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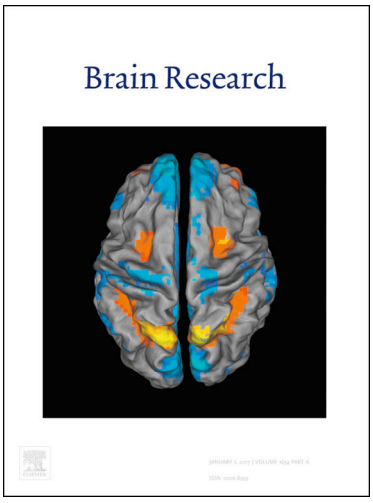

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The Roc domain of LRRK2 as a hub for protein-protein interactions: a focus on PAK6 and its impact on RAB phosphorylation

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Abstract

Leucine-rich repeat kinase 2 (LRRK2) has taken center stage in Parkinson's disease (PD) research as mutations cause familial PD and more common variants increase lifetime risk for disease. One unique feature in LRRK2 is the coexistence of GTPase/Roc (Ras of complex) and kinase catalytic functions, bridged by a COR (C-terminal Of Roc) platform for dimerization. Multiple PD mutations are located within the Roc/GTPase domain and concomitantly lead to defective GTPase activity and augmented kinase activity in cells, supporting a crosstalk between GTPase and kinase domains. In addition, biochemical and structural data highlight the importance of Roc as a molecular switch modulating LRRK2 monomer-to-dimer equilibrium and building the interface for interaction with binding partners. Here we review the effects of PD Roc mutations on LRRK2 function and discuss the importance of Roc as a hub for multiple molecular interactions relevant for the regulation of cytoskeletal dynamics and intracellular trafficking pathways. Among the well-characterized Roc interactors, we focused on the cytoskeletal-related kinase p21-activated kinase 6 (PAK6). We report the affinity between LRRK2-Roc and PAK6 measured by microscale thermophoresis (MST). We further show that PAK6 can modulate LRRK2-mediated phosphorylation of RAB substrates in the presence of LRRK2 wild-type (WT) or the PD G2019S kinase mutant but not when the PD Roc mutation R1441G is expressed. These findings support a mechanism whereby mutations in Roc might affect LRRK2 activity through impaired protein-protein interaction in the cell.

Introduction

Leucine-rich repeat kinase 2 (LRRK2) is a large, multi-domain kinase whose mutations have been associated with the development of familial and sporadic Parkinson's disease (PD), but also with an increased susceptibility to leprosy, Crohn's disease, and several forms of cancer (Bae and Lee, 2015; Cogo et al., 2017; Lebovitz et al., 2021; Saunders-Pullman et al., 2010). In addition, LRRK2 expression levels have been recently shown to influence survival in progressive supranuclear palsy (Jabbari et al., 2021). LRRK2 belongs to the ROCO protein family, characterized by the presence of a Roc-COR bidomain, where Roc mediates enzymatic activity by acting as a GTPase, while COR functions as a platform for dimerization (Gotthardt et al., 2008; Guaitoli et al., 2016). The catalytic core is completed by a serine-threonine kinase domain, and it is surrounded by a number of scaffold domains which mediate protein-protein interactions, namely Armadillo (ARM), Ankyrin (ANK), Leucine-rich repeat (LRR) at the N-terminus and the C-terminal WD40 (Figure 1A).

The current picture about the intra- and intermolecular regulation of LRRK2 activation and LRRK2-mediated signaling cascades is still incomplete. This is partly due to the complex structural organization of the molecule, which functions in equilibrium between a monomeric and a dimeric conformation, where each molecule comprises two enzymatic domains deeply cross talking to each other and with the neighboring regions both *in cis* and *in trans* (Soliman et al., 2020). An additional layer of complexity relates to the expression of LRRK2 in multiple tissues, including the brain, immune system, lungs and kidneys (Biskup et al., 2007; Wallings and Tansey, 2019). In the brain, LRRK2 is expressed in neurons, astrocytes and microglia and there is evidence of cell-type specific splicing variants (Giesert et al., 2013; Miklossy et al., n.d.; Trabzuni et al., 2013). Thus, the widespread expression of LRRK2 combined with its complex enzymatic and scaffolding architecture may account for the multitude of cellular pathways and functions that have been linked to this protein over the years.

This manuscript will first review the multifaceted nature of Roc, spanning from its enzymatic properties to its impact on LRRK2 kinase activity, structure, dimer formation and binding with protein partners. In the second part, we will present multiple results on the interaction between LRRK2-Roc and the actin cytoskeletal-related kinase p21-activated kinase 6 (PAK6), as an example of how mutations in Roc can impact LRRK2 kinase activity in the cell through aberrant protein-protein interactions.

Roc domain: an overview of its function and dysfunction

The most characterized signaling output of LRRK2 is represented by its serine-threonine kinase domain, which can autophosphorylate LRRK2 at multiple sites and phosphorylate several cellular substrates (Gloeckner et al., 2010; Greggio et al., 2009; Kamikawaji et al., 2009; Sheng et al., 2012; Steger et al., 2016). A *bona fide* autophosphorylation site, which is employed as an indicator of LRRK2 kinase activity in cells, is Ser1292, located in the 13th repeat of the LRR domain (Sheng et al., 2012). The common PD G2019S mutation, within the activation loop of the kinase domain, increases LRRK2 activity by ~2-3 fold *in vitro* and in cells (Greggio and Cookson, 2009) by locking the kinase in a more active state (Covy and Giasson, 2010; Greggio and Cookson, 2009; Jaleel et al., 2007; Sheng et al., 2012). Based on the gain of function effect of the G2019S mutation, the identification of kinase inhibitors able to reestablish normal catalytic function has been extensively pursued, as it may be translated in the first genetic-based molecular target therapy for PD. This has led to the generation of dozens of LRRK2 compounds, some of which are currently being tested in clinical trials ("Study to Evaluate DNL151 in Subjects With Parkinson's Disease - Full Text View - ClinicalTrials.gov," n.d., "Study to Evaluate DNL201 in Subjects With Parkinson's Disease - Full Text View - ClinicalTrials.gov," n.d.).

What, then, do we know about mutations in Roc and their effects on GTPase and kinase activities? The presence of a phosphate-binding, P-loop motif between amino acids 1341 and 1348 (^{1341}GNTGSGKT^{1348}) enables LRRK2 to bind guanine nucleotides – GTP and GDP – with similar affinity in the μM range (Civiero et al., 2012; Ito et al., 2007). This affinity is 3-4 orders of magnitude lower than that measured for small G proteins, suggesting that LRRK2 does not require GTPase activating proteins (GAPs) and guanine exchange factors (GEFs) for its activity (Gilsbach and Kortholt, 2014). This is in agreement with the lack of convincing evidence of specific GAPs/GEFs assisting Roc activity (Nguyen and Moore, 2017). Indeed, although several GAPs and GEFs (ARHGEF7, ArfGAP1, RGS2) were shown to interact with LRRK2, none of them binds to Roc, suggesting a divergent mechanism with respect to small GTPases (Berwick et al., 2019), as discussed in the next paragraph.

Several pathogenic mutations as well as protective variants map within Roc (N1437H and R1441C/G/H pathological, R1398H protective) and COR (Y1699C pathological) (Figure 1A; Erb and Moore, 2020; Greggio and Cookson, 2009), highlighting the importance of this domain for LRRK2 cellular function. Accordingly, multiple observations agree with a general

reduction of GTPase activity in the presence of Roc-COR pathogenic mutations (Liao et al., 2014; Nguyen and Moore, 2017; Taymans, 2012). The consequence is a prolonged GTP-bound state, which is predicted to result in sustained signaling. Instead, there is general consensus that Roc-COR mutations only show minor variations in kinase activity when assays are performed *in vitro* with recombinant proteins and autophosphorylation or peptide/substrate phosphorylation as readouts (Jaleel et al., 2007; Nguyen and Moore, 2017; Reynolds et al., 2014; Steger et al., 2016). The situation is different when the kinase activity of Roc-COR mutants is probed in cells. To this regard, two landmarks in LRRK2 research were achieved by the development of antibodies able to detect cellular autophosphorylation at Ser1292 and by the identification of a subset of RAB GTPases as physiological substrates of LRRK2 kinase activity: these new tools and readouts revealed that the Roc-COR mutations display augmented kinase activity when assessed in cells and in tissues, particularly in terms of phosphorylation towards cellular substrates (Iannotta et al., 2020; Sheng et al., 2012; Steger et al., 2016). Interestingly, Steger and coauthors showed that the impact of mutations located outside of the kinase domain is greater than the effect of the G2019S in the kinase domain (Steger et al., 2016) and similar observations were made by Sheng and colleagues who examined cellular autophosphorylation (Sheng et al., 2012). In a recent study addressing autophosphorylation and RAB10 phosphorylation in the tissues where LRRK2 is mostly expressed, namely brain, lungs and kidneys, our group observed that the phosphorylation of RAB10 by LRRK2 is significantly increased in the presence of Roc-COR mutations as compared to the G2019S substitution, while minor effects were detected when measuring autophosphorylation at Ser1292 (Iannotta et al., 2020). This is an important lesson on the intrinsic limitation of *in vitro* kinase assays with LRRK2: they have been fundamental to determine some basic properties of LRRK2 catalytic activity, but they provide limited information when assessing the interplay among different domains, since cellular cofactors and binding partners are absent during *in vitro* reactions. Of interest, Roc-COR mutants display altered subcellular localization, e.g. increased localization to the Golgi, centrosomes and microtubules (Beilina et al., 2014; Giesert et al., 2013; Kett et al., 2012; Madero-Pérez et al., 2018b, 2018a; Wu et al., 2019), suggesting a prolonged substrate availability and interaction with binding partners. In other words, if slower GTP hydrolysis due to PD mutations freezes LRRK2 at signaling stations, the predicted effect is prolonged signaling. This notion is supported by recent analyses of LRRK2 kinase activity when tethered to membranes, showing that localization contributes to activity against RAB substrates (Gomez et al., 2019).

The elevated kinase activity measured for Roc mutants in the cellular context might suggest either that the GTPase activity is a direct, upstream regulator of LRRK2 kinase activity or that it can impact on kinase activity indirectly, e.g. by altering LRRK2 subcellular localization and substrate availability and interaction. Supporting the first notion, the substitution of key residues in the phosphate binding P-loop region of Roc – *via* introduction of the hypothesis-testing K1347A or T1348N mutations – disrupts GTP/GDP binding and concomitantly kinase activity (Biosa et al., 2013; Ito et al., 2007; Liu et al., 2011; Taymans et al., 2011), suggesting that guanine nucleotide binding capacity is the actual requirement for LRRK2 kinase activity. However, the decreased steady state levels of these artificial mutations (Biosa et al., 2013; Lewis et al., 2007) complicate the interpretation. For instance, it cannot be ruled out that nucleotide loss promotes an unstable conformation that is targeted for degradation or mis-localized due to impaired signaling. Several *in vitro* studies attempted to establish a direct link between GTP binding/hydrolysis and kinase activity with mixed outcomes (Taymans et al., 2011; Tsika and Moore, 2013). At present, the evidence that Roc-COR mutations (R1441C/G/H and Y1699C) with reduced GTPase activity *in vitro* (Daniëls et al., 2011; Ito et al., 2007; Lewis et al., 2007; Li et al., 2007; Liao et al., 2014) exhibit increased RAB phosphorylation in cells supports a model where the GTP/GDP cycle is important to direct LRRK2 toward its physiological substrates by favoring monomer-to-dimer transition and the binding with a variety of Roc partners, as discussed in the next paragraphs.

Conversely, a direct kinase-to-Roc crosstalk is supported by the identification of multiple *in vitro* autophosphorylation sites in the Roc domain, including T1343, T1348, S1403, T1404, T1410, T1491, and T1503 (Gloeckner et al., 2010; Greggio et al., 2008; West et al., 2007), although cellular validation of these phospho-sites is lacking. This could be related to the low sensitivity of the available detection tools, the low level of basal autophosphorylation, or both. Nevertheless, autophosphorylation of the Roc domain is thought to play an important role in the regulation of the LRRK2 G-protein cycle, although different phosphorylation sites might exert different effects on GTPase activity (Athanasopoulos et al., 2018). While the exact consequence of each autophosphorylation event on Roc is not clear yet, a study performed with isolated Roc provides evidence that autophosphorylation at multiple sites within the P-loop enhances GTP hydrolysis (Liu et al., 2016).

In addition to self-phosphorylation, the LRRK2 phospho-state is finely tuned by heterologous phosphorylation. One key cluster of phosphorylated serines is located at the N-terminus of LRRK2 and includes Ser910 and Ser935 (Nichols et al., 2010). These sites, targeted by

casein kinase 1α (CK1α) (Chia et al., 2014), PKA (Muda et al., 2014) and the IkB family of kinases (Dzamko et al., 2012), have been widely exploited as an indirect indication of LRRK2 kinase inhibition. Indeed, these sites are not autophosphorylation sites as in a survey of multiple LRRK2 mutants that affect LRRK2 kinase activity, the rate of phosphorylation at these residues does not correlate to kinase activity (Ito et al., 2014). Our own data also show, for instance, that the artificial kinase dead mutant D2017A preserves phosphorylation (Figure 4B,C). The fact that these residues become dephosphorylated as a consequence of LRRK2 kinase inhibition (Deng et al., 2011; Dzamko et al., 2012; Zhao et al., 2012), was initially explained by an indirect, feedback effect on a kinase or phosphatase regulated by LRRK2 kinase activity. However, recent studies comparing the structurally different Type-I and Type-II LRRK2 kinase inhibitors suggest that Type-II compounds can reduce LRRK2 kinase activity against substrates without impacting on Ser935 phosphorylation. As an explanation, the authors hypothesize this is due to the different conformation states induced by the inhibitors (Tasegian et al., n.d.), a concept that finds support in structural studies and will be discussed further in the next section. This has important implications since phosphorylation at Ser910 and Ser935 is known to regulate the binding with the family of 14-3-3 adaptor proteins, which is essential for mediating the subcellular localization of LRRK2, its kinase activity and substrates/interactors availability (Dzamko et al., 2010; Nichols et al., 2010). Additional clusters of phosphorylation are present in the Roc domain. PKA, for example, phosphorylates S1444, generating an *in vitro* docking site for 14-3-3 proteins whose binding leads to a decrease in LRRK2 kinase activity. Importantly, phosphorylation of this residue is impaired by the presence of PD mutations on the R1441 hotspot (Muda et al., 2014), providing one additional mechanistic explanation for the elevated kinase activity measured for Roc mutants. Phosphorylation is counter-regulated by phosphatases, the most validated ones being PP1 and PP2A (Lobbestael et al., 2013; Taymans, 2017). LRRK2 kinase inhibition has been shown to recruit PPP1CA to the LRRK2 complex and LRRK2 dephosphorylation induced by pharmacological kinase inhibition is blocked by PPP1CA inhibitors (Lobbestael et al., 2013).

From structure to function of LRRK2-Roc: learning from simpler ROCO proteins

LRRK2 is classified as an unconventional GTPase, although interaction with classical regulatory proteins for small GTPases, namely ArfGAP1 and ARHGEF7, has been reported (Haebig et al., 2010; Xiong et al., 2012). Initially, LRRK2 was proposed to act as a G-protein activated by nucleotide-dependent dimerization (GAD), extrapolating from the structure of

the bacterial *C. tepidum* ROCO (*Ct*ROCO) proteins (Gotthardt et al., 2008). However, additional studies addressing the complex regulation of LRRK2 G-protein cycle followed over the years, revealing that LRRK2 and, more in general, ROCO proteins have a unique activation mechanism different from both GADs and classical monomeric G-proteins (Wauters et al., 2018). Due to the large molecular weight of LRRK2 and the poor yields achieved from the purification of the full-length protein, structural and functional investigations have initially focused on isolated domains or on bacterial or amoeboid homologs of LRRK2, or have exploited a combination of the two to reconstruct the first model of full-length LRRK2 (Deyaert et al., 2017; Gilsbach et al., 2012; Gotthardt et al., 2008; Guaitoli et al., 2016; Herbst and Lewis, 2021; Rudi et al., 2015; Van Egmond et al., 2008). Importantly, ROCO protein homologs of LRRK2 may have evolved differently both at the structural and functional level, highlighting the need for a detailed representation of human LRRK2 at the atomic level. In this direction, cryo-electron microscopy (EM) and electron tomography (ET) based *in vitro* and *in situ* studies have recently expanded the resolution and the structural knowledge on truncated and full-length human constructs, providing important information about oligomerization interfaces and the close proximity of the catalytic moieties, in support of the crosstalk between Roc and the kinase domains (Deniston et al., 2020; Myasnikov et al., 2021; Watanabe et al., 2020), as discussed in detail throughout this paragraph.

The continuous progress in defining LRRK2 structure will possibly drive our understanding of how GTP hydrolysis in the Roc domain participates in the regulation of LRRK2 activity, oligomeric state and, consequently, function. The current working model from *Ct*ROCO postulates a monomer to dimer cycling of ROCO proteins depending on nucleotide load. Specifically, *Ct*ROCO proteins are dimers in the nucleotide-free or GDP-bound forms, while switching into monomers upon GTP-binding. Whether this model applies to human, full-length LRRK2 remains to be demonstrated. Active LRRK2 is thought to be dimeric and localized to membranes (Berger et al., 2010; Civiero et al., 2012; Greggio et al., 2008; James et al., 2012; Sejwal et al., 2017; Sen et al., 2009). Therefore, although *in vitro* studies suggest that GTP hydrolysis is the key step inducing dimerization, additional factors must be taken into account in the cellular environment. Among these, the occurrence of concentration disequilibrium between the cytosol and the membranous compartments might modulate the preference for one of the two localizations (Berwick et al., 2019). In addition, it is possible that the permanence to a GTP-bound dimeric conformation is favored by

interaction with membrane partners such as RAB29 (Beilina et al., 2020; Purlyte et al., 2018). Hence, from a structural perspective, capturing the kinase active, which is possibly membrane-associated, as well as the subcellular-specific conformations represents the next challenge in the field.

A detailed definition of LRRK2 structure will additionally help dissect the impact of PD mutations on monomer-to-dimer equilibrium, its binding with partners and the signaling properties of the protein. The first model of full-length LRRK2 based on cross-linking experiments and electron microscopy (EM) maps (Guaitoli et al., 2016) confirmed the earlier studies on ROCO proteins that LRRK2 dimerization occurs *via* the interaction of two Roc-COR domains (reviewed in Wauters et al., 2019). In this study, LRRK2 is suggested to be predominantly dimeric in solution. The model also proposes very compact folding of the dimer, which has both kinase and GTPase activities and where the scaffold domains ANK, LRR and WD40 participate in the intramolecular regulation of the catalytic activities by being in close contact to each other. In this frame, the behavior of Roc mutants, displaying decreased GTPase activity and increased kinase activity in cells, overall supports the complexity and multilayer regulation of LRRK2. The tightly packed, homodimeric nature of LRRK2 has been further confirmed through cryo-EM and cryo-ET analyses using either full-length LRRK2 or the catalytic fragment RCKW (Roc-COR-kinase-WD40) (Deniston et al., 2020; Sejwal et al., 2017; Watanabe et al., 2020). RCKW-LRRK2 was observed to be predominantly monomeric in solution, occasionally dimeric, and able to form trimers at high concentrations (Deniston et al., 2020). The concomitant studies performed in 2020 took advantage of the observation that overexpressed mutant or pharmacologically inhibited LRRK2 partially relocalizes to microtubules (Ramírez et al., 2017; Schmidt et al., 2019). Not only did they confirm the involvement of COR and WD40 domains in dimerization, but they additionally highlighted the role of Roc as the binding interface and proposed that the interaction with microtubules is modulated by the conformation of the kinase domain. Specifically, an open conformation decreases oligomerization and the binding to microtubules, maintaining LRRK2 predominantly in the cytosol, whilst a closed conformation favors the interaction (Deniston et al., 2020). The physiological significance of the microtubule-bound form of LRRK2 still requires further investigations (Herbst and Lewis, 2021; Leschziner and Reck-Peterson, 2021). Nevertheless, these structures represent a huge step forward in the process of structure-to-function correlation. Similarly, the newly developed cryo-EM structures of full-length human LRRK2, both in monomeric and dimeric

states and with resolution below 4Å, provide a pathophysiologically relevant representation of LRRK2 native conformations, as they reveal the presence of essential scaffold elements and disease hotspots, and will help in the understanding the impact of pathological mutations, aiding the design of conformation-specific inhibitors (Myasnikov et al., 2021). The observation that different compounds inhibiting LRRK2 kinase activity have diverse structural impact on the conformation of the kinase domain, differentially affecting the ability of LRRK2 to modulate microtubule-based motors, corroborates a mechanism whereby both tight allosteric regulation and compartmentalization play a key role on the regulation of LRRK2-signaling. Because Type-I inhibitors are known to stabilize the open conformation of their target kinase, in contrast with Type-II inhibitors that stabilize the closed conformation, it will now be essential to focus drug-design on the latter, in order to avoid possible long-term interference with microtubule traffic (Leschziner and Reck-Peterson, 2021) and, potentially, other organelle-specific off-target cellular effects. Of note, although it is possible that the cytosolic, monomeric, GTP-bound LRRK2 is functional under specific circumstances, the current hypothesis is that dimeric, physiologically active LRRK2 predominantly localizes at membranes, and membrane recruitment is an essential requirement for LRRK2 activation (Berger et al., 2010; Gomez et al., 2019; James et al., 2012). The presence of LRRK2 both at membranous compartments and in association with the cytoskeleton, with evidence of re-localization in the presence of pathological mutations or upon pharmacological inhibition, may not be mutually exclusive. We have previously discussed the intriguing possibility of LRRK2 acting as a physical and functional bridge between vesicles and the cytoskeleton (Civiero et al., 2018) through its different interaction domains. Accordingly, most LRRK2 interactions with the cytoskeleton are known to occur *via* Roc (Civiero et al., 2018; Gandhi et al., 2008), whereas RAB29 mediates LRRK2 recruitment to intracellular membranes through the ANK domain (Purlyte et al., 2018), although recent data with endogenous RAB29 challenged this mechanism (Kalogeropulou et al., 2020). Whether a simultaneous interaction between the cytoskeleton and membrane-associated partners occurs in the cell remains an open question.

Recent evidence suggests that Roc-COR mutations not only can influence the enzymatic activities, but also affect the monomer-dimer equilibrium (Deyaert et al., 2017), suggesting an additional layer of complexity and supporting the heterogeneity of mechanisms and cellular/tissue phenotypes observed in the presence of LRRK2 PD mutations mapping in different domains (Bonet-Ponce et al., 2020; Iannotta et al., 2020; Wallings et al., 2019).

Along this line, it is also likely that other domains – e.g. the LRR at the N-terminus and the C-terminal WD40 – participate in the dimerization process (Deniston et al., 2020; Jorgensen et al., 2009; Myasnikov et al., 2021; Watanabe et al., 2020; Zhang et al., 2019).

Regarding the effect of mutations on monomer-dimer equilibrium, a univocal relationship between dimerization and GTP binding/GTP hydrolysis still needs to be established, since multiple reports have shown controversial results on the effect that mutations as well as protective variants have on dimerization, GTPase activity, and LRRK2 subcellular localization (Daniëls et al., 2011; Deyaert et al., 2017; Leandrou et al., 2019; Nixon-Abell et al., 2016; Wu et al., 2019). Part of this controversy can be justified with the use of different constructs and models; therefore, additional studies are required to gain further insight into the process. Since LRRK2 has to go through the whole GTPase cycle to become fully activated, and ROCO/LRRK2 proteins need therefore to cycle through both monomeric and dimeric conformations, stabilization of either state by PD-mutations could lead to reduced GTPase activity (Wauters et al., 2019).

To conclude, we expect that variations in the LRRK2 steady-state phosphorylation, as well as the presence of mutations or variants affecting the kinetics of guanine nucleotide binding and dissociation within Roc will likely affect dimer/monomer formation, impacting on both LRRK2 localization and kinase activity. This, in turn, will have direct consequences on the availability of substrates to bind and phosphorylate.

The LRRK2 Roc domain interactome

After 15 years of intense research, an impressive number of interactors and substrates have been nominated for LRRK2 (approximately 2400 unique interactors reported in a recent study focused on human and mouse LRRK2 protein interaction data curated into the IntAct database (Gloeckner and Porras, 2020). This vast dataset may be explained by the so called “date-hub hypothesis” (Manzoni, 2017) according to which LRRK2 might be extremely flexible depending on the macromolecular complexes it forms in different cell-types, developmental stages and under specific stimuli. This hypothesis is likely due to the high degree of structural and regulatory complexity of LRRK2, together with its differential expression profile across tissues (Biskup et al., 2007). Within a single neuron, the formation of protein complexes may be different in the soma as compared to the synaptic compartment or the axonal/dendritic processes, further amplifying the spectrum of possibilities. Supporting this view, a recent *in silico* analysis of LRRK2 interactomes across some of the

tissues where LRRK2 is highly expressed, namely brain, kidneys and lungs, revealed specific interaction profiles based on the different levels of expression of binding partners within the tissue assessed (Verma et al., 2021). More specifically, through evaluation of protein-protein interaction datasets in combination with gene expression profiles, the authors identified a subset of brain-specific LRRK2 binding partners, displaying no or low interaction likelihood in peripheral tissues, with important implications in the design of therapeutic strategies having reduced on-target side effects (Verma et al., 2021). We further hypothesized that the elevated degree of molecular complexity characterizing LRRK2 contributes to the stratification of associated cellular signaling events. To investigate this aspect in detail, we performed a binding region-specific analysis of LRRK2 protein interactors, utilizing manually curated data from the IntAct database (Orchard et al., 2014). Protein interaction data curated to this depth are limited, however we mapped the binding region of 93 interactors (Supplementary File 1), in relation to the LRRK2 domain topology (Figure 1A). The results highlight that 85% of these interactors bind LRRK2 *via* the Roc domain or a Roc-containing region. This analysis provides further evidence that the Roc/GTPase domain represents an interaction platform with distinct cellular components to orchestrate a multitude of signaling programs. Next, to test the intriguing possibility that the spatial separation of binding partners within the LRRK2 structure correlates with a functional specialization, we performed functional enrichment analysis, based on gene ontology biological process (GO BP) annotations, on N-terminal and Roc-COR specific interactors. Figure 1B highlights common as well as region-specific functional associations of LRRK2 interactors. Specifically, N-terminal interactors showed enrichment for terms related to apoptosis, mitochondria and intracellular transport, whilst Roc-COR interactors showed enrichment for terms related to endocytosis, autophagy, RNA metabolism, synaptic signaling and cytoskeleton-associated processes (Supplementary File 2). The presence of shared enriched GO BP terms between the two LRRK2 regions (e.g. intracellular organization and protein localization) can be explained by the ability of key interactors to bind at multiple sites. One substantial example in this respect is represented by 14-3-3 proteins, which are fundamental in regulating LRRK2 stability, localization and activity and can bind to both the Ser910/935 cluster and the Roc domain (Civiero et al., 2017; Nichols et al., 2010). We performed pull-down assays between Flag-tagged LRRK2 expressed in HEK293T cells and the seven members of the 14-3-3 protein family purified from *E. coli*, highlighting the existence of isoform-specificity in the interaction, with γ (gamma) and η (eta) isoforms displaying the highest affinity (Figure 1C). This is in agreement with results reported

independently by Manschwetus et al., 2020, who additionally provided K_D values – spanning from low nanomolar to micromolar – for the binding of the seven isoforms to different LRRK2 phospho-sites (Manschwetus et al., 2020). Moreover, as discussed in the next paragraphs, the regulatory activity of 14-3-3 proteins can be itself regulated through phosphorylation by the LRRK2-Roc interactor PAK6, supporting one more time the complex, multilevel regulation of LRRK2 cellular activity.

On the other hand, the selective enrichment of specific categories of binding partners in one of the two regions supports our initial hypothesis of a "molecular compartmentalization". In this regard, we confirmed that one key component of the LRRK2 interactome is represented by cytoskeletal elements, which preferentially bind Roc. The biochemical and structural indication of LRRK2 bound to microtubules (Figure 1B; Deniston et al., 2020; Gandhi et al., 2008; Gillardon, 2009; Law et al., 2014), the identification of several cytoskeletal, cytoskeletal-related and motor proteins as Roc interactors (Civiero et al., 2018) and the multiple lines of evidence linking LRRK2 with vesicle dynamics *via* RAB phosphorylation (Steger et al., 2016) suggest a picture in which LRRK2 sits at the interface between the actin/microtubule cytoskeleton and intracellular vesicle dynamics (Civiero et al., 2018).

In neurons, microtubules are mainly involved in sustaining the intracellular trafficking between the soma and the synapse, and the LRRK2 G2019S mutation was shown to impair axonal traffic of autophagosomes (Boecker et al., 2021). Instead, neurite outgrowth and synapse formation and maintenance are mainly associated with actin dynamics (Parisiadou and Cai, 2010). LRRK2 binds actin and promotes its polymerization (Bardai et al., 2018; Dwyer et al., 2020; Häbig et al., 2013; Meixner et al., 2011). In addition, LRRK2 binds or phosphorylates a number of proteins involved in the regulation of actin dynamics. Among them, LRRK2 phosphorylates the actin remodeling protein WAVE2 (Kim et al., 2018) and the ERM protein moesin, which, together with ezrin and radixin, links the actin cytoskeleton to the plasma membrane (Jaleel et al., 2007; Parisiadou et al., 2009). ERM are localized at the actin-rich sites in filopodia where they control neurite outgrowth by regulating filopodia architecture (Mangeat et al., 1999). The phosphorylation state of ERM and F-actin content are increased in G2019S LRRK2 neurons and neurite outgrowth defects have been extensively reported in these LRRK2 mutant neurons (Civiero et al., 2017; MacLeod et al., 2006). These data support a role for LRRK2 in the regulation of cytoskeletal dynamics, either by directly binding to core components of the cytoskeletal scaffold or *via* indirect modulation of regulatory elements.

Interaction between Roc and the actin cytoskeletal kinase PAK6

P21-activated kinase 6 (PAK6) is a guanine nucleotide-dependent interactor of Roc that we identified and extensively validated (Beilina et al., 2014; Civiero et al., 2017, 2015). PAKs comprise a family of 6 serine-threonine kinases which play a central role in signal transduction. One of the best-characterized functions of these kinases is their role in actin cytoskeleton reorganization via the LIM kinase-cofilin pathway. When activated, PAKs phosphorylate LIMK1, which phosphorylates cofilin causing inhibition of its actin-severing activity and stabilization of filamentous actin (F-actin) (Civiero et al., 2015; Minden, 2012). PAK6, a poorly characterized family member, is highly expressed in the brain, with elevated expression in the dopaminergic fibers of the *substantia nigra pars compacta*, a brain region preferentially affected in PD (Mahfouz et al., 2016). Of note, double PAK5/PAK6 knockouts exhibit cognitive and locomotor activity defects and neurite shortening (Nekrasova et al., 2008). Our published work showed that the kinase activity of PAK6 stimulates neurite complexity *in vivo* in the striatum in a LRRK2-dependent manner, a finding that functionally links the two kinases with downstream impact on actin cytoskeleton-dependent neurite remodeling (Civiero et al., 2015). Here, we extended our analysis of the functional interplay between LRRK2 and PAK6 probing the expression of the two kinases during postnatal mouse development in WT and BAC-LRRK2 G2019S mice, which overexpress mouse LRRK2 carrying the G2019S mutation (Civiero et al., 2017; Li et al., 2010). As shown in Figure 2, LRRK2 and PAK6 exhibit a similar pattern of expression which increases from post-natal day 0 (P0) up to P28 when the highest expression is reached for both kinases, suggesting they may become important at later developmental stages. Strikingly, BAC-G2019S brains displayed dramatically reduced PAK6 expression across all time points, hinting that augmented LRRK2 activity/expression might provide negative feedback towards PAK6 expression. Given that PAK6 promotes neurite outgrowth upstream of LRRK2 (Civiero et al., 2015), this finding may explain, at least in part, the reduced number and length of neuritic processes observed in these mice (Civiero et al., 2017; Sepulveda et al., 2013) and predicts that PAK6 activity may be beneficial under pathological LRRK2 expression. Future studies addressing the expression pattern of proteins both related and unrelated to LRRK2 and PAK6 during postnatal mouse development and how these are affected by LRRK2 knockout will not only confirm the specificity of this finding, but also elucidate additional participants in the cascade.

Assessment of the impact of Roc-COR nucleotide loading state in mediating interaction with PAK6

In cells, the interaction between LRRK2 and PAK6 is mediated by a multiplicity of factors. First, the binding occurs at the level of Roc and depends on LRRK2 guanine-nucleotide loading state (Beilina et al., 2014; Civiero et al., 2015). As discussed previously, this is likely affecting the equilibrium between the monomeric versus dimeric LRRK2 fractions and, in turn, the cellular localization of the protein, which is also regulated by the binding of 14-3-3 proteins at Ser910/935 and within Roc (Berwick et al., 2019; Muda et al., 2014; Nichols et al., 2010).

In order to define in detail the biochemical basis of the LRRK2 interaction with PAK6, and to separate the contribution of guanine-nucleotide loading within Roc *per se* versus the participation of other factors, e.g. stabilization of the interaction by other domains and/or 14-3-3 binding, we calculated the affinity between the PAK6 and the isolated Roc-COR fragment *via* Microscale Thermophoresis (MST). In particular, we characterized the interaction between Flag-PAK6, purified from mammalian HEK293T cells, and MBP-fused WT Roc-COR – produced with good yield in *E. coli* as a stable truncated dimer (PAK6: 100nM; Roc-Cor 16nM to 64µM). Importantly, Roc had been previously identified as the required domain involved in PAK6 recruitment, and COR is essential for the stability of the construct (Civiero et al., 2015). MST allows to quantify molecular interactions in solution between a target and ligand by detecting changes in fluorescence intensity while a temperature gradient is applied over time (Asmari et al., 2018). We compared the binding affinities of PAK6 for Roc-COR loaded with GDP or a non-hydrolysable GTP analogue. Intriguingly, we observed that the binding affinity between GppNHp-loaded Roc-COR and PAK6 is significantly higher when compared to the GDP-loaded form. Indeed, we were unable to calculate affinity values in the presence of GDP, while a K_D of 10 ± 3µM was obtained for GppNHp-Roc-COR (Figure 3).

Therefore, these data suggest that the affinity between isolated PAK6 and Roc-COR recombinant proteins is significantly increased in the presence of GTP analogues, supporting our previous findings from protein arrays and pull-down assays (Beilina et al., 2014; Civiero et al., 2015), and that the affinity of PAK6:Roc-COR interaction falls in the µM range. One essential aspect to consider in future investigations is that data obtained from isolated Roc-COR fragments are not sufficient to rule out the hypothesis that other factors

participate in the modulation of the interaction, e.g. the binding of 14-3-3s to p-Ser935 outside Roc-COR.

PAK6 is unable to modulate LRRK2-mediated RAB phosphorylation in the presence of mutant Roc

We previously demonstrated that, in addition to being a LRRK2-Roc interactor, PAK6 negatively regulates LRRK2 steady-state phosphorylation at Ser935 by phosphorylating a subset of 14-3-3 proteins at Ser58/59 (Civiero et al., 2017). We also reported that overexpression of a constitutively active form of PAK6 (S531N) corrects the neurite outgrowth phenotype of BAC-G2019S neurons only if 14-3-3γ can be phosphorylated at Ser59 (Civiero et al., 2017). This predicts that PAK6 kinase activity should reduce LRRK2 kinase activity. To test this hypothesis, we set out to measure the impact of PAK6 activity on cellular phosphorylation of two well-established RAB substrates: RAB8a and RAB10 (Lis et al., 2018; Steger et al., 2017). To this end, we overexpressed GFP-LRRK2 and 2xMyc-PAK6 S531N in HEK293T and evaluated Flag-RAB8a phosphorylation by PhosTag gels, a technique previously validated by Ito et al., 2016. As shown in Figure 4A, co-expression of PAK6 S531N dramatically reduces Ser935 phosphorylation, as expected (Civiero et al., 2017), and importantly prevents RAB8a phosphorylation. To prove that the effect depends on PAK6 kinase activity, we applied the group II PAKs pan inhibitor PF-3758309 (Murray et al., 2010), which reduced PAK6 activity (monitored by measuring autophosphorylation of Ser560) and rescued RAB8a phosphorylation. We then expanded this observation by assessing the effect of different PD mutations on RAB10 phosphorylation in the presence of PAK6. We co-expressed GFP-LRRK2 WT, the Roc mutant R1441G, the kinase mutant G2019S or the hypothesis-testing, kinase inactive D2017A in the presence of Flag-PAK6 WT, inactive (K436M) or active (S531N) and HA-RAB10 in HEK293T cells. As expected, active PAK6 caused an increase in p-Ser59 14-3-3 and a reduction of LRRK2 p-Ser935. This occurs independently on LRRK2 kinase activity as LRRK2 kinase dead is also dephosphorylated in the presence of PAK6 S531N (Figure 4B-D). Intriguingly, R1441G LRRK2 showed a reduction in p-Ser935 levels when PAK6 was co-expressed, regardless of PAK6 activity. This is an unexpected effect that will require further investigation. The basal phosphorylation at Ser935 of this mutant is reduced as compared to LRRK2 WT (Nichols et al., 2010) and our data additionally suggest it is insensitive to PAK6 kinase activity. Indeed, PAK6 S531N reduces LRRK2-mediated RAB10 phosphorylation only in the presence of LRRK2 WT or G2019S, but not R1441G LRRK2. Considering that the mechanism whereby

PAK6 reduces p-Ser935 is through phosphorylation of Ser59 on 14-3-3 (Civiero et al., 2017), the intrinsically lower Ser935 phosphorylation/14-3-3 binding renders this mutant refractory to PAK6 activity. The relevance of PAK6-mediated regulation of LRRK2 signaling in an endogenous system is currently being investigated.

Taken together, these data disclosed two important findings: PAK6 activity reduces LRRK2 cellular phosphorylation toward RAB proteins but it is inefficient against R1441G LRRK2, likely due to its impaired 14-3-3 binding to p-Ser935. Future studies are required to understand whether the effects observed for LRRK2 R1441G are generalizable to other ROC-Cor mutants.

Discussion

Obtaining a comprehensive overview of LRRK2 biochemical properties, regulatory mechanisms, as well as its interactome and the associated cellular signaling pathways is key to gain deeper understanding about mutations-specific pathological mechanisms, with clear implications from a therapeutic point of view. From a functional perspective, both GTP binding and kinase activity were associated with induced neuronal toxicity (West et al., 2007). However, multiple lines of evidence suggest that mutations in the GTPase and kinase domains operate through different biochemical mechanisms with convergent pathological consequences (Iannotta et al., 2020). This implies that, although kinase overactivation appears to be a common feature among PD-mutations and represents a promising therapeutic target, alternative strategies should also be considered, as they might prove more effective and/or less toxic. Indeed, recent preclinical studies raised some concerns about the potential toxicity of chronic therapies based on LRRK2 inhibition, suggesting that alternative routes to reestablish normal LRRK2 function are desirable. In this respect, we focused here on a comprehensive discussion of the current knowledge about the Roc/GTPase domain of LRRK2 and its importance in mediating protein activity and function. Although pharmacological targeting of GTPases is challenging due to the low selectivity of compounds (Moore et al., 2020; Teng et al., 2021), some reports describe GTP binding inhibitors with positive effects on mutant LRRK2-mediated toxicity (Li et al., 2015, 2014; Thomas et al., 2020, 2017). More recently, nanobodies – 15 kDa single-domain antigen-binding fragments derived from heavy chain only antibodies from Camelids – are being explored as a strategy to trap the protein in specific conformations (e.g. kinase inactive, GTP-bound – Leemans et al., 2020; Soliman et al., 2020). However, the modulation of

specific interactions that are altered during disease seems a more finely tuned strategy to reduce the probability of undesired side effects (Cromm et al., 2015; Soliman et al., 2020). In the case of LRRK2, this is particularly appealing since hundreds of interactions have been described over the years. Bioinformatics strategies to analyze these data are helping to prioritize this significant amount of information, and to direct the studies towards those interactors that are potentially more relevant for LRRK2 pathophysiology based on their biological function, cell- and/or tissue-specificity. The analyses on the LRRK2 interactome performed in this study highlight the LRRK2 Roc domain as an interaction hotspot, and additionally suggest a sub-molecular compartmentalization of the interactions by function, pinpointing cytoskeletal and endolysosomal dynamics, among others, as Roc-specific functions. These data support the hypothesis of LRRK2 regulating intracellular traffic *via* bridging vesicles with the cytoskeleton.

We previously described the Roc-mediated interaction between LRRK2 and the kinase PAK6 (Beilina et al., 2014; Civiero et al., 2017, 2015), that is known to participate in morphogenetic processes via stabilization of the F-actin cytoskeleton. We demonstrated that PAK6 can regulate neurite outgrowth dependently on LRRK2, and that it is protective towards the neurite shortening caused by the LRRK2 G2019S mutation, with a mechanism dependent on the phosphorylation of 14-3-3s and the consequent regulation of Ser935 phospho-state (Civiero et al., 2017, 2015). In this work, we aimed at further characterizing this interaction, exploring i) the biochemical details, *via* affinity measurements and ii) the consequences on signaling cascades, through evaluation of phosphorylation of the robustly characterized substrates RAB8a and RAB10. Using MST, we calculated the affinity of the interaction between the Roc-COR fragment of LRRK2 and PAK6, obtaining values for the dissociation constant (K_D) in the low micromolar range. Of note, MST data support previous cellular data demonstrating a positive impact of GTP on PAK6:LRRK2 binding (Civiero et al., 2015). The recent structural studies describe a very compact but dynamic structure with multiple interdomain interactions, and suggest that the whole protein, or at least additional domains, participate in stabilizing the interaction. In addition, homotypic interactions required for dimer formation – occurring *via* COR, WD40 and, likely, LRR – were described in detail. Therefore, we hypothesize that PAK6 could bind the full-length LRRK2 with higher affinity, and future studies will help dissect this aspect further. Based on the current model, stating that GTP binding leads to monomerization, while hydrolysis induces the protein to re-dimerize (Deyaert et al., 2017), our observation could suggest that PAK6 is preferentially

binding monomeric LRRK2. However, it could also support the idea that assessment of binding affinities in the presence of full-length LRRK2 is an essential requirement, especially in the perspective of evaluating the behavior of mutants and their impact on monomer-dimer equilibrium and K_D. In the case of the LRRK2:PAK6 interaction, this aspect acquires a particular relevance, considering the involvement of 14-3-3 proteins in the signaling axis. Indeed, Roc itself as well as the Ser910/935 clusters were described as 14-3-3s docking sites. Intriguingly, Roc mutations show decreased 14-3-3 binding and dephosphorylation at both these sites, suggesting that they might participate in key pathological mechanisms. On this line, we confirmed our previous findings showing that active PAK6 activity diminishes Ser935 phosphorylation, and further demonstrated that this correlates with a reduction of RAB8a and RAB10 phosphorylation, supporting the idea that PAK6 is a negative regulator of LRRK2 kinase activity. Structurally, the Ser935 phospho-state was proposed to depend on the conformation of the kinase domain – phosphorylation in the open conformation, dephosphorylation in the closed conformation (Watanabe et al., 2020) – which might suggest that one consequence of PAK6 binding to Roc is the stabilization of the closed conformation, although the effect on Ser935 phosphorylation is absent in the presence of kinase-dead PAK6 (K436M), indicating that PAK6 kinase activity is a prerequisite. As schematized in Figure 5, this pathway is impaired in the presence of R1441G LRRK2, whose Ser935 is basally dephosphorylated, and thus insensitive to PAK6-mediated effects on RAB10 phosphorylation. Based on these novel findings, we also confirmed that the protective effects of PAK6 toward increased LRRK2 activity can be achieved only if 14-3-3s are bound to LRRK2, a condition satisfied in G2019S but not in Roc mutants (Figures 4&5, Civiero et al., 2017). This statement is corroborated by our previous findings showing that PAK6 can mediate LRRK2 phosphorylation at Ser935 via 14-3-3γ and neuroprotective mechanisms are mediated by 14-3-3s (Civiero et al., 2017). Moreover, we showed that in the brain, the G2019S kinase and R1441C Roc mutations behave differently in terms of phosphorylation at Ser935, autophosphorylation at Ser1292, and phosphorylation against substrates (i.e. RAB10). Specifically, while G2019S LRRK2 partially maintains Ser935 phosphorylation and shows increased autophosphorylation at Ser1292, but displays physiological levels of p-RAB10, p-Ser935 is completely lost in the presence of the R1441C mutation. Whilst the Roc mutant does not display alterations in autophosphorylation state, phosphorylation of RAB10 is significantly increased, possibly suggestive of an augmented access to the substrate (Iannotta et al., 2020).

In conclusion, these results further support the notion that LRRK2 activity is tightly modulated by complex networks of protein-protein interactions, and suggests a differential mechanism through which PD Roc mutations lead to an overactivation of the LRRK2/RAB8a-10 signaling *via* PAK6. They additionally highlight the importance of assessing LRRK2 function in the cellular context.

Materials & Methods

Bioinformatics analyses

Protein-protein interaction data for LRRK2 were derived from the IntAct database, queried October 2020 (Orchard et al., 2014). This dataset was processed with a focus on human LRRK2 data only and curated entries which contained binding region-specific information (keywords: sufficient binding region; binding-associated region and necessary binding region). Additional LRRK2 protein interactors, AP2A1, AP2B1, AP2S1, CLTB, PAK6 and PRKAR2B, were manually integrated (Civiero et al., 2015; Heaton et al., 2020; Schreij et al., 2015). From this dataset, the shortest LRRK2 binding region required for the interaction was then approximately mapped to the LRRK2 domain structure.

Functional enrichment analysis was performed on two subsets of LRRK2 interactors separately: N-terminal interactors (defined as binding LRRK2 within residues 1-1000; n=14) and Roc-COR interactors (defined as binding LRRK2 within residues 1350-1850; n=79), based on Gene Ontology (GO) Biological Process (BP) annotations using g:Profiler with g:SCS significance threshold (Raudvere et al., 2019). These two GO BP enrichment profiles (obtained October 2020) were then compared with respect to the associated adjusted p-values.

MBP fused LRRK2 Roc-COR domain purification

MBP-LRRK2-Roc-COR, employed during MST measurements, was expressed in *E. coli* BL21(DE3) cells and purified by Dextrin-Sepharose HP using MBP buffer consisted of 20mM HEPES (pH 8), 200mM NaCl, 10% Glycerol, 10mM $MgCl_2$, 5mM 2-mercaptoethanol and 0.5mM GppNHp. The bound proteins were washed using the same buffer supplemented with extra 10mM $MgCl_2$ and 5mM ATP before elution with MBP-MST buffer consisted of 50mM HEPES (pH 8), 800mM NaCl, 25mM $MgCl_2$, 0.25% Tergitol type NP-40, 10mM D-maltose and 0.5mM GppNHp.

Cell culture, transfection, protein purification and pull-down assays

HEK293T cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and 1x penicillin-streptomycin-glutamine (Gibco). pcDNA3 carrying 3xFlag-RAB8a, pCMV5-HA-RAB10, pcDNA3 carrying 3xFlag-tagged PAK6, pCMV-Tag3B-2xMyc-PAK6, pCHMWS-3XFlag-LRRK2 or pDEST53 carrying GFP-LRRK2 were transfected or co-transfected using jetPEI (Polyplus transfection) according to manufacturer's protocol. The following proportions were maintained during co-transfections: 25% RAB, 25% PAK6, 50% LRRK2. 48-hour post-transfection, cells were collected, washed once with 1x phosphate buffered saline (PBS) and lysed for 45min in a buffer consisted of 20mM Tris pH 7.5, 150mM NaCl, 5mM $MgCl_2$, 1mM EDTA, 1mM β-glycerophosphate, 2.5mM sodium pyrophosphate, 1mM sodium orthovanadate, 1x Roche cOmplete protease inhibitor cocktail (EDTA free), and 1% Triton-X100 for cellular experiments (Figure 4) or 0.5% Tween-20 for binding affinity experiments. The lysate was cleared by centrifugation at 20,000 x g for 15min and the supernatant was incubated with either Anti-Flag® M2 agarose beads (Sigma) or GFP-trap (Chromotek) overnight at 4°C with rotation. Afterwards, the beads were washed 10 times in 5 steps of wash buffers with 20mM Tris pH 7.5, 5mM $MgCl_2$, 500mM NaCl and 1% Triton-X100/0.5% Tween-20 to 20mM Tris pH 7.5, 5mM $MgCl_2$, 150mM NaCl and 0.02% Triton-X100/Tween-20. Purified PAK6 was then eluted in the last wash buffer by competition with an excess (150µg/ml) of 3xFlag peptide (Sigma).

Pull-down assays between LRRK2 and 14-3-3 proteins were performed as previously described (Civiero et al., 2017). Briefly, the seven 14-3-3s isoforms were cloned in pET28a(+) vector in fusion with an N-terminal His-tag for expression in *E. coli* cells (by PCR amplification of the sequences from mammalian expression plasmids kindly provided by Prof. Dario Alessi, University of Dundee). 6xHis-14-3-3s were expressed in BL21(DE3) bacterial cells and purified in batch by IMAC with ProBond™ resin (Invitrogen). After elution, imidazole was removed, and buffer was exchanged with PBS using a PD10 desalting column (GE healthcare). After quantification, 3mM dithiothreitol (DTT) was added for long-term storage and proteins were quickly frozen and kept at -80°C. 3xFlag-LRRK2 was transfected into HEK293T cells and immuno-purified using Anti-Flag® M2 agarose beads (Sigma). Cells were harvested at 48h post-transfection and lysed in buffer containing 50mM Tris pH 7.5, 1mM sodium orthovanadate, 5mM sodium pyrophosphate, 50mM sodium fluoride, 0.27M sucrose, 1 mM EDTA, 1x Roche cOmplete protease inhibitor cocktail (EDTA

free), and 1% Triton X-100. Lysates were incubated with Anti-Flag® M2 agarose beads (Sigma) overnight. Flag-purified LRRK2 bound to the resin was incubated for 2h with purified His-14-3-3 isoforms (1µM), rotating. Immunocomplexes were washed three times with lysis buffer supplemented with 0.25M NaCl, resuspended in sample buffer and subjected to SDS-PAGE and western blot (see following paragraph).

SDS-PAGE and western blot

For Western Blot (WB) analysis, 10 to 30µg of total protein samples were resolved on NuPAGE, Novex precast Bis-Tris 4–20% gels (Invitrogen), in MOPS running buffer, NuPAGE, Novex precast Bis-Tris 12% gels (Invitrogen) in MES running buffer (Invitrogen) or 7.5% Tris-glycine polyacrylamide gels in SDS/Tris-glycine running buffer, according to the size resolution required. The resolved proteins were transferred to polyvinylidenedifluoride (PVDF) membranes (Bio-Rad) or nitrocellulose membranes (Whatman), through a Trans-Blot® Turbo™ Transfer System (Bio-Rad). PVDF membranes were subsequently blocked in Tris-buffered saline plus 0.1% Tween (TBS-T) plus 5% non-fat milk for 1 hour at 4°C and then incubated over-night at 4°C with primary antibodies in TBS-T plus 5% non-fat milk. Membranes were then washed in TBS-T (3x10 minutes) at room temperature (RT) and subsequently incubated for 1 hour at RT with horseradish peroxidase (HRP)-conjugated α-mouse or α-rabbit IgG. Blots were then washed in TBS-T (4x10 min) at RT and rinsed in TBS-T, and immunoreactive proteins were visualized using Immobilon® Forte Western HRP Substrate (Merck Millipore) at the Imager Chemi Premium (VWR). Densitometric analysis was carried out using the Image J software or ImageQuant (LiCor). The antibodies used for WB are as follows: polyHistidine, HRP conjugated (A7058 Sigma), LRRK2 p-Ser935 (ab133450 abcam), LRRK2 p-Ser1292 (ab203181 abcam), Total LRRK2 (GFP (7.1 and 13.1 Roche) and N241 Neuromab); PAK6/LRRK2 (Flag M2 or Flag-HRP, F1804 Sigma); p-PAK4/5/6 (Sigma, SAB4504052); Total (HA 6E2 Cell Signaling) and p-RAB10 Thr73 (ab230261 abcam), pan Total (sc-629 Santa Cruz) and p-14-3-3 (Ser58/59) (ab30554 abcam) and Tubulin (11H10 Cell Signaling).

Microscale Thermophoresis (MST)

Human PAK6 was purified as previously described and labelled with red fluorescent dye NT-647-NHS in the Monolith NT protein labelling kit according to manufacturer's protocol. The unreacted dye was removed from labelled PAK6 by the gravity flow desalting column provided in the kit with the MBP-MST buffer. MST were measured by Monolith NT.115

(NanoTemper). Serial dilutions of MBP-LRRK2-Roc-COR were prepared in MBP-MST buffer and mixed with NT-647-NHS labelled PAK6 at a final concentration of 100nM. The mixtures were incubated on ice for 30min, centrifuged at 10,000 x g at R.T. for 1min and loaded into Monolith premium capillaries. LED laser power was set at 35% for fluorescent detection and IR laser power was set at 80% for MST measurements.

Data analysis software

MST data were analysed by PALMIST software (Scheuermann et al., 2016) and the graphs were created by GUSSI software (Brautigam, 2015). Relative band intensities in western blots were measured with the freeware ImageJ software. Graphs and statistical analysis were realized using GraphPad PRISM 7.

Figure legends

Figure 1 – Region-specific protein interactors of LRRK2. (A) LRRK2 protein interactors mapped by binding region to the LRRK2 domain structure. Filled bars indicate approximate binding regions accompanied alongside residue boundaries for the shortest fragment sufficient for binding. Data obtained from the IntAct database (queried Oct 2020; Orchard et al., 2014) with additional curation for the AP2A1, AP2B1, AP2S1, CLTB, PAK6 and PRKAR2B interactions (Civiero et al., 2015; Heaton et al., 2020; Schreij et al., 2015). * indicates precise residues of binding region were unavailable. Pathological (red) and protective (green) variants mapped on the LRRK2 sequence are also shown. (B) Differential enrichment of Gene Ontology (GO) Biological Process (BP) terms associated with N-terminal vs Roc-COR LRRK2 protein interactors. Dots correspond to specific GO BP terms; dashed lines indicate statistical significance threshold for enrichment, p=0.05; analysis performed using g:Profiler with g:SCS significance threshold (performed Oct 2020; Raudvere et al., 2019). (C) Pull-down assay between 3x-Flag-LRRK2 purified from HEK293T cells and the seven 14-3-3 isoforms purified from *E. coli* by IMAC reveals that 14-3-3γ and η bind LRRK2 with the highest affinity.

Figure 2 – Time-course of Lrrk2 and Pak6 expression during postnatal development. Expression levels of Lrrk2 and Pak6 were assessed in WT and BAC-G2019S mouse brain lysates from postnatal day 0 (P0) to P28.

Figure 3 – Affinity measurements of the interaction between LRRK2 Roc-COR and PAK6. Microscale thermophoresis of fluorescently labelled PAK6 (100nM) against MBP-

fused LRRK2 Roc-COR domain (16 nM to 64 µM). Upper panel: The normalized fluorescence intensity of all MST traces is plotted against different concentrations of MBP-LRRK2-Roc-COR. Lower panel: The change of relative fluorescence during thermophoresis. Data was analyzed by PALMIST software and the graphs were created by GUSSI software (Brautigam, 2015; Scheuermann et al., 2016). The calculated K_D is 10 ± 3 µM. Error bars show the S.D. of three measurements. The residuals between the data and the fit are shown at the bottom of the graph.

Figure 4 – Modulation of LRRK2-mediated RAB phosphorylation by PAK6 kinase activity. (A) GFP-LRRK2 and Flag-RAB8a were co-expressed in HEK293T cells in the absence or presence of 2xMyc-PAK6 S531N (hyperactive). A pan inhibitor of type II PAKs was used to verify that the observed reduction in RAB8a phosphorylation is PAK6-mediated. (B) Plasmids expressing GFP, GFP-LRRK2 wild-type (WT), LRRK2 kinase inactive (D2017A), GFP-LRRK2-G2019S and GFP-LRRK2-R1441G along with HA-Rab10 were transfected into HEK293T cells along with plasmids encoding PAK6 variants WT, kinase inactive (K436M) or hyper-active (S531N). Triton-X100 soluble fractions were analyzed for Total LRRK2 (GFP and N241) and LRRK2 p-Ser935; PAK6 (Flag); Total (HA) and p-RAB10 Thr73, Total and p-14-3-3 (Ser59) and Tubulin. IN=inclusion of 1mM MLi2 in the culture media for 2hrs. (C-D) Quantitation of phospho vs total set to WT LRRK2 co-expressed with RAB10 and Flag empty vector (N=at least 3 ± SEM; one-way ANOVA with multiple comparisons; *p≤0.05; **p≤0.005; ***p≤0.0005; ****p≤0.0001).

Figure 5 – Proposed effect of PAK6 kinase activity on LRRK2 mediated RAB phosphorylation. In the absence of active PAK6, LRRK2 can phosphorylate RAB proteins upon recruitment to specific subcellular compartments. Both the G2019S kinase and R1441G Roc mutants display increased phosphorylation of RAB, although the mechanisms are different (Iannotta et al., 2020) (upper panel). In the presence of PAK6, RAB phosphorylation is reduced through a mechanism involving PAK6-mediated phosphorylation of 14-3-3s (Civiero et al., 2018, 2017), which is effective only in the context of LRRK2 WT

and G2019S, whose Ser935 is phosphorylated, but not for the R1441G LRRK2, which is significantly dephosphorylated at Ser935 (lower panel).

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Highlights

- The Roc/GTPase domain of LRRK2 is a biochemical and molecular signaling hub
- Most of LRRK2 interactome binds Roc
- The spatial separation of binding partners within the LRRK2 structure correlates with a functional specialization
- PAK6 is a GTP-dependent Roc interactor able to modulate LRRK2-mediated RAB phosphorylation
- Parkinson's mutations within Roc impair the ability of PAK6 to regulate LRRK2 activity