRK2 kinase substrate, with T73 as the relevant phosphorylation site. Expression of active and phosphodeficient RAB8A as well as of Rabin8/RAB11, both RAB8A activators, rescued the endolysosomal EGFR trafficking deficits caused by pathogenic LRRK2. These data suggested that pathogenic LRRK2 may cause an increase in RAB8A phosphorylation, thereby inactivating this RAB GTPase. Consequently, phosphomimetic variants of RAB8A were unable to rescue the phenotype while knockdown of RAB8A mimicked the trafficking deficits. Since RAB8A is located at the ERC and known to mediate endocytic recycling events, the delay in EGF degradation could be at least partially caused by its accumulation in an alternative endocytic recycling compartment. Indeed, G2019S LRRK2 was found to cause an accumulation of fluorescent EGF at the fast endocytic RAB4-positive recycling compartment, where degradation and recycling pathways are known to merge (Braun et al., 2015). This phenotype was also mimicked by RAB8A siRNA-mediated knockdown as compared to control. Furthermore, G2019S LRRK2 expression also caused a decrease in levels of surface EGFR in steady-state conditions and a significantly slower recycling rate of the receptor back to the PM as compared to kinase-inactive G2019S-K1906M LRRK2.

Finally, and as previously described for the endolysosomal trafficking impairment mediated by pathogenic LRRK2, active RAB7A expression was found to rescue the endocytic recycling defects caused by pathogenic LRRK2. Thus, active but not inactive RAB7A expression rescued the surface levels of the EGFR and reverted its accumulation at a RAB4-positive compartment as observed by either G2019S LRRK2 expression or RAB8A knockdown. Lastly, dominant-negative RAB7A expression mimicked the described defects, which were also rescued by active RAB8A expression. Thus, RAB7 plays an the essential role in the mutant LRRK2 induced

trafficking phenotypes described above even though it does not serve as a LRRK2 kinase substrate. Altogether, these data suggest that the increased kinase activity of G2019S LRRK2 causes RAB8A inactivation mediated by phosphorylation which then indirectly causes RAB7A inactivation, thereby contributing to both recycling impairments and endolysosomal degradation impairments that leads to the accumulation of EGF in an alternative RAB4-positive endocytic recycling compartment (**Fig. 12**).

RAB10 serves as the most prominent LRRK2 kinase substrate, and RAB29 plays an important role in recruiting LRRK2 to distinct subcellular membranes, as described above. However, the roles of RAB10 and RAB29 in the endolysosomal trafficking deficits mediated by pathogenic LRRK2 remain unknown. The present work aimed to investigate the role of these two RAB proteins as relevant for the trafficking deficits mediated by G2019S LRRK2.

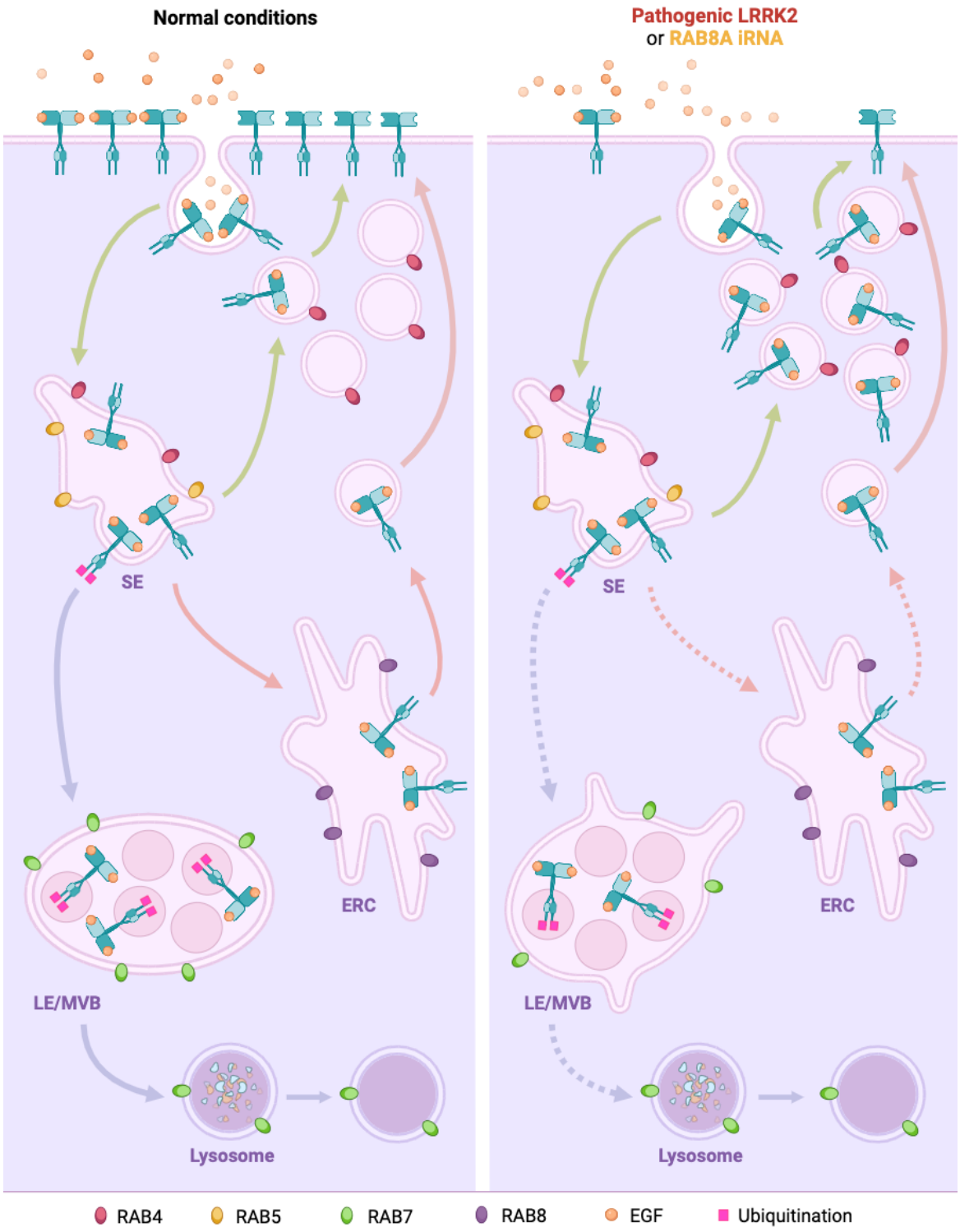

Fig. 12. Schematic model of trafficking deficits mediated by pathogenic LRRK2 or knockdown of RAB8. *Left: Under healthy conditions, EGFR is internalized and then sorted from the EE to LE/MVB and lysosome for degradation, or it can follow a recycling pathway back to the PM via fast RAB4-mediated (green arrows) or slow RAB11-mediated (orange arrows) transport. Right: Pathogenic LRRK2 expression or knockdown of RAB8 decrease RAB7 activity, which causes deficits in the endolysosomal degradative pathway (purple arrows) including trafficking from SE to LE/MVB, LE/MVB morphology and further transport of cargo to lysosomes.. LRRK2-mediated phosphorylation or knockdown of RAB8A/RAB10 also delays trafficking to and from the ERC, which results in the accumulation of the EGFR in a RAB4-positive recycling compartment. SE, sorting endosome; LE/MVB, late endosome/multivesicular body; and ERC, early recycling compartment.*

Objectives

1. Determine whether previously described LRRK2-mediated endolysosomal trafficking deficits are rescued upon RAB10 expression, and whether this is dependent on the activation or phosphorylation status of RAB10.
2. Analyze whether mutant LRRK2 and RAB8A knockdown-mediated endolysosomal trafficking deficits are mimicked by RAB10 knockdown.
3. Determine the effects of endolysosomal trafficking alterations due to the loss of RAB10 on the accumulation of endocytosed EGF in a RAB4-positive intracellular membrane compartment.
4. Evaluate whether the EGFR trafficking deficits mediated by RAB10 knockdown are rescued upon active RAB7A expression.
5. Determine the consequence of RAB10 loss on RAB7 activity levels.
6. Analyze whether RAB8A and RAB10 behave in a functionally redundant fashion with respect to the observed EGFR trafficking phenotypes.
7. Investigate the role of RAB29 expression on the endolysosomal defects due to mutant LRRK2-expression, loss of RAB8, loss of RAB10 or dominant-negative RAB7A expression.
8. Analyze whether the accumulation of EGF in a RAB4 positive compartment due to mutant LRRK2 expression, knockdown of RAB8A or knockdown of RAB10 is rescued upon RAB29 expression.
9. Assess the ability of RAB29 expression to recruit LRRK2 to the Golgi apparatus.
10. Study the relevance of RAB29 expression levels for LRRK2 recruitment to the Golgi and for the efficient rescue of EGFR trafficking deficits caused by G2019S LRRK2.
11. Evaluate whether RAB29 knockdown mimics the EGF trafficking deficits caused by mutant LRRK2 expression, loss of RAB8 or loss of RAB10.
12. Determine the dependency of mutant LRRK2-mediated deficits in EGF trafficking and its rescue upon RAB29 expression on the integrity of the Golgi apparatus.

Material and Methods

DNA Constructs and Site-Directed Mutagenesis

Triple flag-tagged LRRK2 constructs, as well as N-terminally tagged RAB8A, RAB10, RAB29, RAB7A, and RAB4 constructs, have been previously described (Civiero et al., 2012; Lara Ordónez et al., 2019; Madero-Pérez et al., 2018; Rivero-Ríos et al., 2019). The siRNA-resistant form of RAB10 was generated by introducing three silent mutations into the target sequence of the seed region of the RAB10-siRNA (Ambion, Thermo Fisher, Waltham, MA, USA, ID s21391), altering the original sequence by site-directed mutagenesis (QuickChange, Stratagene, San Diego, CA, USA) with primers 5'-GTCTATTCCTATGGTGGAAATAAATGTCGTGTTGAAGGCATCATCCGAAAAACGA-3' and 5'-TCGTTTTTCGGATGATGCCTTCAACACGACATTTATTTCCACCATAGGAATAGAC-3'. The identity of the construct was verified by sequencing of the entire coding region. DNA was prepared from bacterial cultures grown at 37 ºC using PureYield™ Plasmid Midiprep System (Promega, Madison, WI, USA) according to the manufacturer's instructions.

Cell Culture and Transfection

HeLa cells (ATCC; American Tissue Culture Collection) were cultured in 100 mm dishes in full medium (DMEM containing 10% fetal bovine serum, non-essential amino acids, high glucose) at 37 ºC in 5% CO_2. Confluent cells were harvested with 0.05% trypsin/0.02 mM EDTA in PBS, and subcultured at a ratio of 1:2–1:3. Cells were plated onto six-well plates and transfected the following day (70–80% confluence) using Lipofectamine 2000 (LF2000) (Invitrogen, Carlsbad, CA, USA) for 4 h in DMEM, followed by replacement with full medium. Double-transfections were performed using 2 µg of LRRK2 plasmids and 500 ng of RAB plasmids of interest. Alternatively, and where indicated, cells were transfected using Jetprime in full medium overnight according to manufacturer's instructions using the same DNA concentrations as for transfections with LF2000. The following day, transfected cells were replated at a 1:2 ratio onto coverslips in 24-well plates, and proteins were expressed for 48 h before analysis.

Knockdown of RAB10, RAB8A, or RAB29 by RNAi

HeLa cells were plated into six-well plates, and transfected the following day at 70–80% confluence using 25 nM siRNA and 4 µL of jetPRIME transfection reagent (Polyplus-Transfection SA, catalog number 114-15) in 200 µL of jetPRIME buffer overnight in full medium. For knockdown experiments in the presence of GFP-tagged RAB construct

expression, cells were transfected with 500 ng of the indicated RAB constructs using LF2000 for 4 h in DMEM, followed by replacement with full medium, and transfection with siRNA using jetPRIME overnight, as indicated above. In all cases, cells were passaged the next day and processed 48 h after transfection. RNAi reagents included the following: Silencer Select Negative Control Number 1 siRNA (Ambion, Thermo Fisher, catalog number 4390843), Silencer Select RAB8A (Ambion, Thermo Fisher, ID s8679), Silencer Select RAB10 (Ambion, Thermo Fisher, ID s21391), and Silencer Select RAB29 (Ambion, Thermo Fisher, ID s17082). In all cases, knockdown efficacy of the various siRNA reagents was confirmed by Western blotting with the appropriate antibodies. The precise sequences of the RNAi reagents employed are shown in **Table 3**.

siRNA	Sequence (5'->3')	Source	Order nr.
Silencer Select Negative Control Number 1 siRNA	GGAUGAUGCCUUCAAUACUtt	Ambion, Thermo Fisher	4390843
Silencer Select RAB8A (ID s8679)	GAGUCAAAAUCACACCGGAtt	Ambion, Thermo Fisher	4390824
Silencer Select RAB10 (ID s21391)	UCACCUCUAUGACACGAUUtt	Ambion, Thermo Fisher	4392420
Silencer Select RAB29 (ID s17082)	GAUUGACCGGUUCAGUAAAtt	Ambion, Thermo Fisher	4392420

Table 3. *List of siRNA reagents employed.*

Immunofluorescence and Laser Confocal Imaging

Where indicated, HeLa cells were incubated with 5 μg/mL brefeldin A (BFA) (Sigma Aldrich, St. Louis, MO, USA) before fixation with 4% paraformaldehyde (PFA) in PBS for 20 min at room temperature. Cells were next washed in 0.5% Triton X-100 in PBS for 3 × 5 min, followed by incubation in blocking buffer (10% goat serum, 0.5% Triton X-100 in PBS) for 1 h. Coverslips were incubated with primary antibody in blocking buffer for 1 h at room temperature, followed by washes in 0.5% Triton X-100 in PBS and incubation with secondary antibodies for 1 h. Coverslips were washed with PBS, and mounted in mounting medium with DAPI (Vector Laboratories, Burlingame, CA, USA). Primary antibodies included rabbit polyclonal anti-β-COP protein (1:200; Thermo Fisher, PA1-061) and mouse

monoclonal anti-flag (1:500; Sigma, F1804), and secondary antibodies included Alexa594-conjugated goat anti-rabbit (1:2000; Invitrogen) or Alexa594-conjugated goat anti-mouse (1:2000; Invitrogen), respectively. To determine the localization of GFP-tagged RAB protein variants in the absence of antibody staining, cells were fixed as described above, but only briefly permeabilized with 0.5% Triton X-100 in PBS for 3 min, followed by washes in PBS and mounting, as described above.

Images were acquired on a Leica TCS-SP5 confocal microscope using a 63 × 1.4 numerical aperture oil UV objective (HCX PLAPO CS). Single excitation for each wavelength separately was used for all acquisitions (488 nm argon laser line and a 500–545 nm emission band pass; 543 HeNe laser line and a 556–673 nm emission band pass; 405 nm UV diode and a 422–466 nm emission band pass). The same laser settings and exposure times were employed for acquisition of individual experiments, and 10 to 15 image sections of selected areas acquired with a step size of 0.4 µm. All z-stack images were analyzed and processed using Fiji.

Live-cell fluorescence microscopy to determine colocalization of fluorescent EGF with GFP-RAB4 in the absence or presence of pathogenic LRRK2 or siRNA of RABs was performed. For live-cell fluorescence microscopy aimed at assessing the colocalization of fluorescent EGF with various RAB proteins in the presence or absence of pathogenic LRRK2, reseeded transfected cells onto 35-mm glass-bottom dishes (ibidi) 24 hours post-transfection. To analyze colocalization with fluorescent EGF, cells underwent overnight serum starvation. On the following day, the medium was substituted with phenol-free, serum-free DMEM (Gibco), and cells were exposed to 100 ng/ml Alexa647-EGF (Invitrogen) for 20 minutes at 4 °C to facilitate surface binding of fluorescent EGF. Subsequently, cells were rinsed twice with ice-cold PBS and then incubated for 20 minutes at 37 °C to permit internalization of bound EGF before capturing images. Imaging was conducted on a Leica TCS-SP5 confocal microscope using a 63× 1.4 numerical aperture oil UV objective (HCX PLAPO CS), capturing individual z-stack images corresponding to the cell center. The quantification of colocalization between different GFP-tagged RAB proteins and Alexa647-EGF was performed using the JACoP plugin of Fiji. Following thresholding, the percentage of colocalization was determined by calculating Manders' coefficients (M1 for the red channel (Alexa647-EGF)), and the percentage of colocalization was derived as M1 × 100 (Cookson, 2016). 15–20 independent cells were analyzed per experiment.

Fluorescent EGF Binding and Uptake Assays

Assays were performed essentially as described (Rivero-Ríos et al., 2019). Briefly, transfected HeLa cells were plated onto coverslips the day after transfection, and serum-starved overnight. The following day, medium was replaced with fresh serum-free medium containing 100 ng/mL Alexa555-EGF (Invitrogen) at 4 ◦C to allow for ligand binding in the absence of internalization. In parallel experiments, control cells were washed with PBS, followed by acid stripping (0.5 M NaCl, 0.2 M acetic acid, pH 2.5, 3 min, 4 ◦C) to confirm that fluorescent EGF was merely surface-bound under those conditions. After binding of fluorescent EGF at 4 ◦C, cells were washed twice in ice-cold PBS, and then transferred to prewarmed serum-free medium to allow for uptake, and cells were fixed at the indicated times (10 min and 30 min) to quantify internalized fluorescent EGF. Fixation was performed with 4% PFA in PBS for 15 min at room temperature, cells were softly permeabilized with 0.5% Triton-X100 in PBS for 3 min, and mounted with DAPI. A minimum of 20 and up to 80 independent cells were analyzed for each condition and experiment, and quantification of the number of Alexa555-EGF structures per cell was performed by an observer blind to the conditions (Rivero-Ríos et al., 2019).

Cell Extracts and Western Blotting

HeLa cells were collected 48 h after transfection, washed in PBS, and resuspended in cell lysis buffer (1% SDS in PBS containing 1 mM PMSF, 1 mM Na3VO4, and 5 mM NaF). Lysed extracts were sonicated, boiled and centrifuged at 13,500 rpm for 10 min at 4 ◦C, and the protein concentration of supernatants was estimated using the BCA assay (Pierce). Where indicated, HEK293T cells were transfected as previously described (Rivero-Ríos et al., 2019), and extracts were prepared as described above. Proteins were resolved by SDS-PAGE and electrophoretically transferred to nitrocellulose membranes. Membranes were blocked in blocking buffer (LiCOR Biosciences, Li-COR Odyssey PBS blocking buffer, Lincoln, NE, USA, 927–40000) for 1 h at room temperature, and incubated with primary antibodies in blocking buffer overnight at 4 ◦C. Antibodies used for immunoblotting included rabbit polyclonal anti-GFP (1:2000; Abcam, Cambridge, UK, ab6556), mouse monoclonal anti-GAPDH (1:2000; Abcam, ab9484), mouse monoclonal anti-flag (1:500; Sigma, F1804), mouse monoclonal anti-tubulin (clone DM1A; 1:10,000; Sigma), rabbit monoclonal anti-RAB8A (1:1000; Abcam, ab188574), mouse monoclonal anti-RAB10 (1:1000; Sigma; SAB53000028), rabbit polyclonal anti-RAB7 (1:1000; Sigma, R4779), mouse monoclonal

anti-RAB11 (1:1000; BD Biosciences, Madrid, Spain, 610656), rabbit monoclonal anti-RAB4 (1:1000; Abcam, ab109009), sheep polyclonal anti-RAB29 (1:250; S984D, MRC-PPU Reagents), and rabbit monoclonal anti-phosphoT73-RAB10 (1:1000; Abcam, ab230261). Secondary antibodies included goat anti-rabbit or anti-mouse IRDye 800CW, and goat anti-rabbit or anti-mouse IRDye 680CW (1:14,000), and blots were imaged via near infrared fluorescent detection using Odyssey CLx Imaging System, with quantification performed using the instrument's Image Studio software.

Alternatively, and in all cases employing antibodies raised in sheep, membranes were blocked in blocking buffer (5% milk in 0.1% Tween-20/TBS) for 8 h, and primary antibodies diluted in blocking buffer and incubated overnight at 4 ◦C. Membranes were washed three times in 0.1% Tween-20/TBS, followed by incubation with HRP-conjugated rabbit anti-sheep antibodies and detection using ECL reagents (Roche Diagnostic GmbH, Barcelona, Spain). A series of timed exposures were undertaken to ensure that densitometric analyses were performed at exposures within the linear range, and the films were scanned and analysed using Quantity One (Bio-Rad, Hercules, CA, USA). A list of antibodies and their dilutions for either Western-blotting (WB) or immunocytochemistry (ICC) is provided in **Table 4.**

Primary Antibodies					
Antibody	Species	ICC	WB	Source	Catalogue nr.
anti-β-COP	pRb	1:200	-	Thermo Fisher	PA1-061
anti-Flag	mMs	1:500	-	Sigma	F1804
anti-GFP	pRb	-	1:2000	Abcam	ab6556
anti-GADPH	mMs	-	1:2000	Abcam	ab9484
anti-α-tubulin	mMs	-	1:10000	Sigma	T6199
anti-RAB8A	mRb	-	1:1000	Abcam	ab188574
anti-RAB10	mMs	-	1:1000	Sigma	SAB53000028
anti-RAB7	pRb	-	1:1000	Sigma	R4779
anti-RAB11	mMs	-	1:1000	BD Biosciences	610656
anti-RAB4	mRb	-	1:1000	Abcam	ab109009
anti-RAB29	pSh	-	1:250	MRC-PPU Reagents	S984D
anti-RAB10 (pT73)	mRb	-	1:1000	Abcam	ab230261
Secondary Antibodies					
anti-Rabbit IRDye 800 CW	goat	-	1:14000	LI-COR Biosciences	926-32211
anti-Mouse IRDye 800 CW	goat	-	1:14000	LI-COR Biosciences	926-32210
anti-Rabbit IRDye 680 CW	goat	-	1:14000	LI-COR Biosciences	926-68071
anti-Mouse IRDye 680 CW	goat	-	1:14000	LI-COR Biosciences	926-68070

Table 4. *List of antibodies employed.*

GST-RILP Pulldown Assays for Determination of Active RAB7

GST-RILP pulldown experiments were conducted in accordance with established procedures (references 24 and 42). In summary, the GST-RILP vector underwent transformation into Escherichia coli strain BL21. A 250 ml LB culture, initiated with a 1-ml overnight culture and grown at 37 °C until reaching an OD of 0.6–0.8, was induced with Isopropyl 1-thio-β-D-galactopyranoside (EMDBiosciences) at a concentration of 0.5 mM. The bacteria were induced for protein production for 3–4 h at 28 °C. Subsequently, bacterial cells were pelleted, washed with cold PBS, and the cell pellets were frozen at -20 °C.

The frozen pellets were later resuspended in 5 ml of ice-cold purification buffer (25 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.5 mM EDTA, 1 mM DTT, 0.1% Triton X-100, and 1 mM PMSF). The lysates underwent sonication and were cleared by centrifugation. The supernatant was diluted with an additional 5 ml of ice-cold purification buffer. GST-RILP was then purified using 300 µl of a pre-equilibrated 50% slurry of GSH-Sepharose 4B beads (GE Healthcare) with incubation for 1 h at 4 °C on a rotary wheel. The beads were washed with purification buffer, resuspended to a 50% slurry, and stored at 4 °C. A sample (5 µl) was separated by SDS-PAGE and subjected to Coomassie Brilliant Blue staining to assess protein purity, while protein concentration was estimated using a BCA assay (Pierce). The beads were utilized with cell lysates within 2 days of preparation.

Transfected HEK293T cells (one 10-cm-diameter dish per assay) were harvested by centrifugation, washed in PBS, and resuspended in pulldown buffer (20 mM HEPES, pH 7.4, 100 mM NaCl, 5 mM MgCl2, 1% Triton X-100, and 1 mM PMSF). Lysates were cleared by centrifugation at 13,500 rpm for 10 min at 4 °C. GST-RILP pulldown assays were performed in 1 ml of pulldown buffer containing 300 µg of cell lysate and 60 µl of 50% slurry beads pre-equilibrated in pull-down buffer. The beads were incubated on a rotary wheel overnight at 4 °C, washed twice with ice-cold pulldown buffer, and bound proteins were eluted by adding 40 µl of 1× sample buffer/β-mercaptoethanol and boiling for 4 min at 95 °C before separation by SDS-PAGE. Alternatively, active RAB7 levels were assessed using a RAB7 activation assay kit (NewEast Biosciences) following the manufacturer's specifications and as previously described (reference 43). In brief, cells were transfected as outlined above, and lysates (2 mg of total protein) underwent immunoprecipitation with a conformation-specific anti-RAB7-GTP mouse monoclonal antibody (NewEast Biosciences, 26923) bound to protein A/G–agarose. The precipitated RAB7-GTP was detected by immunoblotting with a nonconformation-specific rabbit polyclonal anti-RAB7 antibody (1:1000; Sigma, R4779).

As a positive control, extracts were incubated with 100 μM GTPγS (NewEast Biosciences, 30302) for 30 min at 30 °C to activate all available RAB7 before immunoprecipitation.

Statistical Analysis

All data are expressed as means ± S.E.M. One-way analysis of variance (ANOVA) with Tukey's post hoc test was employed, and significance was set at $p < 0.05$. Significance values for all data are indicated in the Fig. legends, and all statistical analyses and graphs were performed using Prism software version 7.0 (GraphPad, San Diego, CA, USA).

Results

G2019S LRRK2-Mediated Endolysosomal Trafficking Defects are Rescued by Active RAB10 and Mimicked by Knockdown of RAB10

To determine whether RAB10 modulates the pathogenic LRRK2-mediated endolysosomal trafficking deficits, we used the EGFR trafficking assay (Gómez-Suaga et al., 2014b; Rivero-Ríos et al., 2019). Upon ligand binding using high concentrations of EGF, the EGFR is internalized by clathrin-mediated endocytosis and sorted to lysosomes for degradation (Katzmann et al., 2002). The surface availability of the receptor can be determined by quantifying the binding of fluorescent EGF to cells at 4 ◦C, and the endocytic trafficking and degradation by quantifying the amount of endocytosed fluorescent EGF at 37 ◦C over time, respectively. HeLa cells were co-transfected with flag-tagged G2019S LRRK2 and either with GFP or with GFP-tagged RAB10 variants, and binding and degradation of fluorescently labelled EGF quantified (**Fig. 13A,B**).

As previously described (Gómez-Suaga et al., 2014b; Rivero-Ríos et al., 2019), expression of flag-tagged G2019S LRRK2 reduced the binding of fluorescent EGF at 4 ˚C, and impaired the clearance/degradation of internalized fluorescent EGF upon incubation of cells at 37 ˚C (**Fig. 13A–D**). GFP-tagged wildtype RAB10, or GTP-locked, constitutively active RAB10-Q68L were both localized to a tubular perinuclear compartment, while GDP-locked inactive RAB10-T23N was largely cytosolic (**Fig. 14A**). Both wildtype RAB10 and RAB10-Q68L were expressed to similar degrees, and did not interfere with the co-expression of G2019S LRRK2 (**Fig. 14B**). Expression of GFP-tagged RAB10 variants on their own was without effect on EGF binding or degradation (**Fig. 13E,F**). However, when co-expressed with pathogenic G2019S LRRK2, active RAB10-Q68L fully rescued the decrease in EGF binding and the impairment in EGFR degradation, which was not observed with wildtype RAB10 or with the inactive RAB10 variant (**Fig. 13C,D**), suggesting that pathogenic LRRK2 may cause the inactivation of RAB10.

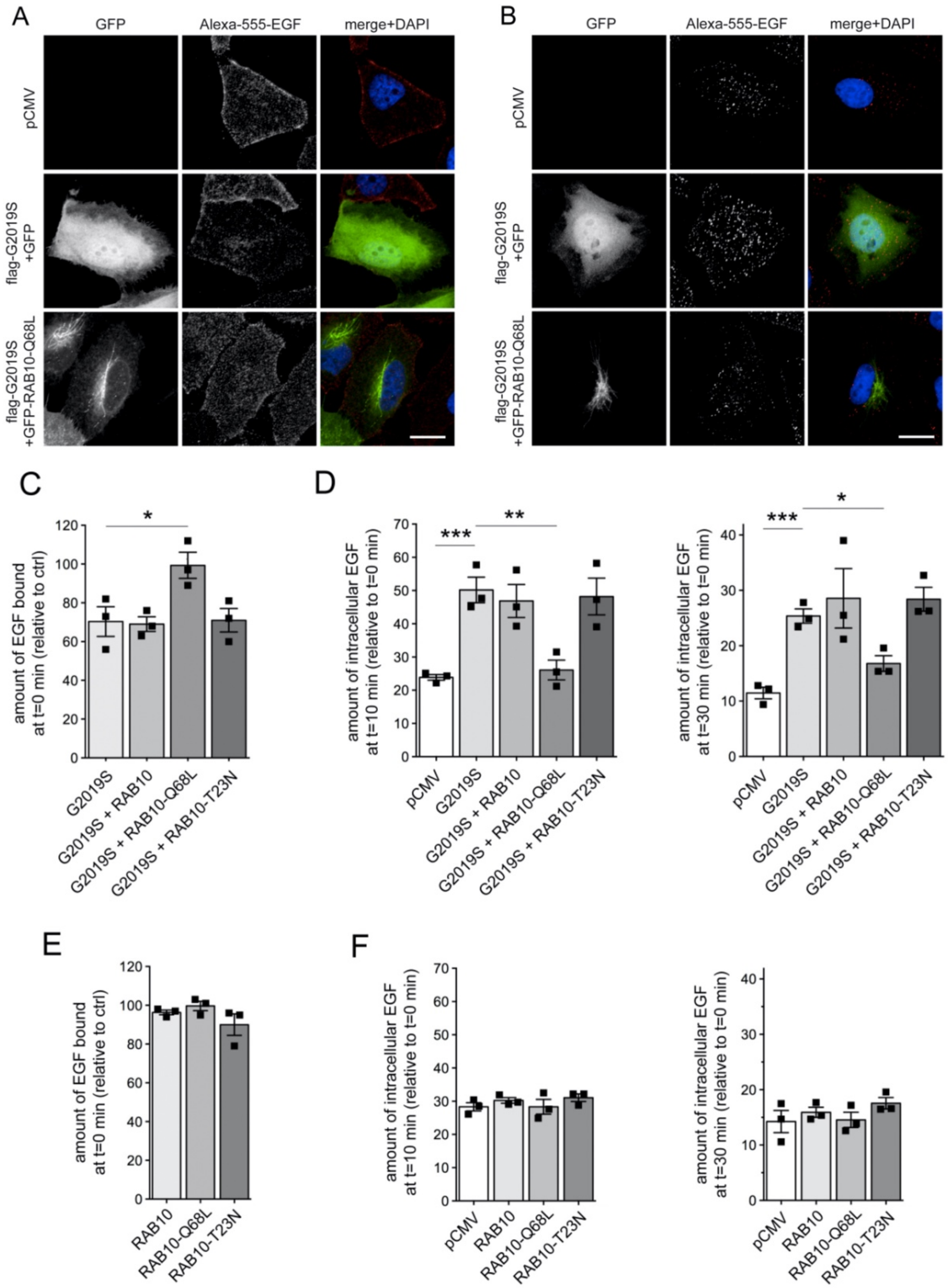

Fig. 13. Active RAB10 rescues the G2019S leucine-rich repeat kinase 2 (LRRK2)-mediated deficit in epidermal growth factor (EGF) binding and degradation. *(**A**) HeLa cells were transfected with either pCMV, or cotransfected with flag-tagged G2019S LRRK2 and GFP or GFP-tagged RAB10-Q68L as indicated. Cells were incubated with Alexa555-EGF for 20 min at 4 ˚C, followed by washing to remove unbound fluorescent EGF before fixation (t = 0 min). Scale bar, 10 µm. (**B**) Same as in (**A**), but upon incubation and washing, cells were shifted to 37 ˚C for 10 min to allow for the internalization and degradation of fluorescent EGF. Scale bar, 10 µm. (**C**) Cells were co-transfected with G2019S LRRK2*

*and either GFP, or GFP-tagged RAB10 constructs as indicated, and the amount of surface-bound fluorescent EGF was quantified. N = 3 experiments; * p < 0.05. (**D**) Cells were co-transfected as indicated, and the amount of internalized Alexa555-EGF in transfected cells was quantified after 10 min (left) and 30 min (right) of internalization, with values normalized to the amount of fluorescent EGF binding at t = 0. N = 3 experiments; * p < 0.05; ** p < 0.01; *** p < 0.005. (**E**) The amount of surface-bound fluorescent EGF was quantified at t = 0 min from cells transfected with the indicated GFP-tagged RAB10 constructs, and normalized to EGF surface binding of pCMV-transfected cells (ctrl). N = 3 experiments. (**F**) The amount of fluorescent EGF was quantified after 10 min (left) and 30 min (right) upon internalization, and normalized to the amount of Alexa555-EGF binding for each condition at t = 0 min, thus reflecting the percentage of internalized bound fluorescent EGF. N = 3 experiments. All bars represent mean ± s.e.m.*

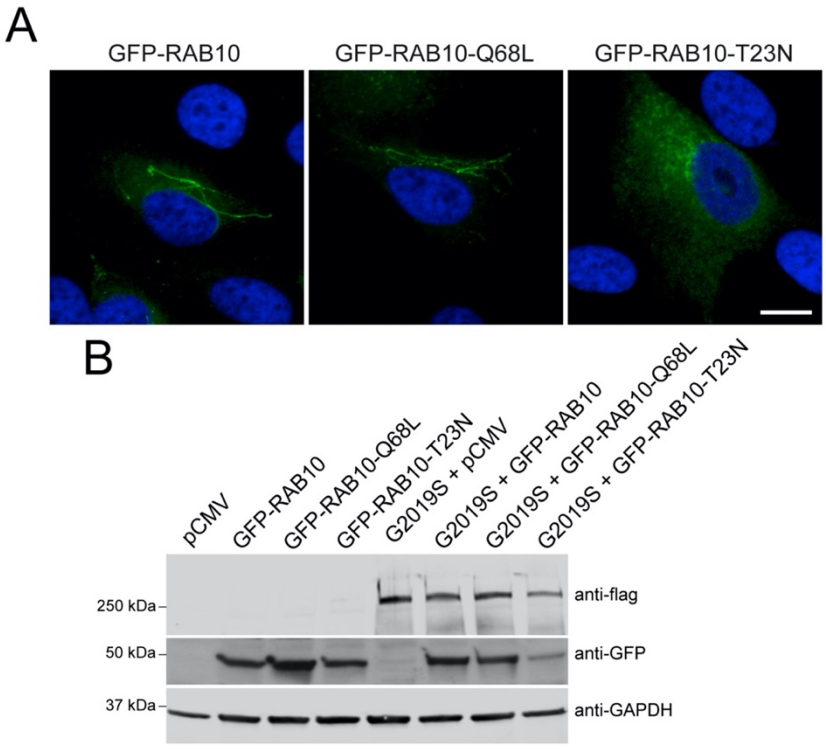

Fig. 14. Subcellular localization and expression levels of RAB10 variants. *(A) HeLa cells were transfected with the different GFP-tagged RAB10 constructs as indicated, and fixed but only briefly permeabilized before mounting with DAPI to maintain proper membrane association. Scale bar, 10 µm. (B) HeLa cells were transfected with empty pCMV vector, with GFP-tagged RAB10 constructs, or cotransfected with flag-G2019S LRRK2 along with GFP or GFP-tagged RAB10 constructs as indicated, and cell extracts (30 µg) analyzed by Western blotting for flag, GFP, and GAPDH as loading control.*

As another means to analyze the effect of RAB10 inactivation on EGF binding and EGFR trafficking, we performed siRNA experiments. Knockdown of RAB10 caused a pronounced decrease in RAB10 protein levels 48 h post-transfection (**Fig. 15A,B**), while the steady-state levels of several other RAB proteins including RAB8A (**Fig. 15A,B**) or RAB7A remained unchanged (**Fig. 16**). The knockdown of RAB10 was accompanied by a significant decrease in EGF surface binding and EGFR degradation (**Fig. 15C,D**). A siRNA-resistant version of RAB10, but not wildtype siRNA-sensitive RAB10, rescued the effect of RAB10 knockdown on EGF binding and EGFR trafficking (**Fig. 15E–G**), indicating that the effects were owing to the specific knockdown of RAB10. Thus, siRNA of RAB10 mimics the endolysosomal trafficking deficits mediated by G2019S LRRK2 expression.

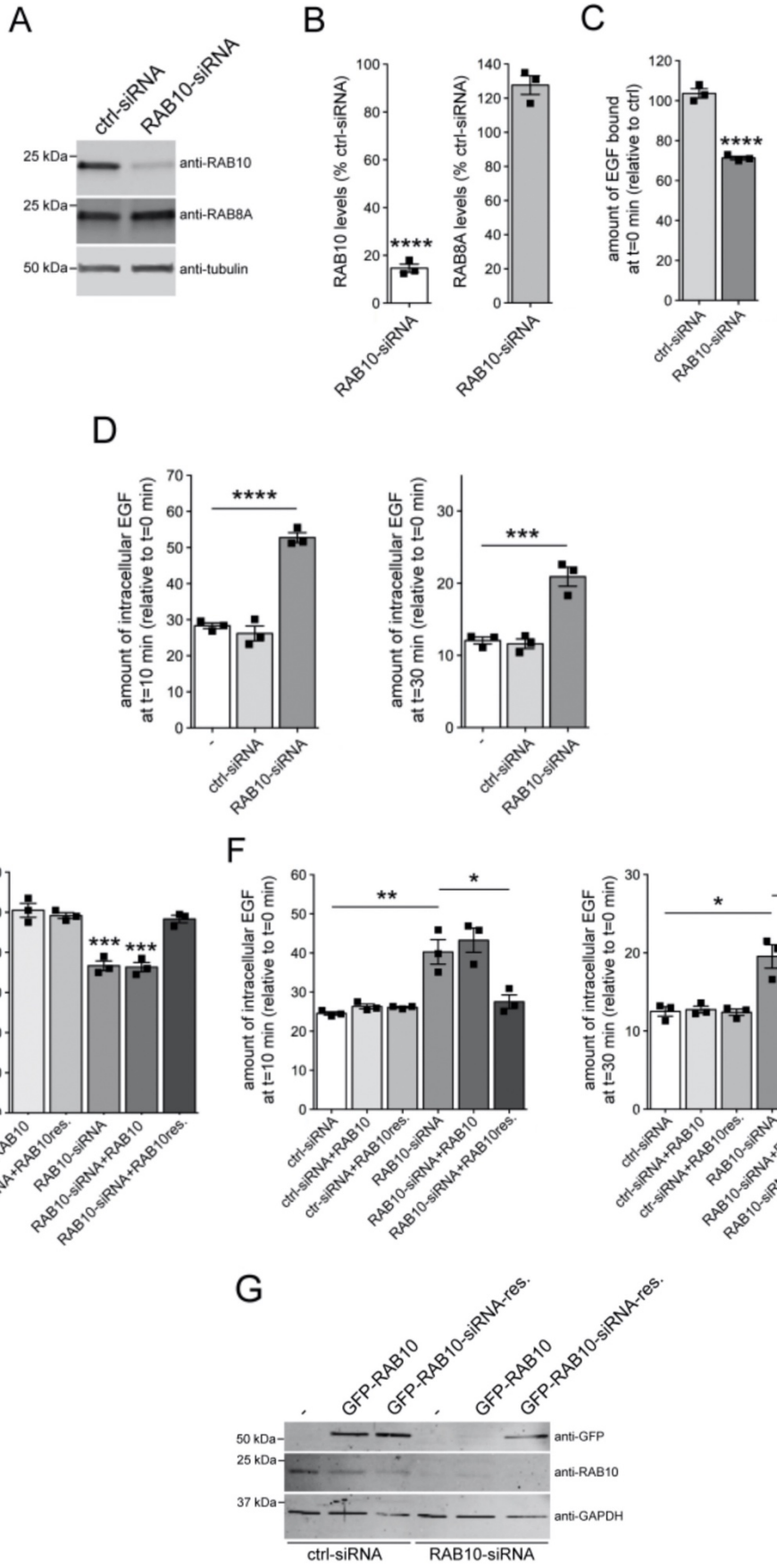
A
ctrl-siRNA
RAB10-siRNA
25 kDa
anti-RAB10
25 kDa
anti-RAB8A
50 kDa
anti-tubulin
B
RAB10 levels (% ctrl-siRNA)
****
RAB10-siRNA
RAB8A levels (% ctrl-siRNA)
RAB10-siRNA
C
amount of EGF bound at t=0 min (relative to ctrl)
****
ctrl-siRNA
RAB10-siRNA
D
amount of intracellular EGF at t=10 min (relative to t=0 min)
****
-
ctrl-siRNA
RAB10-siRNA
amount of intracellular EGF at t=30 min (relative to t=0 min)
***
-
ctrl-siRNA
RAB10-siRNA
E
amount of EGF bound at t=0 min (relative to ctrl)
***
***
ctrl-siRNA+RAB10
ctr-siRNA+RAB10res.
RAB10-siRNA
RAB10-siRNA+RAB10
RAB10-siRNA+RAB10res.
F
amount of intracellular EGF at t=10 min (relative to t=0 min)
**
*
ctrl-siRNA
ctrl-siRNA+RAB10
ctr-siRNA+RAB10res.
RAB10-siRNA
RAB10-siRNA+RAB10
RAB10-siRNA+RAB10res.
amount of intracellular EGF at t=30 min (relative to t=0 min)
*
*
ctrl-siRNA
ctrl-siRNA+RAB10
ctr-siRNA+RAB10res.
RAB10-siRNA
RAB10-siRNA+RAB10
RAB10-siRNA+RAB10res.
G
-
GFP-RAB10
GFP-RAB10-siRNA-res.
-
GFP-RAB10
GFP-RAB10-siRNA-res.
50 kDa
anti-GFP
25 kDa
anti-RAB10
37 kDa
anti-GAPDH
ctrl-siRNA
RAB10-siRNA

Fig. 15. Knockdown of RAB10 mimics the EGF trafficking deficits observed upon G2019S LRRK2 expression. *(A) HeLa cells were either transfected with ctrl-siRNA or RAB10-siRNA, and cell extracts were analyzed by Western blotting for RAB10 protein levels, RAB8A protein levels, or tubulin as loading control. (B) Quantification of RAB10 and RAB8A protein levels in the presence of RAB10-siRNA, normalized to the levels in the presence of ctrl-siRNA. Bars represent mean ± s.e.m. (N = 3 independent experiments; **** p < 0.001). (C) Cells were either left untreated (-), or treated with ctrl-siRNA or RAB10-siRNA, and the amount of surface-bound fluorescent EGF was quantified. N = 3 independent experiments; **** p < 0.001. (D) Cells were either untreated (-), or treated with ctrl-siRNA or RAB10-siRNA, and internalized fluorescent EGF was quantified at 10 min (left) and 30 min (right). N = 3 independent experiments; *** p < 0.005; **** p < 0.001. (E) Cells were either transfected with ctrl-siRNA or RAB10-siRNA in the absence or presence of wildtype or siRNA-resistant GFP-tagged RAB10 as indicated, and the amount of surface-bound fluorescent EGF was quantified. N = 3 independent experiments. *** p < 0.005 (F) Cells were either transfected with ctrl-siRNA or RAB10-siRNA in the absence or presence of wildtype or siRNA-resistant GFP-RAB10, and internalized fluorescent Alexa555-EGF quantified upon 10 min (left) or 30 min (right) of internalization. N = 3 independent experiments. * p < 0.05; ** p < 0.01. (G) Cells were either transfected with ctrl-siRNA or RAB10-siRNA, and with either wildtype or siRNA-resistant GFP-tagged RAB10 as indicated, and cell extracts (30 µg) analyzed by Western blotting for GFP-RAB10 levels, endogenous RAB10 levels, and GAPDH as loading control.*

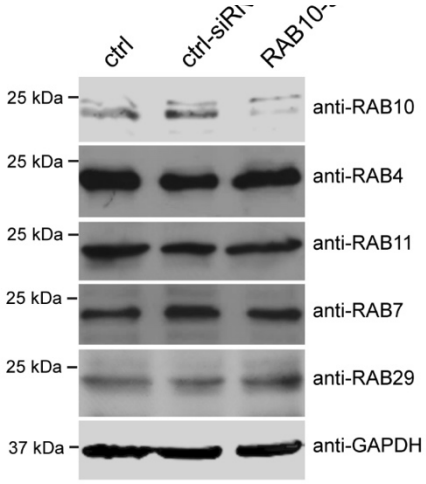

Fig. 16. Specificity of RAB10 knockdown. *HeLa cells were treated with either ctrl-siRNA or RAB10-siRNA, and extracts subjected to Western blotting against various RAB proteins as indicated, with GAPDH as loading control.*

Knockdown of RAB10 Causes a Decrease in RAB7 Activity and Mistargeting of EGF into an RAB4 Compartment

We previously reported that knockdown of RAB8A mimicked the effects of G2019S LRRK2 on endolysosomal trafficking by decreasing the activity of RAB7 (Rivero-Ríos et al., 2019), a crucial regulator of endolysosomal trafficking pathways (Ceresa & Bahr, 2006; Vanlandingham & Ceresa, 2009). The decrease in RAB7 activity upon either G2019S LRRK2 expression or knockdown of RAB8A was associated with the accumulation of EGF in a RAB4-positive endocytic

compartment, and all trafficking deficits were rescued upon expression of active RAB7A (Gómez-Suaga et al., 2014b). Similarly, the deficits in EGF surface binding and EGFR degradation induced upon siRNA of RAB10 were rescued when overexpressing active, GTP-locked RAB7A (RAB7A-Q67L), but not wildtype or GDP-locked RAB7A (RAB7A-T22N) (**Fig. 17A,B**), even though all RAB7A versions were expressed to comparable degrees (**Fig. 17C**). In addition, knockdown of RAB10 was associated with the redistribution of EGF into a vesicular recycling compartment colocalizing with RAB4, which was rescued upon active RAB7A expression (**Fig. 18A,B**).

To gain direct evidence for a decrease in RAB7A activity upon siRNA of RAB10, we performed effector pulldown assays. Cells were treated with either control siRNA or with RAB10-siRNA, and cell lysates were subjected to pulldowns with the RAB7-binding domain of RILP to selectively isolate active, GTP-bound RAB7 (Peralta et al., 2010; Rivero-Ríos et al., 2019). Studies of this type further indicate that the fraction of endogenous active RAB7 was significantly reduced upon RAB10-siRNA (**Fig. 17D,E**). Because either knockdown of RAB8A or of RAB10 phenocopied the effects of pathogenic G2019S LRRK2 expression, we wondered whether these RAB proteins may act in a functionally redundant manner with respect to EGF trafficking and EGFR degradation. Indeed, the decrease in EGF binding and EGFR degradation mediated by knockdown of RAB10 was rescued upon expression of active, but not wildtype or inactive RAB8A (**Fig. 19A,B**). Conversely, the deficits in EGF binding and EGFR degradation upon siRNA of RAB8A were rescued upon expression of active, but not wildtype or inactive RAB10 (**Fig. 19C,D**). Thus, impairing the function of either RAB8A or RAB10 causes endolysosomal trafficking deficits identical to G2019S LRRK2 expression, which are associated with a decrease in RAB7 activity and culminate in the accumulation of EGF in a non-degradative, RAB4-positive recycling compartment.

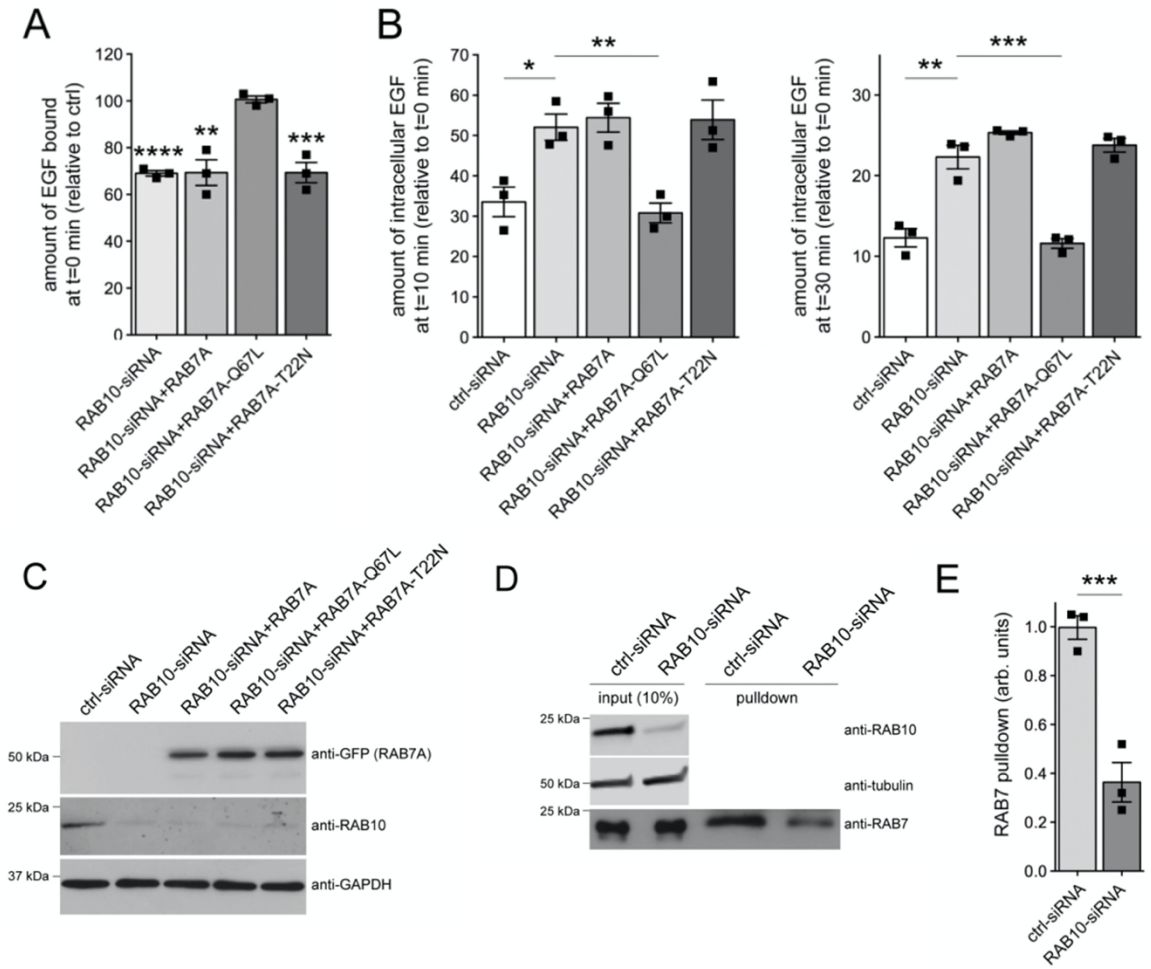

Fig. 17. Knockdown of RAB10 decreases RAB7 activity. *(A) HeLa cells were either transfected with ctrl-siRNA, or with RAB10-siRNA in the absence or presence of GFP-tagged RAB7A constructs as indicated, and surface-bound fluorescent EGF was quantified. N= 3 independent experiments. ** p < 0.01; *** p < 0.005; **** p < 0.001. (B) Cells were either transfected with ctrl-siRNA, or with RAB10-siRNA in the absence or presence of GFP-tagged RAB7A constructs as indicated, and internalized fluorescent EGF was quantified at 10 min (left) and 30 min (right) upon internalization. N = 3 independent experiments. * p < 0.05; ** p < 0.01; *** p < 0.005. (C) Cells were treatedwith ctrl-siRNA or RAB10-siRNA, and transfected with the indicated RAB7A constructs, and cell extracts (30 µg) were analyzed by Western blotting for GFP-RAB7A protein levels, endogenous RAB10 protein levels, and GAPDH as loading control. (D) Cells were either treated with ctrl-siRNA or RAB10-siRNA. The RAB7-binding domain of RILP coupled to GST was used to pull down the GTP-bound form of RAB7 from cell lysates (300 µg), and 10% of input was run alongside pulldowns to show equal levels of total RAB7 protein in ctrl-siRNA or RAB10-siRNA-treated cells. The levels of RAB10 and tubulin were analyzed on a separate gel. (E) Quantification of the type of experiments is depicted in (D), with the amount of RAB7 isolated by GST-RILP expressed relative to input. N = 3 independent experiments. *** p < 0.005..*

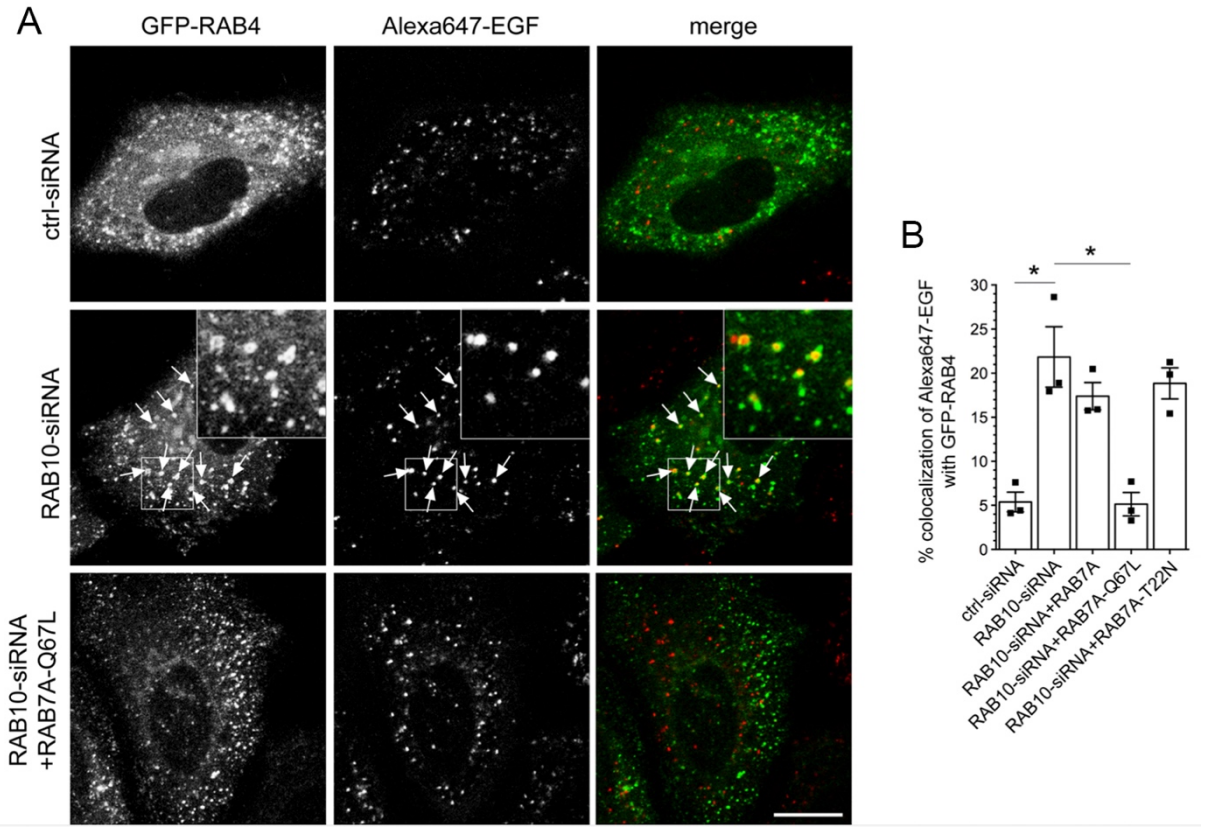

Fig. 18. Knockdown of RAB10 causes accumulation of EGF in a RAB4-positive compartment rescued upon active RAB7A expression. *(A) Example of HeLa cells cotransfected with GFP-RAB4 and either ctrl-siRNA or RAB10-siRNA with or without RAB7A-Q67L expression as indicated. Live pictures were taken 20 min upon EGF internalization, and arrows point to GFP-RAB4-positive vesicles containing Alexa647-EGF. An independent picture (543 HeNe laser line) was acquired to confirm coexpression of the distinct mRFP-tagged RAB7A constructs in all cases. Scale bar, 10 µm. (B) Quantification of the colocalization of Alexa647-EGF with GFP-RAB4 in either ctrl-siRNA or RAB10-siRNA-treated cells, in the absence or presence of the distinct RAB7A constructs as indicated (Manders´coefficient 1 x 100) from around 20 cells per experiment. N=3 independent experiments; *, $p < 0.05$.*

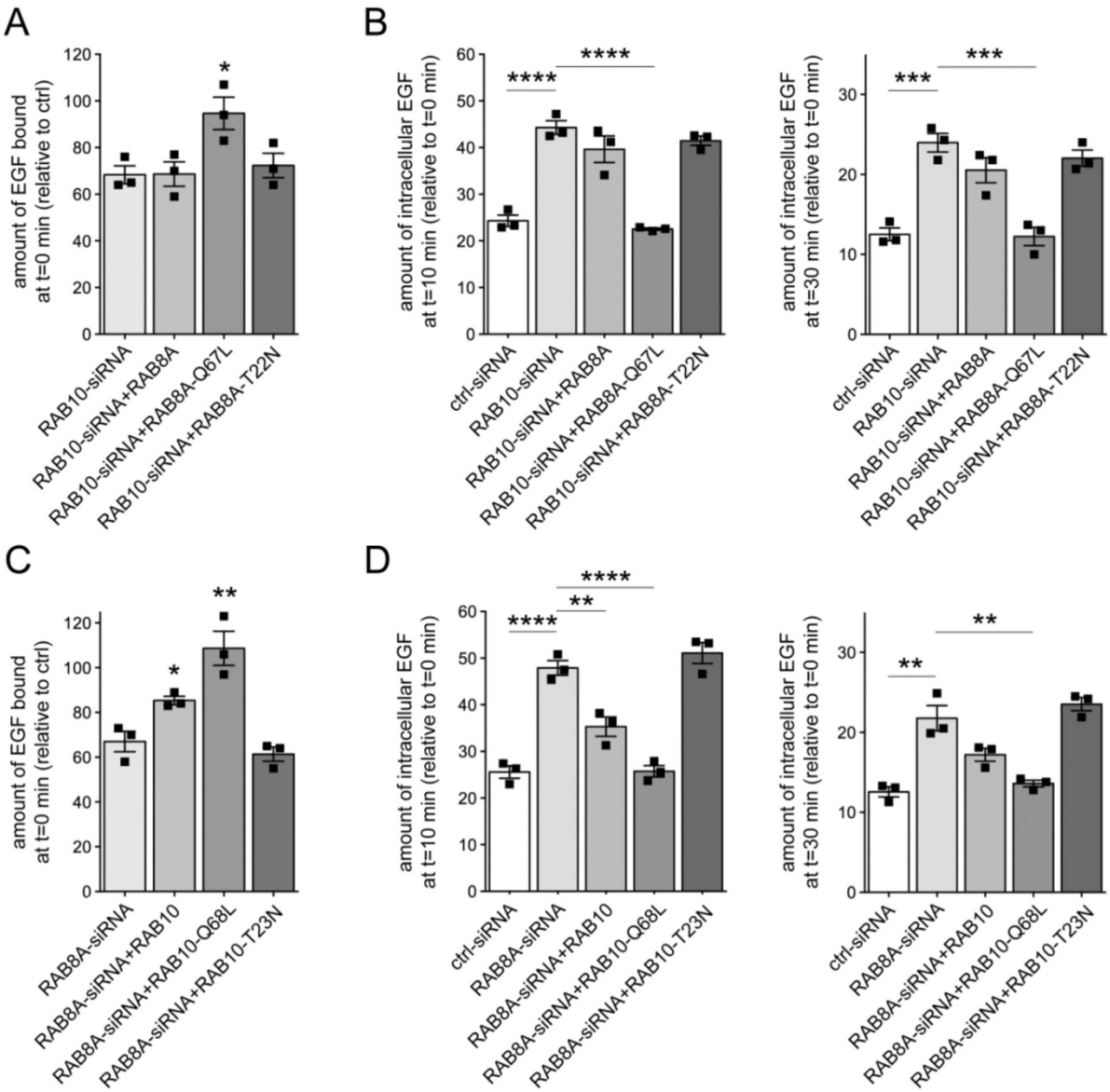

Fig. 19. RAB8A and RAB10 are functionally redundant in regulating endolysosomal EGF trafficking. *(A) HeLa cells were either transfected with ctrl-siRNA or RAB10-siRNA, and co-transfected with GFP-tagged RAB8A constructs as indicated, and the amount of surface-bound fluorescent EGF was quantified. N = 3 independent experiments; * p < 0.05. (B) Cells were transfected with ctrl-siRNA or RAB10-siRNA, and co-transfected with GFP-tagged RAB8A constructs as indicated, and internalized fluorescent EGF was quantified at 10 min (left) and 30 min (right). N = 3 independent experiments; *** p < 0.005; **** p < 0.001. (C) HeLa cells were either transfected with ctrl-siRNA or RAB8A-siRNA, and co-transfected with GFP-tagged RAB10 constructs as indicated, and the amount of surface-bound fluorescent EGF was quantified. N = 3 independent experiments; * p < 0.05; ** p < 0.01. (D) Cells were transfected with ctrl-siRNA or RAB8A-siRNA, and co-transfected with GFP-tagged RAB10 constructs as indicated, and internalized fluorescent EGF was quantified at 10 min (left) and 30 min (right). N = 3 independent experiments; ** p < 0.01; **** p < 0.001.*

G2019S LRRK2-Mediated Endolysosomal Trafficking Defects are Rescued by RAB29 Expression

Previous studies indicate that overexpression of RAB29 causes recruitment of LRRK2 to the trans-Golgi network (TGN), which in turn activates LRRK2 as assessed by RAB10 phosphorylation (Beilina et al., 2014; Z. Liu et al., 2018; Madero-Pérez et al., 2018; Purlyte et al., 2018). Surprisingly, while GFP-tagged RAB29 largely colocalized with a Golgi marker, coexpression of RAB29 with flag-tagged G2019S LRRK2 revealed that this did not result in the efficient recruitment of LRRK2 to the Golgi complex (**Fig. 20A**). We next assessed the effect of RAB29 expression on the pathogenic LRRK2-mediated endolysosomal trafficking deficits. When coexpressed with G2019S LRRK2, RAB29 rescued the LRRK2-mediated decrease in EGF binding and the impairment in EGFR degradation (**Fig. 20B,C**)**.** In contrast, two mutant RAB29 versions previously described to cause RAB29 inactivation (Beilina et al., 2014; Z. Liu et al., 2018; Madero-Pérez et al., 2018) did not rescue the LRRK2-mediated endolysosomal trafficking deficits (**Fig. 20B,C**). Furthermore, expression of the various RAB29 constructs on their own was without effect (**Fig. 20D,E**), even though the RAB29 variants were expressed to similar degrees and did not change expression levels of G2019S LRRK2 (**Fig. 20F**). Finally, wildtype, but not mutant RAB29 versions were also found to rescue the G2019S LRRK2-mediated accumulation of EGF in a RAB4-positive endocytic compartment (**Fig. 21A**).

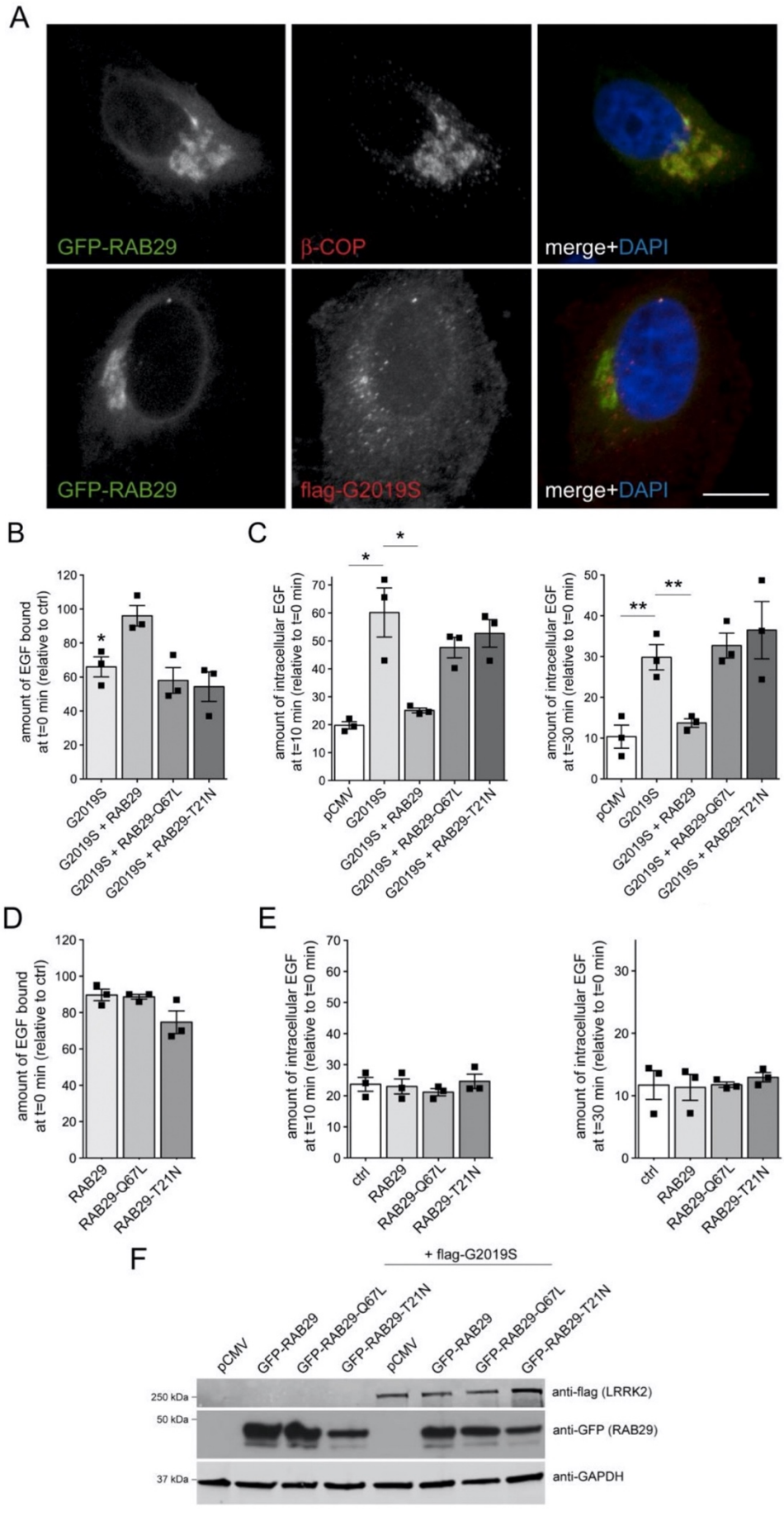
A
GFP-RAB29
β-COP
merge+DAPI
GFP-RAB29
flag-G2019S
merge+DAPI
B
amount of EGF bound at t=0 min (relative to ctrl)
G2019S
G2019S + RAB29
G2019S + RAB29-Q67L
G2019S + RAB29-T21N
C
amount of intracellular EGF at t=10 min (relative to t=0 min)
amount of intracellular EGF at t=30 min (relative to t=0 min)
pCMV
G2019S
G2019S + RAB29
G2019S + RAB29-Q67L
G2019S + RAB29-T21N
D
amount of EGF bound at t=0 min (relative to ctrl)
RAB29
RAB29-Q67L
RAB29-T21N
E
amount of intracellular EGF at t=10 min (relative to t=0 min)
amount of intracellular EGF at t=30 min (relative to t=0 min)
ctrl
RAB29
RAB29-Q67L
RAB29-T21N
F
+ flag-G2019S
pCMV
GFP-RAB29
GFP-RAB29-Q67L
GFP-RAB29-T21N
250 kDa
50 kDa
37 kDa
anti-flag (LRRK2)
anti-GFP (RAB29)
anti-GAPDH

Fig. 20. RAB29 rescues the G2019S LRRK2-mediated EGF trafficking deficits*. (**A**) Top: Example of HeLa cell transfected with GFP-tagged RAB29 using Lipofectamine 2000 (LF2000), and stained with Golgi marker (β-COP) and DAPI. Bottom: Example of HeLa cell co-transfected with GFP-tagged RAB29 and flag-tagged G2019S LRRK2 using LF2000, and stained with flag antibody and DAPI. Scale bar, 10 µm. (**B**) Cells were transfected with either pCMV, or cotransfected with flag-tagged G2019S LRRK2 and GFP or GFP-tagged RAB29 constructs as indicated, and surface-bound fluorescent EGF quantified. N = 3 independent experiments. * p < 0.05. (**C**) Cells were transfected with either pCMV, or cotransfected with flag-tagged G2019S LRRK2 and GFP or GFP-tagged RAB29 constructs, and internalized fluorescent EGF was quantified at 10 min (left) and 30 min (right) upon internalization. N = 3 independent experiments. * p < 0.05; ** p < 0.01. (**D**) HeLa cells were transfected with the indicated GFP-tagged RAB29 constructs using Lipofectamine 2000 (LF2000), and surface-bound fluorescent EGF quantified. N = 3 independent experiments. (**E**) Cells were transfected with the indicated GFP-tagged RAB29 constructs, and internalized fluorescent EGF was quantified at 10 min (left) and 30 min (right) upon internalization. N = 3 independent experiments. (**F**) HeLa cells were transfected with empty pCMV vector, with GFP-tagged RAB29 constructs, or with flag-G2019S LRRK2 along with GFP-tagged RAB29 constructs as indicated, and cell extracts (30 µg) analyzed by Western blotting for flag, GFP, and GAPDH as loading control.*

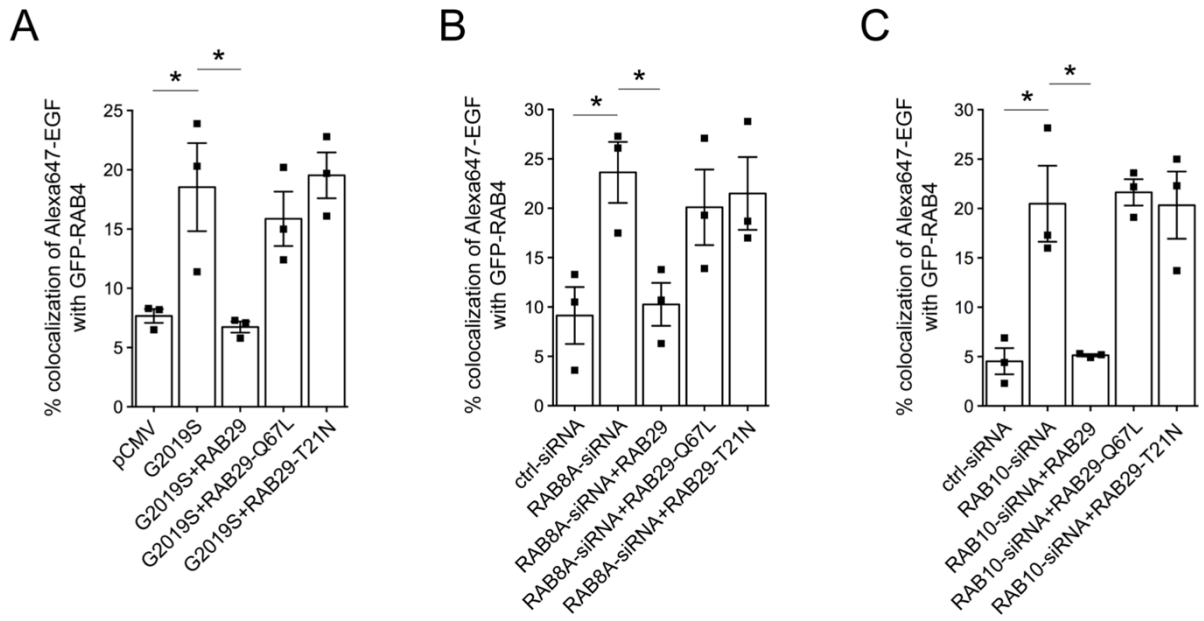

Fig. 21. RAB29 rescues the accumulation of EGF in a RAB4-positive compartment due to G2019S LRRK2 expression, knockdown of RAB8A or knockdown of RAB10. *(A) Quantification of the colocalization of Alexa647-EGF with GFP-RAB4 in flag-tagged G2019S LRRK2-expressing cells in either the absence or presence of mRFP-tagged RAB29 constructs as indicated (Manders´coefficient 1 x 100) from around 20 cells per experiment. N=3 independent experiments; *, p < 0.05. (B) Quantification of the colocalization of Alexa647-EGF with GFP-RAB4 in cells treated with ctrl-siRNA or RAB8A-siRNA in either the absence or presence of mRFP-tagged RAB29 constructs as indicated (Manders´coefficient 1 x 100) from around 20 cells per experiment. N=3 independent experiments; *, p < 0.05. (C) Quantification of the colocalization of Alexa647-EGF with GFP-RAB4 in cells treated with ctrl-siRNA or RAB10-siRNA in either the absence or presence of mRFP-tagged RAB29 constructs as indicated (Manders´coefficient 1 x 100) from around 20 cells per experiment. N=3 independent experiments; *, p < 0.05.*

We reasoned that the previously reported RAB29-mediated recruitment and activation of LRRK2 may be dependent on overexpression levels. Indeed, when employing another transfection reagent to achieve higher expression levels, GFP-RAB29 expression caused a pronounced recruitment of G2019S LRRK2 (**Fig. 22**). Under these conditions, while expression of RAB29 variants on their own still was without effect (**Fig. 23A,B**), co-expression of wildtype RAB29 with G2019S LRRK2 failed to rescue the LRRK2-mediated deficits in EGF binding and EGFR degradation (**Fig. 23C,D**), in contrast to what was observed with low-level RAB29 expression in parallel experiments (**Fig. 23E,F**). Thus, GFP-RAB29 is largely localized to the Golgi complex independent of expression levels. However, and at least as analyzed here, the RAB29-mediated recruitment of LRRK2 to the Golgi complex is only evident upon higher expression levels, and is associated with an impairment of the RAB29-mediated rescue of the endolysosomal trafficking deficits owing to G2019S LRRK2 expression.

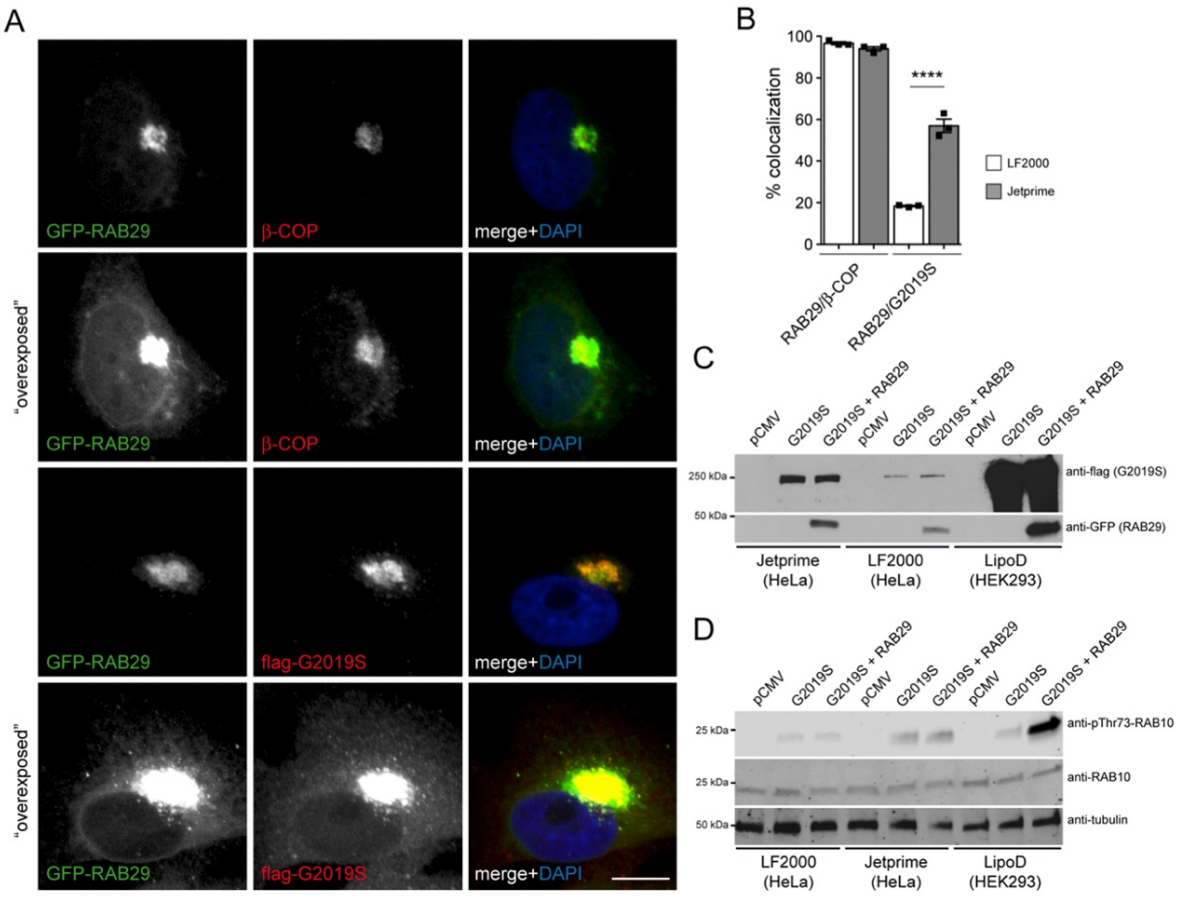

Fig. 22. RAB29 recruits G2019S LRRK2 to the Golgi complex upon higher expression levels. *(**A**) Top: Example of HeLa cell transfected with GFP-tagged RAB29 using Jetprime, and stained with Golgi marker (β-COP) and DAPI. "Overexposed": same cell acquired using confocal settings employed for Lipofectamine 2000 (LF2000)-transfected cells (see Fig. 20A). Bottom: Example of HeLa cell co- transfected with GFP-tagged RAB29 and flag-tagged G2019S LRRK2 using Jetprime, and stained with flag antibody and DAPI. "Overexposed": same cell acquired using confocal settings employed for LF2000-transfected cells (see Fig. 5A). Scale bar, 10 µm. (**B**) Quantification of colocalization of RAB29 with the Golgi marker β-COP, or colocalization of G2019S LRRK2 with RAB29, in cells cotransfected with flag-tagged G2019S LRRK2 and GFP-tagged RAB29 using either LF2000 or Jetprime. One hundred random transfected cells were scored for colocalization per experiment (N=3 independent experiments). ****, p < 0.001. (**C**) HeLa or HEK293 cells were transfected with either empty pCMV, or co-transfected with flag-tagged G2019S LRRK2 and either GFP or GFP-tagged RAB29 using the indicated lipofection reagents, and extracts (30 µg) analyzed for flag-tagged LRRK2 and GFP-RAB29 expression levels as indicated. (**D**) Cell extracts were analyzed for endogenous phospho-RAB10 levels, total RAB10 levels and tubulin as loading control.*

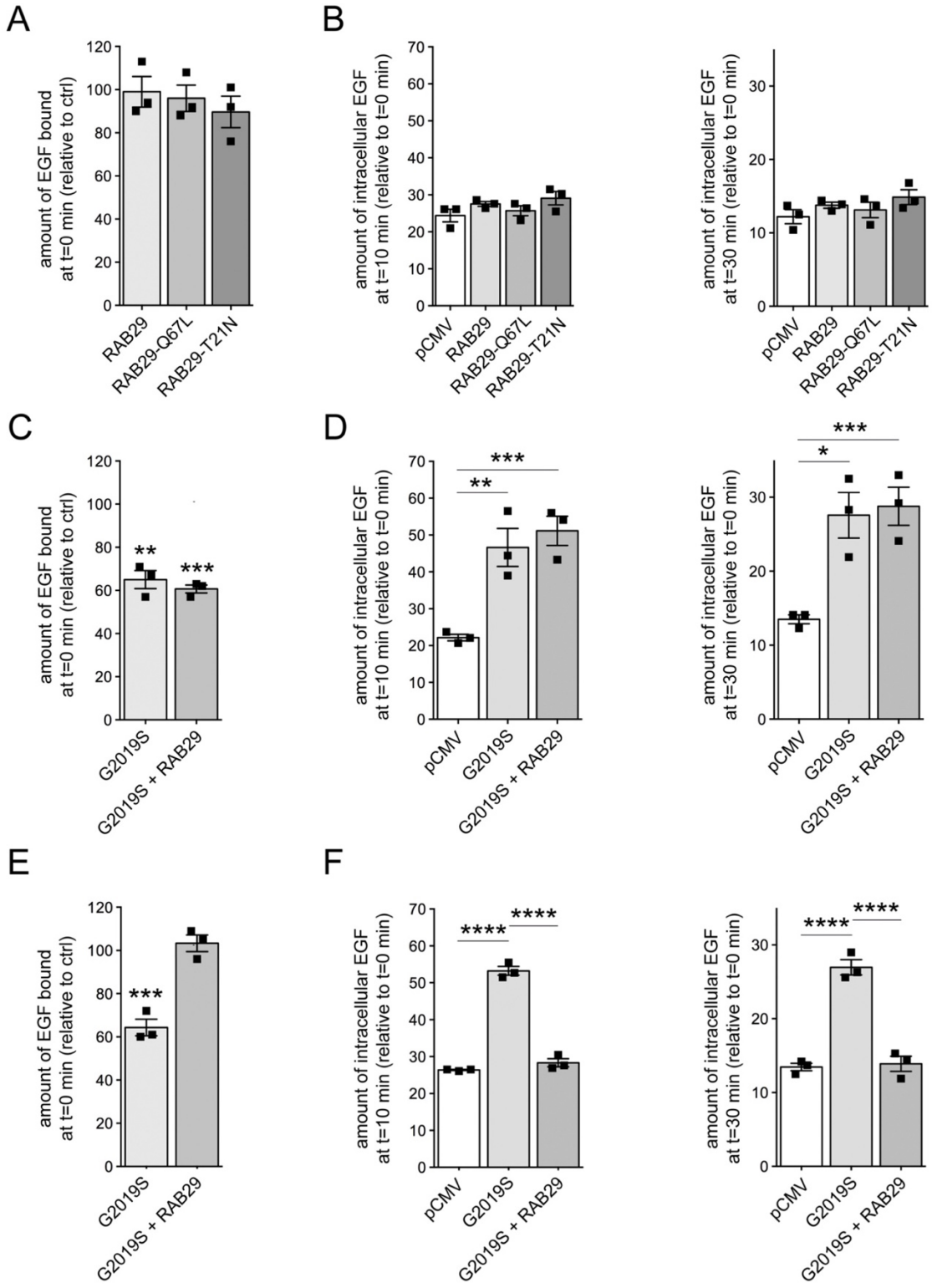

Fig. 23. Rescue of G2019S LRRK2-mediated EGF trafficking deficits by RAB29 is impaired upon higher expression levels. *(**A**) HeLa cells were transfected with pCMV or with the indicated GFP-tagged RAB29 constructs using Jetprime, and surface-bound fluorescent EGF quantified. N=3 independent experiments. (**B**) HeLa cells were transfected with pCMV or with the indicated GFP-tagged RAB29 constructs using Jetprime, and internalized fluorescent EGF was quantified at 10 min (left) and 30 min (right) upon internalization. N=3*

*independent experiments. (**C**) In parallel experiments, HeLa cells were transfected with pCMV, or co-transfected with flag-tagged G2019S LRRK2 and either GFP or GFP-tagged RAB29 using Jetprime, and surface-bound fluorescent EGF quantified. N=3 independent experiments. **, p < 0.01; ***, p < 0.005. (**D**) HeLa cells were transfected with pCMV, or co-transfected with flag-tagged G2019S LRRK2 and either GFP or GFP-tagged RAB29 using Jetprime, and internalized fluorescent EGF was quantified at 10 min (left) and 30 min (right) upon internalization. N=3 independent experiments. *, p < 0.05; **, p < 0.01; ***, p < 0.005. (**E**) In parallel experiments, HeLa cells were transfected with pCMV, or co-transfected with flag-tagged G2019S LRRK2 and either GFP or GFP-tagged RAB29 using LF2000, and surface-bound fluorescent EGF quantified. N=3 independent experiments. ***, p < 0.005. (**F**) HeLa cells were transfected with pCMV, or co-transfected with flag-tagged G2019S LRRK2 and either GFP or GFP-tagged RAB29 using LF2000, and internalized fluorescent EGF was quantified at 10 min (left) and 30 min (right) upon internalization. N=3 independent experiments. ****, p < 0.001.*

RAB29 Expression Rescues the Endolysosomal Trafficking Deficits Mediated by G2019S LRRK2 or Knockdown of Either RAB8A or RAB10

We next assessed whether the LRRK2-mediated endolysosomal trafficking deficits may be owing to RAB29 inactivation. Knockdown of RAB29 was without effect on EGF binding and EGFR degradation (**Fig. 24A-D**), in contrast to what we observed with knockdown of RAB8A (Rivero-Ríos et al., 2019) or RAB10 (**Fig. 15**). Furthermore, transient disruption of the Golgi complex by brefeldin A (BFA) treatment did not alter EGF binding or EGFR trafficking per se (**Fig. 25A–C**), suggesting that the LRRK2-mediated endolysosomal trafficking deficits are neither mimicked by RAB29 inactivation nor dependent on Golgi integrity. Importantly, while BFA treatment caused the perinuclear dispersal of a Golgi marker as well as of GFP-RAB29 (**Fig. 25D,E**), it did not interfere with the RAB29-mediated rescue of the deficits in EGF binding and EGFR degradation owing to the presence of G2019S LRRK2 (**Fig. 25F,G**). Thus, the RAB29-mediated rescue of the trafficking deficits owing to G2019S LRRK2 expression does not require an intact Golgi complex.

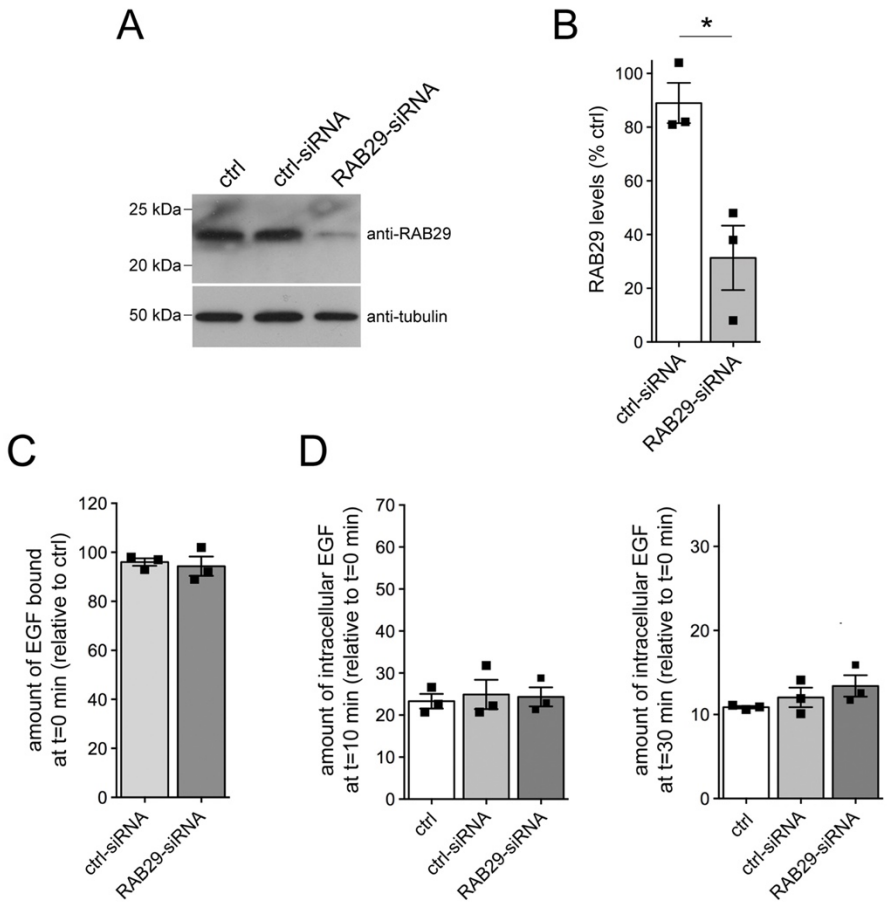

Fig. 24. siRNA-mediated knockdown of RAB29 does not affect EGF trafficking. *(**A**) HeLa cells were either left untreated, transfected with ctrl-siRNA or RAB29-siRNA as indicated, and cell extracts were analyzed by Western blotting for RAB29 levels and tubulin as loading control. (**B**) Quantification of the levels of RAB29 in the presence of ctrl-siRNA or RAB29-siRNA, normalized to the levels in untreated cells. Bars represent mean ± s.e.m. (N=3 independent experiments; *, p < 0.05). (**C**) Cells were either left untreated (ctrl), or treated with ctrl-siRNA or RAB29-siRNA, and the amount of surface- bound fluorescent EGF quantified. N=3 independent experiments. (**D**) Cells were either untreated (ctrl), or treated with ctrl-siRNA or RAB29-siRNA, and internalized fluorescent EGF was quantified at 10 min (left) and 30 min (right). N=3 independent experiments.*

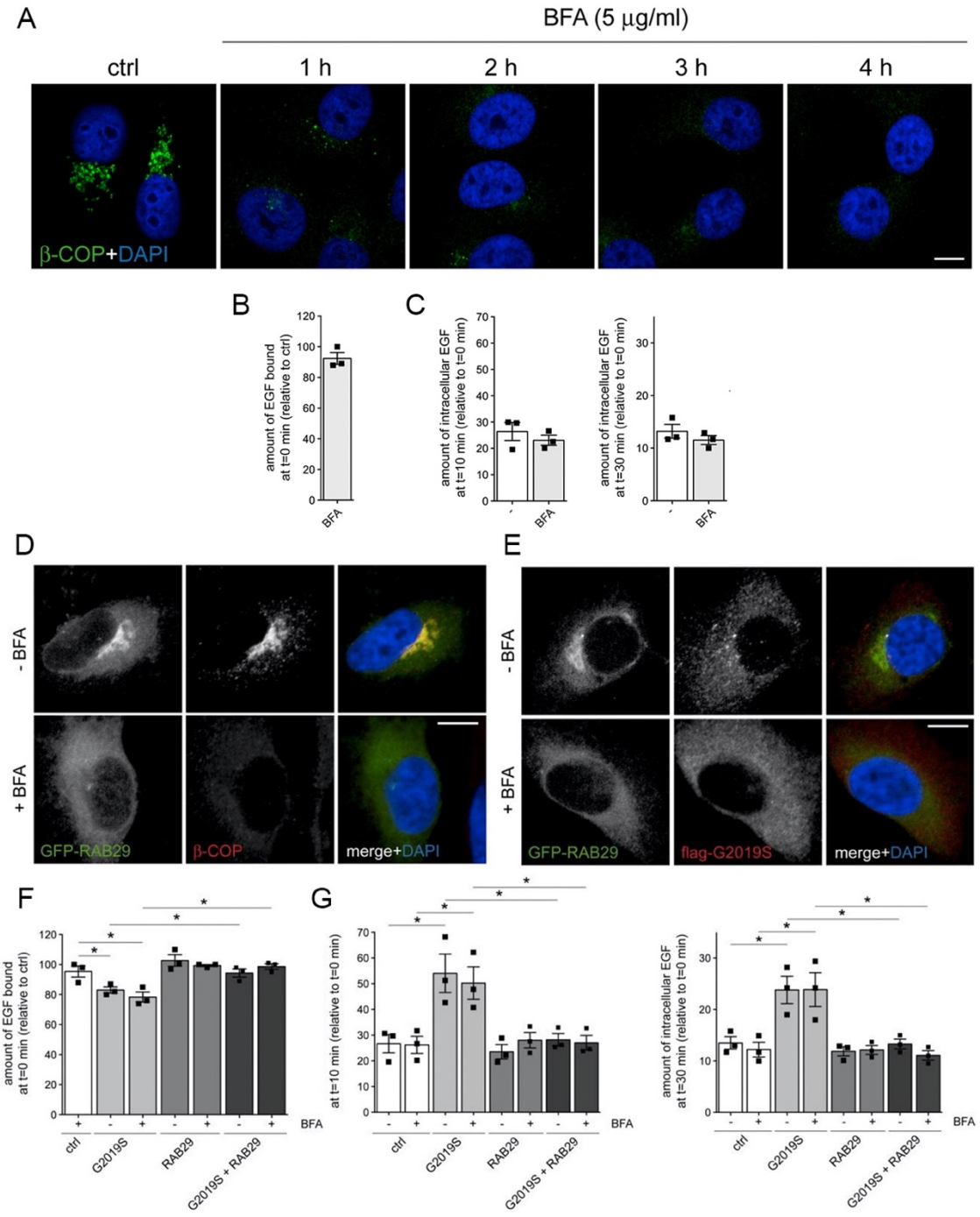

Fig. 25. The G2019S LRRK2-mediated deficit in EGF trafficking and its rescue upon RAB29 expression are independent of Golgi integrity. *(A) Example of HeLa cells either treated with vehicle (ctrl) or with brefeldin A (BFA) (5 µg/ml) for the indicated amounts of time, followed by staining with a Golgi marker (β-COP) and DAPI. Scale bar, 10 µm. (B) Hela cells were either left untreated, or treated for 2 h with BFA as indicated, and the amount of surface-bound fluorescent EGF quantified. N=3 independent experiments. (C) Hela cells were either left untreated, or treated for 2 h with BFA as indicated, and internalized fluorescent EGF was quantified at 10 min (left) and 30 min (right) upon internalization. N=3 independent experiments. (D) Example of HeLa cell transfected with GFP-RAB29 using Lipofectamine 2000 (LF2000), and stained for β-COP and DAPI in either the absence or*

*presence of BFA treatment (5 μg/mL, 2 h) as indicated. Scale bar, 10 μm. (E) Example of HeLa cell cotransfected with GFP-RAB29 and flag-tagged G2019S LRRK2 using LF2000, and stained for flag and DAPI in either the presence or absence of BFA treatment (5 μg/mL, 2 h) as indicated. Scale bar, 10 μm. (F) HeLa cells were either transfected with pCMV (ctrl), flag-tagged G2019S LRRK2, GFP-tagged RAB29, or cotransfected with flag-tagged G2019S LRRK2 and GFP-tagged RAB29 using LF2000 as indicated, and either treated with or without BFA (5 μg/mL, 2 h) before determination of the amount of surface-bound fluorescent EGF. N = 3 independent experiments; * p < 0.05. (G) HeLa cells were either left untreated (ctrl), transfected with flag-tagged G2019S LRRK2, GFP-tagged RAB29, or cotransfected with flag-tagged G2019S LRRK2 and GFP-tagged RAB29 using LF2000, either treated with or without BFA (5 μg/mL, 2 h) as indicated, and internalized fluorescent EGF was quantified at 10 min (left) and 30 min (right) upon internalization. N = 3 independent experiments. * p < 0.05.*

We reasoned that RAB29 may play additional roles in membrane trafficking apart from those described at the Golgi complex, possibly overlapping with those of either RAB8A and/or RAB10. Indeed, expression of wildtype, but not inactive RAB29 variants rescued the deficits in EGF binding and EGFR degradation induced upon knockdown of either RAB10 (**Fig. 26A–C**) or RAB8A (**Fig. 26D–F**), with the RAB29 variants expressed to similar degrees (**Fig. 26C,F**). Expression of wildtype RAB29 also reverted the accumulation of EGF in a RAB4-positive endocytic compartment induced upon knockdown of either RAB10 or RAB8A (**Fig. 21B,C**). Finally, the trafficking deficits mediated by expression of a dominant-negative RAB7A mutant were rescued by RAB29 expression (**Fig. 26G-I**), as previously described for active RAB8A (Rivero-Ríos et al., 2019). These data indicate that RAB29 can rescue the endolysosomal trafficking deficits induced by either G2019S LRRK2 expression or by knockdown of RAB8A or RAB10 in a manner independent of its localization/function at the Golgi complex.

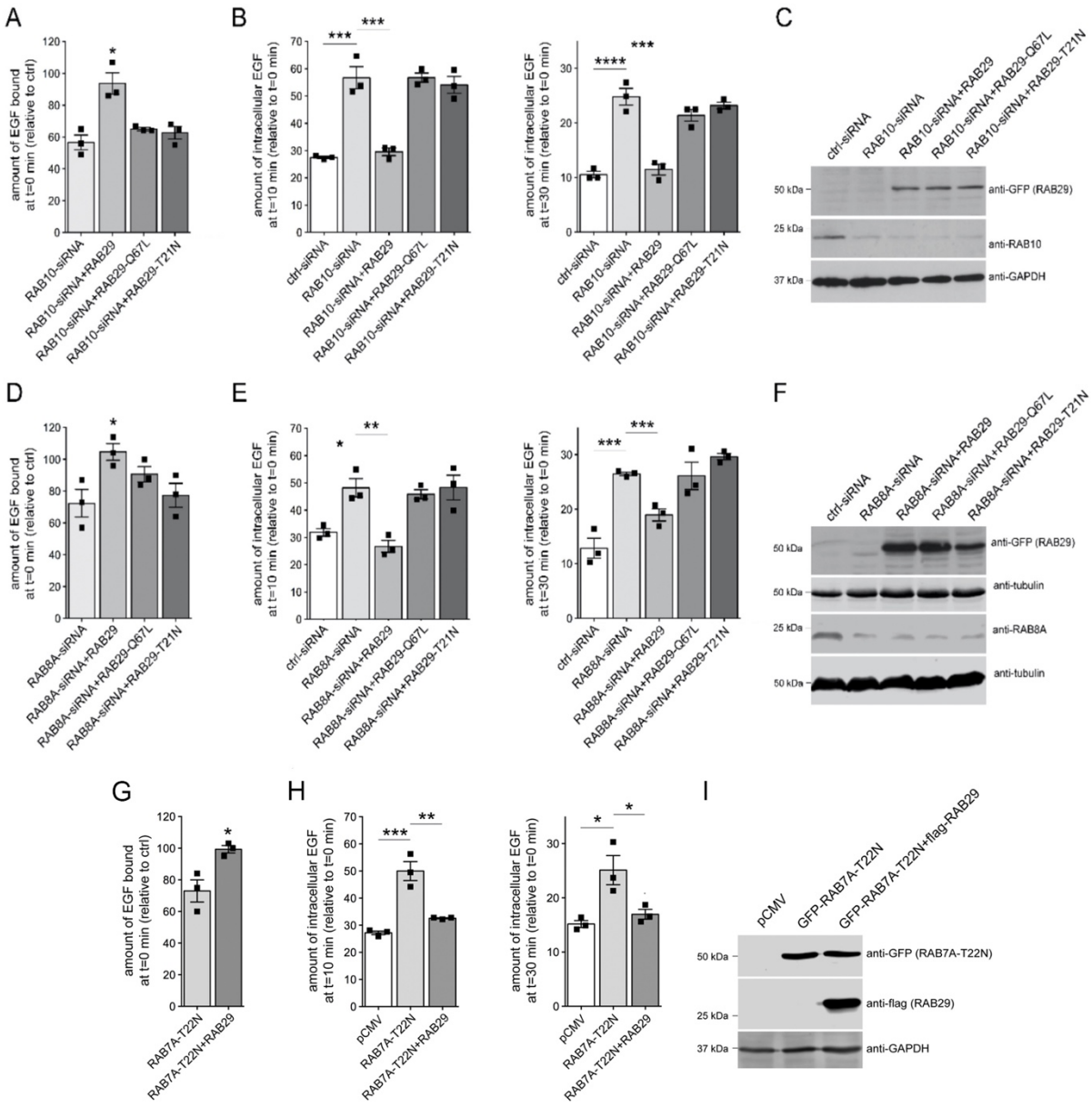

Fig. 26. Deficits in EGF trafficking mediated by knockdown of RAB8A or RAB10 or dominant-negative RAB7A expression are rescued upon RAB29 expression. *(**A**) HeLa cells were either transfected with ctrl-siRNA or RAB10-siRNA, and cotransfected with GFP-tagged RAB29 constructs as indicated, and the amount of surface-bound fluorescent EGF quantified. N = 3 independent experiments; * p < 0.05. (**B**) Cells were transfected with ctrl-siRNA or RAB10-siRNA, and cotransfected with GFP-tagged RAB29 constructs as indicated, and internalized fluorescent EGF was quantified at 10 min (left) and 30 min (right). N = 3 independent experiments; *** p < 0.005; **** p < 0.001. (**C**) Cells were either transfected with ctrl-siRNA or RAB10-siRNA, and transfected with GFP-tagged RAB29 constructs as indicated, and cell extracts (30 µg) were analyzed by Western blotting for GFP-RAB29 levels, endogenous RAB10 levels, and GAPDH as loading control. (**D**) HeLa cells were either transfected with ctrl-siRNA or RAB8A-siRNA, and cotransfected with GFP-tagged RAB29 constructs as indicated, and the amount of surface-bound fluorescent EGF was quantified. N = 3 independent experiments; * p < 0.05. (**E**) Cells were transfected with ctrl-siRNA or RAB8A-siRNA, and cotransfected with GFP-tagged RAB29 constructs as indicated, and internalized fluorescent EGF was quantified at 10 min (left) and 30 min (right). N = 3 independent experiments; * p < 0.05; ** p < 0.01; *** p*

*< 0.005. (**F**) Cells were either transfected with ctrl-siRNA or RAB8A-siRNA, and transfected with GFP-tagged RAB29 constructs as indicated, and cell extracts (30 µg) were analyzed by Western blotting for GFP-RAB29 levels, endogenous RAB8A levels, and tubulin as loading control. (**G**) HeLa cells were transfected with either empty pCMV vector, or dominant-negative GFP-tagged RAB7A (RAB7A-T22N) in the presence or absence of flag-tagged RAB29, and surface-bound fluorescent EGF was quantified. N=3 independent experiments. *, p < 0.05. (**H**) HeLa cells were transfected with the indicated constructs, followed by quantification of internalized fluorescent EGF at 10 (left) and 30 min (right). N=3 independent experiments. *, p < 0.05; **, p < 0.01; ***, p < 0.005. (**I**) HeLa cells were transfected with the indicated constructs, and cell extracts (30 µg) were analyzed by Western blotting for GFP-tagged RAB7A-T22N, flag-tagged RAB29, and GAPDH as loading control.*

DISCUSSION

The present work provides evidence for novel roles for RAB10, a major LRRK2 kinase substrate and RAB29, a highly relevant protein reported to interact with LRRK2, in the endolysosomal EGFR trafficking deficits mediated by pathogenic LRRK2 expression. RAB10 knockdown mimics the previously described endolysosomal trafficking defects of the EGFR mediated by G2019S LRRK2 expression or RAB8A knockdown. This is associated with a decrease in RAB7 activity, and results in the accumulation of the EGFR in a RAB4-positive recycling compartment. In addition, active RAB10 expression rescues the RAB8A knockdown-mediated trafficking impairment of the EGFR and vice versa, pointing towards a functional redundancy between these two LRRK2 substrates. RAB29 expression also is able to rescue the endolysosomal trafficking defects of the EGFR mediated by G2019S LRRK2, even upon disruption of Golgi integrity, highlighting a potential Golgi-independent novel role for this Rab GTPase in endocytic and endolysosomal trafficking steps.

Role of RAB10 in endolysosomal trafficking defects of the EGFR mediated by pathogenic LRRK2

We find here that knockdown of RAB10 mimics the previously described endolysosomal defects in internalization, transport and degradation of the EGFR mediated by G2019S LRRK2 expression or RAB8A knockdown (**Fig. 27**; Rivero-Ríos et al., 2019), and that expression of active RAB10 rescues these trafficking defects. The defects mediated by RAB10 silencing result in the accumulation of the EGFR in a RAB4-positive early recycling compartment as well as a decrease in RAB7 activity, as previously shown for G2019S LRRK2 expression or RAB8A loss. In addition, expression of active RAB8A rescues the EGFR trafficking impairment caused by RAB10 knockdown and vice versa.

These findings are consistent with a model in which pathogenic LRRK2 mediates the endolysosomal trafficking deficits through RAB8A and/or RAB10 phosphorylation, causing their subsequent inactivation and impeding their proper function for the membrane trafficking pathways they are normally involved in. As explained in previous sections, the LRRK2-mediated phosphorylation of RAB GTPases occurs in their highly conserved switch II domains, which are crucial for RAB proteins to interact with their effectors (Zhen & Stenmark, 2015). Therefore, phospho-RABs are expected to have a compromised ability to establish such interactions and thereby support the specific vesicular trafficking pathways which they normally support.

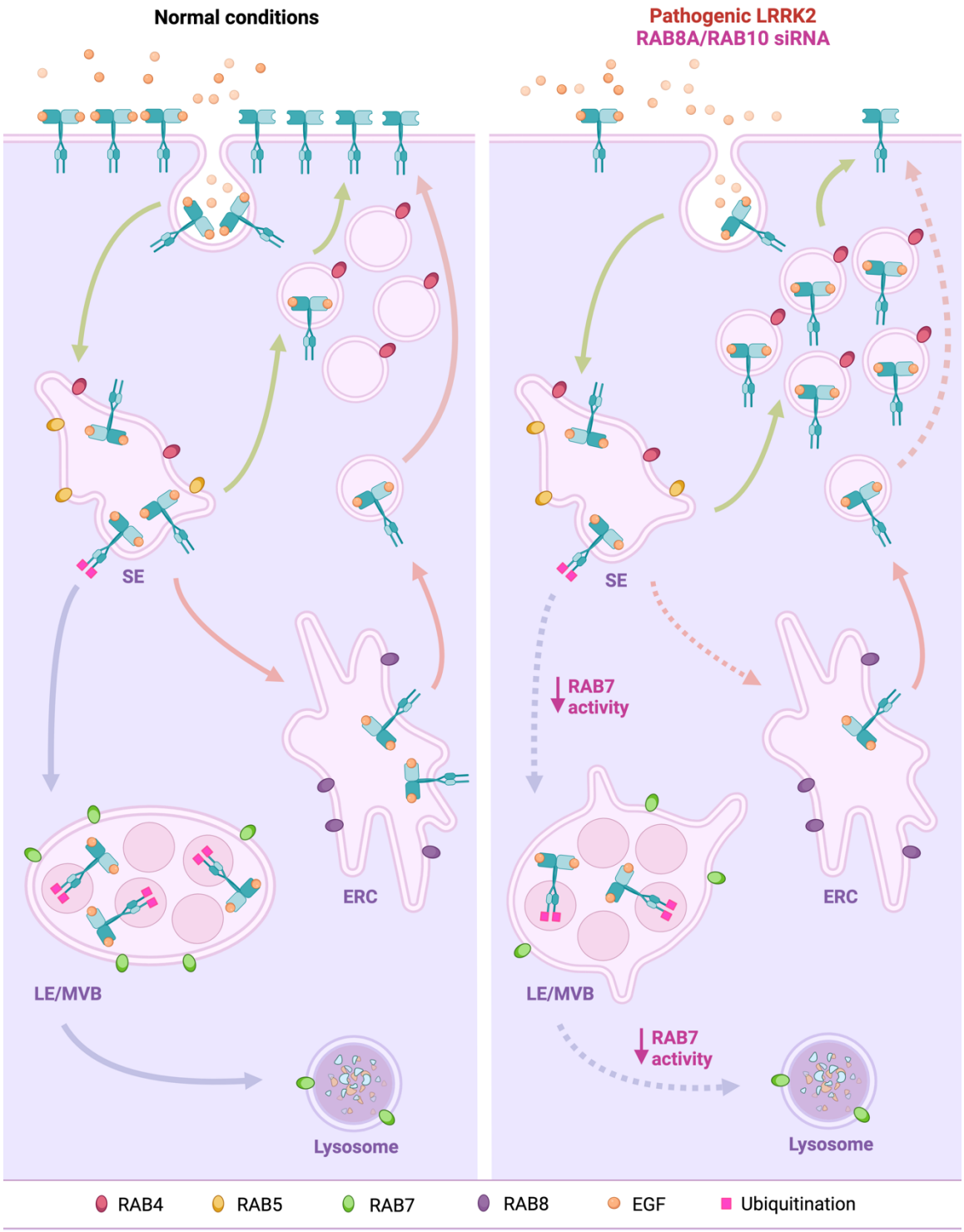

Fig. 27. Schematic model of trafficking deficits mediated by pathogenic LRRK2 or knockdown of RAB10. *Left: Under normal conditions, EGFR is endocytosed and then sorted either to LE/MVB and later to the lysosome to be degraded, or it can be recycled back to the PM via fast RAB4-mediated (green arrows) or slow RAB11-mediated (orange arrows) recycling. Right: Pathogenic LRRK2 expression or knockdown of RAB10 decrease RAB7 activity, causing deficits in the endolysosomal degradative pathway (purple arrows) including transport from SE to LE/MVB and then towards lysosomes.. LRRK2-mediated phosphorylation or knockdown of RAB8A/RAB10 also hinders trafficking to/from the ERC, which results in the accumulation of the EGFR in a RAB4-positive recycling compartment. SE, sorting endosome; LE/MVB, late endosome/multivesicular body; and ERC, early recycling compartment.*

To more directly test the implication of RAB10 phosphorylation in the described endolysosomal trafficking deficits, it would be interesting to perform expression studies with phosphomimetic (RAB10-T73E) and phosphodeficient (RAB10-T73A) variants to determine if, according to the proposed model, phosphodeficient Rab10 would rescue the pathogenic phenotypes while phosphomimetic Rab10 would fail to do so. However, these RAB10 variants have been shown not to be properly localized but rather to be mainly cytosolic (Dhekne et al., 2018), which is why we instead chose the specific knockdown approach here. Transfection efficiencies in our HELA cell model are modest. Therefore, even under pathogenic LRRK2 overexpression conditions, RAB phosphorylation levels are low, which is why we did not pursue western blotting with phospho-state-specific antibodies. In future, it will be important to confirm if the endolysosomal trafficking defects studied here correlate with the phosphorylation of RAB8A and RAB10, for example in cell lines stably expressing pathogenic LRRK2. However, in previous work we have shown that expression of G2019S LRRK2 as well as other pathogenic LRRK2 variants cause an increase in RAB phosphorylation in easily transfectable HEK293T cells (Lara Ordónez et al., 2019), including RAB8A and RAB10. These findings, together with the previously reported reversal of the EGFR trafficking phenotypes by specific LRRK2 kinase inhibitors (Rivero-Ríos et al., 2019), point towards a LRRK2-phosphorylation-mediated inactivation of its RAB GTPase substrates which then causes the trafficking deficits as shown here.

Our data also provide evidence that RAB8A and RAB10 play redundant roles for the endolysosomal trafficking and degradation pathways of the EGFR. In agreement with these findings, previous studies have shown that these two GTPases colocalize in a perinuclear tubular endocytic recycling compartment (Babbey et al., 2010; Etoh & Fukuda, 2019; Lara Ordónez et al., 2019) and are both implicated in certain vesicular trafficking pathways such as polarized transport, ciliogenesis and neurite outgrowth-related vesicular transport (Homma & Fukuda, 2016; Sato et al., 2014; Schuck et al., 2007; Tao et al., 2019). In future, it will be interesting to determine whether RAB8A and RAB10 also play redundant roles in other, disease-relevant cell models such as in dopaminergic neurons, primary cultured astrocytes or peripheral patient-derived cells. Indeed, functional redundancy of RAB8 and RAB10 has not been observed in all cell types. For instance, in a mouse model, RAB10 knockout has been shown to result in early embryonic lethality, which indicates that RAB8A is unable to compensate for an essential role of RAB10 (Lv et al., 2015). In addition, mice double-knockout for RAB8A/B show impaired apical transport which cannot be compensated by RAB10. At the same time, cells deficient in RAB8A/B show no alterations in cilia morphology, but additional knockdown of RAB10 causes a significant decrease in the

percentage of ciliated MEF cells (Sato et al., 2014). These findings suggest that apart from potential cell type specificity, the functional redundancy between RAB8 and RAB10 may be limited to certain vesicular trafficking steps such as ciliogenesis. In a similar way, our findings show that RAB8 and RAB10 play redundant roles for the EGFR trafficking pathways as assessed in HELA cells.

Crosstalk between endocytic recycling and endolysosomal degradation modulated by pathogenic LRRK2

The endolysosomal trafficking deficits of the EGFR mediated by pathogenic LRRK2 expression or by silencing of either RAB8A or RAB10 ultimately result in the accumulation of the EGFR in a RAB4-positive early recycling compartment. This provides evidence for a crosstalk between recycling and degradative trafficking pathways, in agreement with various previous studies (Braun et al., 2015; Goueli et al., 2012; McCaffrey et al., 2001). These studies often have combined analysis of EGFR trafficking with that of transferrin receptor (TfR) trafficking. The TfR is required for delivery of iron into cells. Upon binding to the Fe3+-transferrin complex, the TfR is endocytosed to deliver Fe3+ to endosomes, and the TfR-apotransferrin complex is recycled back to the PM. Given its importance in iron metabolism which is crucial for cell homeostasis, the TfR plays key roles in various diseases such as cancer, autoimmune or neurodegenerative diseases. Importantly, the TfR follows similar recycling pathways as those described for the EGFR, including fast recycling through a RAB4 compartment (Daro et al., 1996; Ullrich & Molecular, 1996). For example, studies have shown that expression of dominant-negative (GDP-locked) RAB4 leads not only to a significant decrease of recycling and degradation of the EGFR, but also causes morphological alterations of transferrin-positive recycling compartments in HELA cells (McCaffrey et al., 2001). In addition, the expression of TBC1D16, which acts as a RAB4 GAP, results in a reduction of TfR recycling back to the PM and abnormal EGFR trafficking (Goueli et al., 2012). Finally, interfering with the integrity of the RAB8-positive early recycling compartment impairs both TfR transport to the ERC and degradation of the EGFR (Braun et al., 2015). Altogether, these data indicate a crosstalk between the endocytic recycling and endolysosomal degradation pathways as relevant to both EGFR and TfR trafficking and involving the RAB4-positive fast recycling compartment.

Alterations in iron metabolism are of considerable interest in relation to PD pathogenesis. A variety of studies have shown increased total iron levels in the SN in PD

patients as compared to age-matched healthy control patients (R. J. Ward et al., 2014), and alterations in ferritin and TfR levels in neural exosomes may serve as potential biomarkers for PD diagnosis (Z.-T. Chen et al., 2023). Intracellular iron overload has been shown to correlate with endolysosomal defects which correspond to abnormal autophagy, resulting in higher levels of cytosolic oxidative stress and cell death in different cell models, which suggest an important role of iron in PD pathogenesis (Fernández et al., 2016). However, mechanisms underlying abnormal iron accumulation still remain mostly unclear, highlighting the importance of investigating the TfR trafficking dynamics in a PD-relevant cellular context. Interestingly, a recent study shows that in iPSC-derived microglia from G2019S LRRK2 PD patients, the TfR is mislocalized to perinuclear RAB8-positive lysosomes in response to proinflammatory stimuli. In addition, G2019S LRRK2 knock-in mice show augmented iron deposition in microglia upon intrastriatal LPS injection (Mamais et al., 2021). These data point towards a role for pathogenic LRRK2 in regulating TfR-mediated iron endocytosis which is altered in a PD proinflammatory context, ultimately causing iron accumulation. Therefore, in future experiments it will be interesting to determine whether pathogenic LRRK2 also impairs recycling of the TfR, possibly by causing its accumulation in a RAB4-positive recycling compartment similar to what we observed with the EGFR, and in this manner cause intracellular iron accumulation which contributes to PD pathogenesis.

Here, we used the well-characterized EGFR to study the effects of G2019S LRRK2 on endocytic recycling and endolysosomal trafficking. Apart from the TfR, other disease-relevant receptors and transporters also recycle through a RAB4-positive-compartment, including the glutamate transporter 1 (GLT1). Extracellular clearance of glutamate is also relevant to PD, as glutamate transporter 1 (GLT1) downregulation causes the appearance of PD phenotypes such as astrocytic reactivity, motor deficits and neurodegeneration in mouse and rat models (Ren et al., 2022; Y. Zhang et al., 2020). Recent studies indicate trafficking deficits of the astrocytic GLT1 in primary astrocytes from G2019S LRRK2 knock-in mice (Iovino et al., 2022). The authors show that the pathogenic LRRK2 kinase activity delays GLT1 recycling back to the PM by causing its accumulation in an enlarged RAB4-positive compartment, likely mediated by enhanced RAB8A and RAB10 phosphorylation. This results in redirecting part of the GLT1 to the lysosomal compartment for degradation, and a consequent decrease in GLT1 levels in brains from both G2019S LRRK2 knockin mice and postmortem brain extracts from G2019S LRRK2 PD patients (Iovino et al., 2022). These findings indicate that mechanisms similar to those observed for the trafficking of the EGFR mediated by pathogenic LRRK2 as described in this thesis may be applicable to disease-relevant cell types and receptors/transporters.

Indeed, a prediction from our present results is that pathogenic LRRK2 may affect trafficking of any receptor or transporter which undergoes Rab4-mediated recycling events, which includes the D2 dopamine receptor (Y. Li et al., 2012). This receptor can follow either a slow RAB11-mediated dopamine activity-dependent recycling pathway or a fast RAB4-mediated constitutive recycling pathway, which maintains steady-state surface expression levels of the D2 receptor. Therefore, it would be of interest to analyze if pathogenic LRRK2 also causes defects in D2 DA receptor trafficking, leading to a decrease in surface levels and its accumulation in a RAB4 compartment. This would be in consonance with studies showing abnormal intracellular D2 accumulation in SH-SY5Y cells upon pathogenic LRRK2 expression (Rassu et al., 2017). Consistent with this possibility, D2 receptor agonists have a neuroprotective effect with respect to synaptic activity in G2019S LRRK2 knock-in mouse models (Tozzi et al., 2018). highlighting the possibility that D2 receptor recycling may be affected in a similar way as EGFR recycling,

Lastly, the G protein-coupled AT1 angiotensin II receptor (AGTR1) is another receptor of interest in a PD context which undergoes both RAB11- and RAB4-positive-compartment-dependent recycling (Hunyady et al., 2002). AGTR1 has been recently evidenced as a potentially relevant receptor in a PD context, since its expression levels are specifically upregulated in a population of DA neurons at the ventral SNc, which are the most susceptible to neurodegeneration in PD (Kamath et al., 2022). Therefore, it would be interesting to analyze the endolysosomal trafficking dynamics of AGTR1 in DA neurons expressing pathogenic LRRK2, since altered degradative and/or recycling trafficking of this receptor may underlie the observed alterations in protein levels, possibly as a compensatory effect.

Decreased RAB7 activity mediated by pathogenic LRRK2

As determined by a pulldown approach, the levels of GTP-bound or active RAB7 were found to be decreased upon G2019S LRRK2 expression or RAB8A or RAB10 silencing. RAB7A is known to be implicated in several endolysosomal system-related functions including trafficking from LE/MVB to the lysosome, late endosome maturation and lysosome reformation. Thus, decreasing RAB7 activity will have several consequences for endolysosomal trafficking. However, RAB7 is not a substrate for the LRRK2 kinase activity neither in vitro nor in intact cells (Martin Steger et al., 2016), suggesting that pathogenic LRRK2 expression causes a reduction in RAB7 activity in an indirect manner.

In order to regulate membrane identity of vesicular trafficking compartments as well as cargo directionality, RAB GTPases are often implicated in a complex feedback mechanism named RAB cascades. In this process, the upstream RAB protein can recruit and interact with a specific effector that acts at the same time as a GEF or activator for the downstream RAB protein. One of the best studied RAB cascades is the RAB5/RAB7 cascade also called RAB switch, responsible for regulating endosome maturation, in which RAB5 has been proven necessary and sufficient to activate RAB7 in a GEF-dependent manner (Langemeyer et al., 2020). Therefore, it would be of interest to determine whether a similar RAB cascade acts as a regulatory mechanism underlying the decrease in RAB7 activity, perhaps associated with RAB8A and RAB10 inactivation.

In previous work we showed that pathogenic LRRK2 expression caused a decrease in the GTP-bound active fraction of RAB7 as addressed by effector pulldown experiments, but no change in the total protein levels of RAB7. We also reported that pathogenic LRRK2 caused defects in endolysosomal trafficking by delaying the RAB5/RAB7 switch at the endosome which resulted in the formation of RAB7-positive tubules and an abnormal morphology of the LE (Gómez-Suaga et al., 2014a). Furthermore, expression of the RAB7 GAP or inactivating protein TBC1D15 mimicked this phenotype, and defects were rescued when expressing a TBC1D15-insensitive GTP-locked RAB7 mutant, evidencing that RAB7 activity is key for these trafficking deficits.

The precise mechanism for RAB7 inactivation mediated by pathogenic LRRK2 remains unclear. In further support that pathogenic LRRK2 causes Rab7 inactivation, it would be interesting to determine whether the trafficking deficits mediated by LRRK2 are rescued upon expression of RAB7 GEFs, such as the Mon1-Ccz1 tether complex which is directly implicated in endosome maturation and motility (Bhattacharya et al., 2023). It is possible that LRRK2 is able to phosphorylate RAB7 under certain cellular conditions, perhaps especially those occurring in a LRRK2-PD context such as those in DA neurons which are highly susceptible to cellular stress. Alternatively, LRRK2 may regulate the activity of its homolog LRRK1, which has been shown to phosphorylate RAB7 (Hanafusa et al., 2019; Malik et al., 2021) and to operate in association with LRRK2 to regulate autophagy (Toyofuku et al., 2015). Indeed, some functional redundancy has been suggested for these two homologous proteins (Biskup et al., 2007b), and LRRK2 may modulate its homolog LRRK1 by either direct or indirect means, thereby affecting RAB7 phosphorylation levels and decreasing its overall activity. Defining the link between pathogenic LRRK2 expression, RAB8A and RAB10 phosphorylation and RAB7 inactivation requires thorough further investigations.

Novel regulatory role for RAB29 in endolysosomal trafficking

The absence of alterations on EGFR endolysosomal trafficking upon knockdown of RAB29, in contrast to what was observed for RAB8A and RAB10 knockdowns, suggests that RAB29 function is not necessary for the endolysosomal trafficking pathway of the EGFR. Previous studies have shown that knockout of RAB29 alters retrograde trafficking from the LE to the Golgi Apparatus, thereby disrupting the proper transport of lysosomal enzymes and impairing lysosomal homeostasis (Beilina et al., 2020; Kuwahara et al., 2016; MacLeod et al., 2013). However, such alterations in the transport of lysosomal hydrolases have only been observed under RAB29 knockout conditions (Beilina et al., 2020; Kuwahara et al., 2016), suggesting that continuous missorting of cargo may be necessary to generate such alterations. In addition, whilst the presence of RAB29 may be necessary for proper trafficking between the LE and the Golgi Apparatus, our data suggest that RAB29 may not be required for proper endolysosomal trafficking of the EGFR. However, it remains possible that RAB29 knockdown only causes a transitory decrease in RAB29 levels, which may be insufficient to cause detectable endolysosomal trafficking deficits, and experiments in RAB29 knockout cells will be required to address this possibility.

Conversely, RAB29 overexpression rescued the endolysosomal trafficking defects mediated by pathogenic LRRK2, RAB8A or RAB10 knockdown and dominant-negative RAB7 expression. Previous studies have shown that increasing RAB29 protein levels can rescue pathogenic LRRK2-mediated defects in Golgi to lysosome trafficking and neurite outgrowth, resulting in enhanced survival of dopaminergic neurons in *Drosophila Melanogaster* PD models. In addition, transcriptomic studies show that RAB29 PD risk variants may correlate with lower RAB29 expression levels (MacLeod et al., 2013). These as well as our data are consistent with the idea that increasing RAB29 levels may be beneficial in reverting the trafficking deficits mediated by pathogenic LRRK2.

In contrast to our data, other studies suggest that RAB29 recruits LRRK2 to the Golgi Apparatus. The authors propose that upon RAB29-mediated recruitment, LRRK2 becomes active and phosphorylates its substrates including RAB8 and RAB10 in a feed-forward mechanism, with phospho-RAB8/RAB10 further retaining LRRK2 at membranes and thereby facilitating further LRRK2-mediated phosphorylation events (Vides et al., 2022). However, this model would implicate a detrimental effect of overexpression of either RAB29, RAB8A or RAB10 on their own, as potentially recruiting, sequestering and

potentiating the LRRK2 kinase activity, in direct contradiction to our data. We did not observe EGFR trafficking defects upon individual RAB29, RAB8 or RAB10 expression, and we observed a beneficial effect of active RAB10 or RAB29 expression in the context of pathogenic LRRK2. However, the model of RAB29-mediated LRRK2 recruitment and activation has recently also been challenged by Kolageropoulou and colleagues, who evidenced that RAB29 is not necessary for LRRK2 activation under endogenous conditions (Kalogeropulou et al., 2020). Therefore, the proposed feed-forward mechanism for LRRK2 activation may only work under conditions of high overexpression levels. Indeed, our data suggest that the beneficial effect of RAB29 is only observed under moderate expression levels and in the absence of detectable recruitment of LRRK2 to the Golgi complex. In contrast, and under conditions of high overexpression levels, RAB29 recruits LRRK2 to the Golgi complex, but is unable to rescue the pathogenic LRRK2-mediated trafficking defects of the EGFR (**Fig. 28**). This provides a reconciliatory model by which expression levels may dictate whether RAB29 plays beneficial or detrimental roles for membrane trafficking as observed here.

The function of RAB29 with respect to vesicular trafficking has been broadly related to Golgi Apparatus integrity and Golgi-mediated transport steps. Our work provides evidence that RAB29 expression rescues the trafficking deficits mediated by pathogenic LRRK2 independently of Golgi integrity, thus pointing towards a novel role for RAB29 in

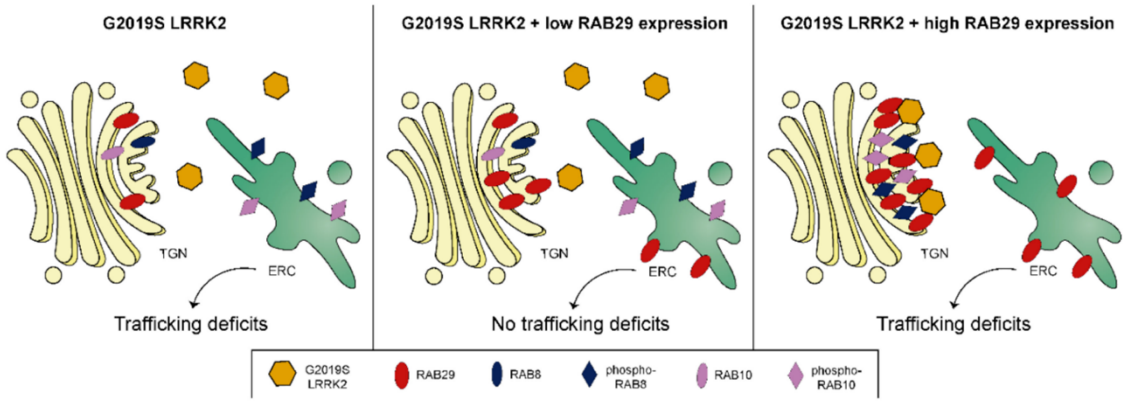

Fig. 28. Schematic representation of the proposed model for RAB29-mediated regulation of G2019S LRRK2 recruitment and rescue of endolysosomal trafficking defects caused by pathogenic LRRK2 expression. *Left: Pathogenic G2019S LRRK2 phosphorylates RAB8A/10, mainly located at the ERC, causing their inactivation and leading to EGFR trafficking deficits. RAB29 is mainly located at the Golgi Apparatus. Middle: Mild levels of RAB29 are able to rescue G2019S LRRK2-mediated trafficking defects involving RAB8A/RAB10 phosphorylation at the ERC. It is possible that a fraction of RAB29 located at the ERC is responsible for this rescue, perhaps by upregulating the remanent of non-phosphorylated RAB8A/10. Right: Higher overexpression levels of RAB29 get to recruit LRRK2 to the Golgi Apparatus, where it phosphorylates RAB8A and RAB10, sequestering them and finally resulting in the exhaustion of non-phosphorylated RAB8A/RAB10. In this conditions, ERC-localized RAB29 is unable to rescue the LRRK2-mediated trafficking impairment.*

endolysosomal membrane trafficking events. In line with this, RAB29 has been previously shown to colocalize and interact with RAB8 and to regulate receptor recycling in T cells and ciliary-targeted endosome transport, with its depletion resulting in receptor accumulation in RAB11-positive endosomes (Onnis et al., 2015). Given that RAB29 expression is able to compensate for the trafficking deficits mediated by RAB8A and RAB10 knockdown, it will be important to determine whether a subset of RAB29 may be located to recycling compartments, where it may play a role in endosomal recycling and endolysosomal trafficking, partially overlapping with the function of RAB8A and RAB10.

In summary, our data provide detailed mechanistic insights into how pathogenic LRRK2 regulates endocytic recycling and endolysosomal degradative trafficking events, with clear implications for potential aberrant trafficking of receptors and transporters as relevant to PD.

CONCLUSIONS

Conclusions

1. Active RAB10 but not WT variant rescues the pathogenic LRRK2-mediated endolysosomal trafficking deficits of the EGFR, including the reduced EGF surface binding and the delayed EGFR degradation.
2. RAB10 knockdown mimics the endolysosomal trafficking deficits mediated by G2019S LRRK2 expression and RAB8A knockdown, while phosphodeficient or active but not phosphomimetic or WT RAB10 rescues such phenotypes.
3. Endolysosomal trafficking alterations caused by the loss of RAB10 cause the accumulation of internalized EGF in a RAB4-positive recycling compartment.
4. GTP-locked active RAB7A expression rescues the decreased EGF binding and delayed EGFR degradation along with the EGF accumulation in a RAB4-positive early recycling compartment mediated by RAB10 knockdown.
5. RAB10 knockdown significantly reduces RAB7A activity as assessed by effector pulldown assays.
6. Expression of active RAB8A rescues the reduction in EGF surface binding and the delayed EGFR degradation caused by RAB10 knockdown and vice versa, active RAB10 rescues the same effects caused by RAB8A knockdown, suggesting that these GTPases act in a redundant manner with respect to the analyzed EGFR trafficking pathway.
7. WT RAB29 but not its inactive variants reverts the endolysosomal defects caused by G2019S LRRK2 expression, loss of RAB8, loss of RAB10 or dominant-negative RAB7A expression.
8. WT RAB29 but not its inactive variants rescues EGF accumulation at a RAB4-positive endocytic recycling compartment caused by either G2019S expression or knockdown of RAB8A or RAB10.
9. Low level expression of WT RAB29 does not efficiently recruit G2019S LRRK2 to the Golgi Apparatus.
10. Higher expression levels of WT RAB29 efficiently recruit G2019S LRRK2 to the Golgi Apparatus but fail to rescue the G2019S LRRK2-mediated defects in EGFR endolysosomal trafficking.
11. RAB29 knockdown mimics the EGFR degradation deficits caused by mutant LRRK2 expression, loss of RAB8A or loss of RAB10.

12. Rescue of the G2019S LRRK2-mediated deficits in EGF trafficking by RAB29 expression is independent on the integrity of the Golgi Apparatus, suggesting a novel role for RAB29 in endolysosomal trafficking.

Conclusiones

1. RAB10 activa, pero no sus variantes silvestre ni fosfomimética, rescata los déficits patogénicos de tráfico endolisosomal del EGFR mediados por LRRK2, incluyendo la reducción de la unión a la superficie del EGF y la degradación ralentizada del EGFR.
2. El silenciamiento de RAB10 mimetiza los déficits de tráfico endolisosomal mediados por la expresión de LRRK2 G2019S y el silenciamiento de RAB8A, mientras que RAB10 fosfodeficiente o activo pero no fosfomimético o silvestre rescata tales fenotipos.
3. Las alteraciones del tráfico endolisosomal causadas por la pérdida de RAB10 provocan una acumulación de EGF, internalizándolo en un compartimento de reciclaje positivo para RAB4.
4. La expresión de RAB7A activo (mediante el bloqueo con GTP) rescata la disminución de la unión a EGF y retrasa la degradación de EGFR, con la consiguiente acumulación de EGF en un compartimento de reciclaje temprano positivo para RAB4, efectos mediados por el silenciamiento de RAB10.
5. El silenciamiento de RAB10 reduce significativamente la actividad de RAB7A evaluada mediante ensayos de *pulldown* de efectores.
6. La expresión de RAB8A activo rescata la reducción en la unión a la superficie del EGF y el retraso en la degradación del EGFR causados por el silenciamiento de RAB10 y de igual modo, RAB10 activo rescata los efectos causados por el silenciamiento de RAB8A, sugiriendo que estas GTPasas actúan de forma redundante con respecto a la vía de tráfico del EGFR analizada.
7. RAB29 silvestre, pero no sus variantes inactivas, revierte los defectos endolisosomales causados por la expresión de LRRK2 G2019S, por la pérdida de RAB8, la pérdida de RAB10 o la expresión RAB7A negativa dominante.
8. RAB29 silvestre, pero no sus variantes inactivas, rescata igualmente, la acumulación de EGF en un compartimento endocítico de reciclaje positivo para RAB4.
9. Bajos niveles de expresión de RAB29 silvestre no reclutan eficientemente LRRK2 G2019S en el aparato de Golgi.
10. Por el contrario, altos niveles de expresión de RAB29 silvestre reclutan eficientemente LRRK2 G2019S en el Aparato de Golgi, pero no rescatan los defectos mediados por LRRK2 G2019S en el tráfico endolisosomal de EGFR.

11. El silenciamiento de RAB29 mimetiza los déficits de degradación del EGFR causados por la expresión de LRRK2 mutante, la pérdida de RAB8A o la pérdida de RAB10.
12. El rescate de los déficits en el tráfico de EGF mediados por LRRK2 G2019S mediante la expresión de RAB29 es independiente de la integridad del aparato de Golgi, lo que sugiere un nuevo papel de RAB29 en el tráfico endolisosomal.

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List of publications

Research articles:

I. **Distinct Roles for RAB10 and RAB29 in Pathogenic LRRK2-Mediated Endolysosomal Trafficking Alterations.** Rivero-Ríos P*, **Romo-Lozano M***, Fernández B, Fdez E, Hilfiker S. *Cells*. 2020 Jul 17;9(7):1719. doi: 10.3390/cells9071719. PMID: 32709066; PMCID: PMC7407826. (equal contribution).

II. **RAB8, RAB10 and RILPL1 contribute to both LRRK2 kinase-mediated centrosomal cohesion and ciliogenesis deficits.** Lara Ordónez AJ, Fernández B, Fdez E, **Romo-Lozano M**, Madero- Pérez J, Lobbestael E, Baekelandt V, Aiastui A, López de Munaín A, Melrose HL, Civiero L, Hilfiker S. *Hum Mol Genet.* 2019 Nov 1;28(21):3552-3568. doi: 10.1093/hmg/ddz201. PMID: 31428781; PMCID: PMC6927464.

III. **The G2019S variant of leucine-rich repeat kinase 2 (LRRK2) alters endolysosomal trafficking by impairing the function of the GTPase RAB8A.** Rivero-Ríos P, **Romo-Lozano M**, Madero- Pérez J, Thomas AP, Biosa A, Greggio E, Hilfiker S. J *Biol Chem*. 2019 Mar 29;294(13):4738- 4758. doi: 10.1074/jbc.RA118.005008. Epub 2019 Feb 1. PMID: 30709905; PMCID: PMC6442034.

Review articles:

IV. **LRRK2 Related Parkinson's Disease Due to Altered Endolysosomal Biology With Variable Lewy Body Pathology: A Hypothesis**. Rivero-Ríos P, **Romo-Lozano M**, Fasiczka R, Naaldijk Y, Hilfiker S. *Front Neurosci.* 2020 Jun 4;14:556. doi: 10.3389/fnins.2020.00556. PMID: 32581693; PMCID: PMC7287096.

V. **Cellular effects mediated by pathogenic LRRK2: homing in on Rab-mediated processes.** Madero- Pérez J, Fdez E, Fernández B, Lara Ordóñez AJ, Blanca Ramírez M, **Romo Lozano M**, Rivero- Ríos P, Hilfiker S. *Biochem Soc Trans.* 2017 Feb 8;45(1):147-154. doi: 10.1042/BST20160392. PMID: 28202668.

VI. 6. **Two-pore channels and Parkinson´s disease: where´s the link?** Rivero-Ríos, P., Fernández, B., Madero-Pérez, J., **Lozano, M.R**. and Hilfiker, S. (2016) *Messenger* 5, 67-75.