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Review Article

Cellular effects mediated by pathogenic LRRK2: homing in on Rab-mediated processes

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Leucine-rich repeat kinase 2 (LRRK2) is a key player in the pathogenesis of Parkinson's disease. Mutations in LRRK2 are associated with increased kinase activity that correlates with cytotoxicity, indicating that kinase inhibitors may comprise promising disease-modifying compounds. However, before embarking on such strategies, detailed knowledge of the cellular deficits mediated by pathogenic LRRK2 in the context of defined and pathologically relevant kinase substrates is essential. LRRK2 has been consistently shown to impair various intracellular vesicular trafficking events, and recent studies have shown that LRRK2 can phosphorylate a subset of proteins that are intricately implicated in those processes. In light of these findings, we here review the link between cellular deficits in intracellular trafficking pathways and the LRRK2-mediated phosphorylation of those newly identified substrates.

Introduction

Mutations in the *leucine-rich repeat kinase 2* (*LRRK2*) gene are the most common cause of familial autosomal-dominant Parkinson's disease (PD), and sequence variants in LRRK2 are known to alter PD risk [1]. While only one pathogenic LRRK2 mutation increases LRRK2 kinase activity *in vitro*, all mutations seem to converge on increasing substrate phosphorylation when assayed in intact cells [2]. Such excess kinase activity is known to cause neurotoxicity, which can be reversed by pharmacologically or genetically decreasing kinase activity [3]. These findings suggest that the kinase activity of LRRK2 may constitute a promising disease-modifying target for LRRK2-related PD, and possibly for the entire PD disease spectrum. Towards this end, highly potent, selective and brain-permeable LRRK2 kinase inhibitors have been developed, but recently reported on-target side effects in peripheral tissues highlight the need for a better understanding of the possible tissue- and cell type-specific role(s) of LRRK2 kinase activity [4]. Here, we discuss the most consistently reported LRRK2-mediated intracellular membrane trafficking deficits in the context of a set of proteins intimately implicated in those events.

LRRK2 and modulation of vesicle trafficking

The observation that LRRK2 is expressed in many tissues [5] is consistent with an important role for this protein common to various cell types. Indeed, a wealth of studies in distinct cell lines, patient-derived cells and animal models indicate that LRRK2 plays an important role in macroautophagy, a process by which cytosolic components, such as damaged organelles and aggregated proteins, are engulfed by special double-membraned vesicles called autophagosomes that subsequently fuse with amphisomes and/or lysosomes for subsequent degradation of engulfed components [6]. Most reports are consistent with pathogenic LRRK2 causing a deficit in the autophagic–lysosomal protein degradation system [7–10], in agreement with the neuropathological accumulation of autophagic vacuoles observed in PD patients [11]. Pathogenic LRRK2 has also been described to affect the perinuclear positioning of endolysosomes that is crucial for efficient flux through the endocytic and autophagic systems,

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retromer-mediated trafficking from the late endosome to the Golgi, as well as clathrin-mediated endocytic events [12–20]. A set of proteins, including Rab7 and Rab9, have been shown to be important for most of the above-mentioned membrane trafficking events [3], raising the possibility that pathogenic LRRK2 may cause those cellular alterations by changing Rab functioning.

The LRRK2–Rab protein link

Indeed, various studies have suggested a link between pathogenic LRRK2 and Rab proteins. Rab proteins comprise the largest family of small GTPases, with over 60 members identified in humans. They are localized to distinct intracellular membranes and are crucial modulators of vesicle budding, tethering, transport and fusion [21–23]. They bring about those events by recruiting distinct sets of effector proteins in a manner dependent on their nucleotide-bound state. Therefore, they have also been termed 'molecular switches', with a GTP-bound active 'on' state and a GDP-bound inactive 'off' state.

LRRK2 can interact with several Rab proteins including Rab5b, Rab7, Rab7L1, Rab32, Rab38, Rab8a, Rab10 and Rab12 [2,12–15,24]. Such interactions may serve to recruit LRRK2 to distinct subcellular organelles to phosphorylate select substrate proteins involved in membrane trafficking events to and from that specific Rab-containing compartment [3]. However, only one study addressed whether the distinct pathogenic LRRK2 mutants may differentially interact with the specific Rab protein when compared with wild-type LRRK2, and at least for Rab7L1, no differences in affinity were observed [15].

LRRK2 has also been described to alter the activity of certain Rab proteins. Studies using overexpression of mutant Rab proteins designed to mimick the GTP-bound (active) or GDP-bound (inactive) forms suggest that pathogenic LRRK2 decreases the amount of active Rab7 and Rab9 [13,16,17]. Rab9 has arisen from a gene duplication event of Rab7 [25] and is involved in various late endosomal trafficking steps largely overlapping with those modulated by Rab7 [26]. Since mutations tailored to mimick the GTP-bound state do not always behave like active Rab protein versions, as recently shown for the case of Rab7L1 [15], we used pulldown assays with a specific effector protein that only binds to Rab7 when in its active, GTP-bound form. Indeed, studies of this type revealed that distinct pathogenic LRRK2 mutants decrease the amount of endogenous, active Rab7 without effects on overall protein levels and consistent with the observed LRRK2-mediated trafficking deficits in and out of the late endosome [16].

A recent seminal report identified a subset of Rab proteins as bona fide LRRK2 kinase substrates *in vivo*, with the most prominent ones being Rab3a, Rab8a, Rab10 and Rab12 [2]. Increased phosphorylation mediated by pathogenic LRRK2 was not associated with altered steady-state protein levels, and in all cases was mapped to an evolutionarily conserved residue in a region of Rab proteins called the switch II region. This region undergoes a conformational change upon GTP binding and is crucial for the interaction of the Rab proteins with many of their regulatory proteins [27]. Phosphorylation in the switch II motif was shown to cause a deficit in the interaction of Rab proteins with several regulatory proteins in a manner largely consistent with a predicted loss-of-function phenotype [2].

While the long-awaited identification of very clear LRRK2 kinase substrates demarcates a milestone in the LRRK2–PD field, it also opens up many questions. Since these studies were performed in fibroblasts, it will be important to corroborate that Rab proteins can serve as LRRK2 kinase substrates in disease-relevant cells such as neurons. Furthermore, it is currently unknown which of the many Rab proteins can be efficiently phosphorylated by LRRK2 *in vivo*, and under which cellular conditions. At least *in vitro*, threonine-containing Rabs were found to be more potently phosphorylated when compared with serine-containing Rabs, confirming the previously reported *in vitro* preference of LRRK2 for threonine residues [28]. Interestingly, alignment of the switch II regions of the various human Rab proteins with respect to the currently identified Rab substrates correlates with the presence of TITT, TITS or SITS (T, threonine; I, isoleucine; S, serine), with the first residue corresponding to the phosphorylatable residue (Figure 1). Based on this, it will be interesting to determine whether these sequences indeed contribute to the substrate preference of LRRK2 for distinct Rab proteins *in vitro*, or whether Rab13 or Rab35 comprise other LRRK2 kinase substrates *in vitro* and possibly *in vivo*.

Importantly, the observation that Rab12 is a poor LRRK2 kinase substrate *in vitro*, but a genuine substrate *in vivo* [2], highlights the existence of additional mechanisms in intact cells that govern the ability of a Rab protein to serve as a kinase substrate. This could be related to altered substrate recognition of LRRK2, for example dependent on the nucleotide-bound status of the Rab protein, or dependent on it being complexed to other proteins. Alternatively, it could be due to a requirement for prior phosphorylation events mediated by cellular kinases and on sites distinct from the LRRK2 phosphorylation site. In this manner, a variety of

Biochemical Society Transactions (2017) **45** 147–154
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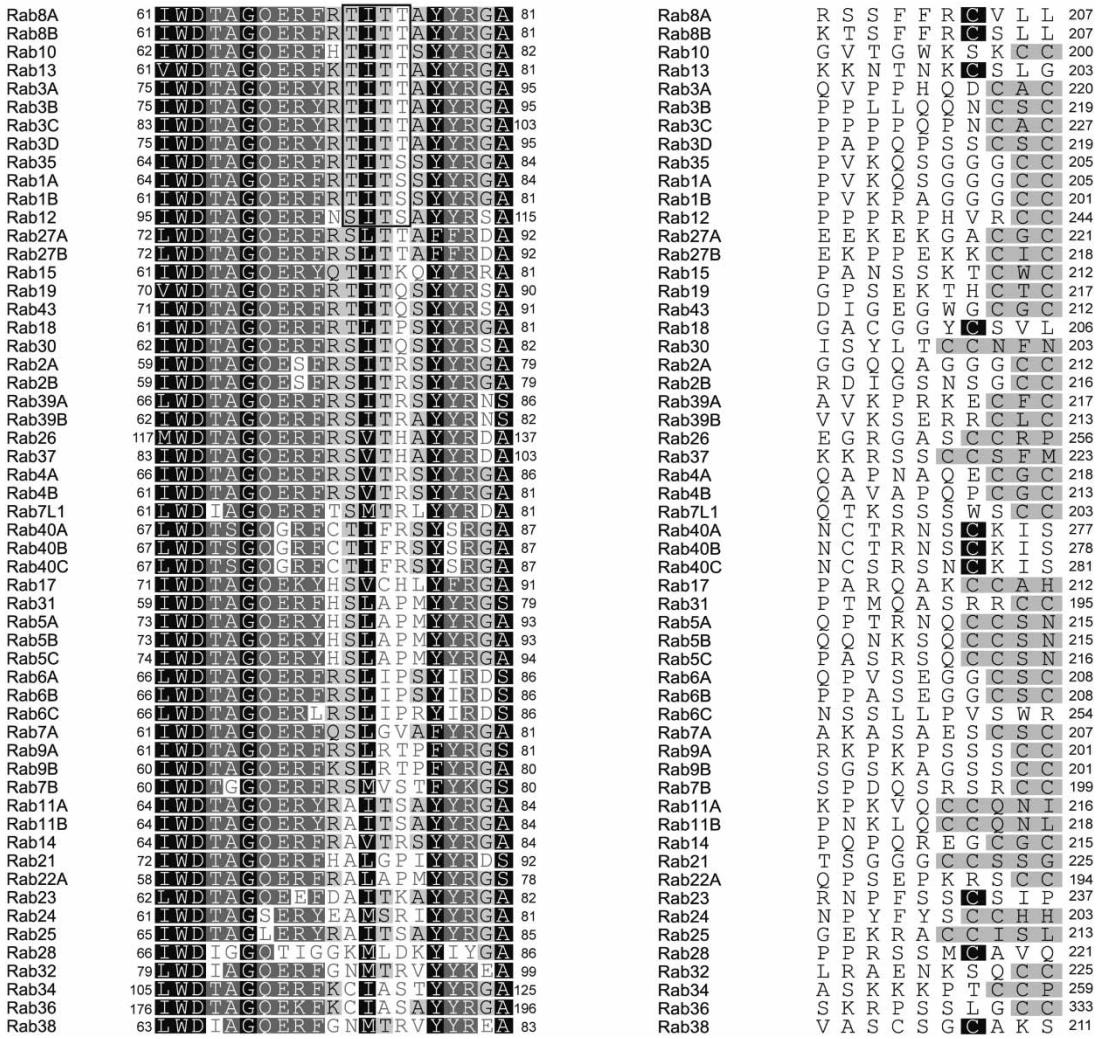

Figure 1. Alignment of human Rab sequences.

Left: sequences were aligned with respect to the switch II region and with respect to the identified *in vitro* and *in vivo* substrates (Rab8a, Rab10, Rab3a and Rab12). Black, 100% similarity; dark gray, 80–100% similarity; light gray, 60–80% similarity; white, <60% similarity. Sequences were ordered according to the presence of T/S-I-T-T/S, starting with T as the phosphorylatable residue given the reported preference for LRRK2 to phosphorylate residues *in vitro*, followed by T/S-L-T-T/S, T-I/L-T and S-I/V/M-T, and finally just containing T, S or no phosphorylatable residue, respectively. Predicted substrates for LRRK2 phosphorylation *in vitro* are boxed. Right: same sequence alignment depicting the C-terminal residues of the respective Rab proteins. The consensus sites for prenylation by GGTII are boxed in gray, with generally both C (cysteine) residues being modified with geranylgeranyl groups. Few Rab proteins contain only one C residue (black) and are thought to be monoprenylated by GGTI.

extracellular and intracellular signaling cascades may alter the efficacy by which a subset of Rab proteins may undergo subsequent LRRK2-mediated phosphorylation. The distinct types of signals, combined with the presence of different subsets of Rab proteins, would then allow for tissue- and even cell type-specific effects. Several Rab proteins have been reported to be phosphorylated by a variety of cellular kinases and on sites different from the LRRK2 phosphorylation site, even though most of the reported phosphorylation events are related to cell cycle-dependent changes in Rab localization [21]. Of note, Rab8a, Rab8b and Rab13 have recently also been reported to be phosphorylated on a site distinct from the LRRK2 phosphorylation site and in a manner dependent on PINK1 (phosphatase and tensin homolog (PTEN)-induced putative kinase 1) kinase activity, even though not directly mediated by PINK1 [29]. Unfortunately, it is currently unknown whether the

Biochemical Society Transactions (2017) 45 147–154
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PINK1-regulated phosphorylation of Rab8 alters its capacity to serve as LRRK2 kinase substrate and vice versa. However, phosphorylation of Rab8 by either PINK1-mediated events or by LRRK2 kinase activity seems to both result in decreased Rab8 activation [2,29]. As it is the loss of PINK1 that causes an autosomal-recessive form of PD [30], this leads to the apparently contradictory finding that decreased phosphorylation of Rab8 upon PINK1 depletion activates, and increased phosphorylation of Rab8 by pathogenic LRRK2 kinase activity inactivates the protein, with both events linked to PD pathogenesis. While many scenarios are possible, at least under certain cellular conditions, loss of PINK1-mediated phosphorylation and increased LRRK2-triggered phosphorylation may cause common alterations in the subcellular localization and/or altered protein interactions in a manner not strictly regulated by the nucleotide-bound activity status of Rab8 (see also below).

Since steady-state phosphorylation levels of a protein are the result of a balance between kinase and phosphatase activities, the phosphatases that dephosphorylate the LRRK2 phosphorylation site in the distinct Rab proteins also warrant further investigation. Indeed, the fast dephosphorylation of Rab10 observed upon LRRK2 kinase inhibition indicates that its steady-state phosphorylation levels are under tight control by currently unknown phosphatases [31]. These may even include noncanonical phosphatases such as PTEN, a lipid phosphatase with weak protein phosphatase activity. Interestingly, PTEN has recently been shown to be required for trafficking of the epidermal growth factor receptor (EGFR) from early to late endosomes by dephosphorylating Rab7 on the equivalent predicted LRRK2 phosphorylation site in the switch II region, and such dephosphorylation seems necessary for Rab7 recruitment and subsequent maturation of the late endosome [32].

The Rab GTPase cycle and possible effects of LRRK2-mediated Rab phosphorylation

Given the universal importance of Rab proteins for all distinct intracellular membrane trafficking steps, the regulation of their localization and activation has been studied in great detail [21,33–35]. Once a Rab protein is synthesized, it is post-translationally modified at the C-terminus by prenyl groups that function as membrane anchors. Most Rab proteins contain a variety of prenylation motifs such as CC, CXC, CCX, CCXX and CCXX, with C for cysteine and X for any amino acid (Figure 1), and generally both cysteine residues are modified by geranylgeranyl groups. The vast majority of Rab proteins are isoprenylated by geranylgeranyl transferase type II (GGTII). The prenylation motifs of the Rab proteins are insufficient for substrate recognition by GGTII, but require prior binding of unprenylated Rabs to the Rab escort proteins (REP1/2) [34]. GGTII then binds to this complex, lipid groups are transferred, GGTII dissociates and the REP/Rab protein complex is delivered to cellular membranes. The initial membrane association of newly synthesized Rab proteins is regulated by REP proteins, but subsequent regulation of membrane association is subject to the action of Rab GDP dissociation inhibitor (GDI1/2), a protein capable of delivering and extracting prenylated Rab proteins from membranes.

Once loosely targeted to a membraneous environment by either REP1/2 or GDI1/2, additional mechanisms contribute to the proper subcellular localization of the Rab proteins. On the one hand, once inserted into the membrane, they can become activated by their respective guanine nucleotide exchange factors (GEFs). The high cytosolic concentrations of GTP ensure that GTP binds to the Rab protein as soon as GDP has been released, thus allowing conversion into an active form resistant to membrane extraction and capable of interacting with various effector proteins [21]. However, as many GEFs seem to be largely cytosolic, other mechanisms must exist to prevent activation of Rab proteins in inappropriate membrane compartments. At least in some cases, proper membrane targeting has been described to involve additional interactions of the hypervariable C-terminal domains of the Rab proteins with specific effector proteins, and/or the action of GDI displacement factors (GDFs), a family of proteins that are localized to distinct intracellular membrane compartments and display preference for a subset of Rab proteins to facilitate GDI1/2 dissociation and concomitant membrane insertion [33].

Upon insertion into their target membrane and conversion into their active, GTP-bound form by their respective GEFs, Rab proteins then act upon their effector proteins to bring about compartment-specific membrane trafficking events. Given their low intrinsic GTPase activity, conversion to their inactive forms is catalyzed by GTPase-activating proteins (GAPs), and almost 40 distinct GAPs with limited specificity for distinct Rab proteins have been described [21]. Finally, once in their GDP-bound forms, Rabs lose their affinity for effector molecules and display increased affinity for GDI1/2, which is followed by membrane extraction.

Superimposed onto this Rab GTPase-mediated cycle crucial for virtually all intracellular membrane trafficking steps, what then may be the possible consequences of pathogenic LRRK2-mediated Rab phosphorylation? Phosphorylation of Rab proteins by LRRK2 occurs in the switch II region that is important for nucleotide

binding. Indeed, both switch I and II regions are disordered in the GDP-bound form of Rab proteins, but adopt an ordered state upon GTP binding [27]. It will be important to determine whether LRRK2 differentially phosphorylates the switch II region of GDP-bound (inactive) versus GTP-bound (active) Rab proteins. In either case, analysis of phosphomimetic versus phosphodeficient Rab mutants revealed a drastically reduced interaction with both GDI1/2 and GEF, and these findings have been the basis for a model in which LRRK2 may cause accumulation of inactive, phosphorylated Rabs in their membraneous target compartments (Figure 2) [2].

While not analyzed in detail, some data also suggest that phosphorylated Rabs may differentially interact with GGTII and REP1/2 [2]. This is not unexpected, as REP1/2, GDI1/2 and GEFs are all known to interact with Rab proteins in ways involving the switch II region [27,33]. However, if independently confirmed, this finding may have important implications for the fate of the phosphorylated Rab protein in terms of subcellular localization, as LRRK2-mediated phosphorylation of a newly synthesized Rab protein may interfere with its binding to REP1/2 for prenylation by GGTII, resulting in hypoprenylation and accumulation in the cytosol (Figure 2).

Phosphorylation and inactivation of membrane-localized Rab proteins may also cause accumulation of the phosphorylated protein in distinct membraneous and/or nonmembraneous compartments. Such alterations in subcellular localization may be especially prominent with Rab proteins that are monoprenylated, as this is thought to correlate with looser membrane association [36]. Only a handful of Rab proteins are monoprenylated, including Rab8a, Rab8b and Rab13, with Rab8a determined to be a LRRK2 kinase substrate (Figure 1) [2]. Consistent with the idea that these Rab proteins may be especially prone to mislocalization, chimeric Rab proteins containing a Rab8 prenylation sequence have been reported to mislocalize to the endoplasmic reticulum (ER) [37], and inactive Rab13 has been shown to associate with nontarget membraneous vesicles via protein–protein interactions [38].

In summary, LRRK2-mediated phosphorylation of Rab proteins may have complex effects on their subcellular localization, including an increase in newly synthesized hypoprenylated proteins in the cytosol, accumulation in

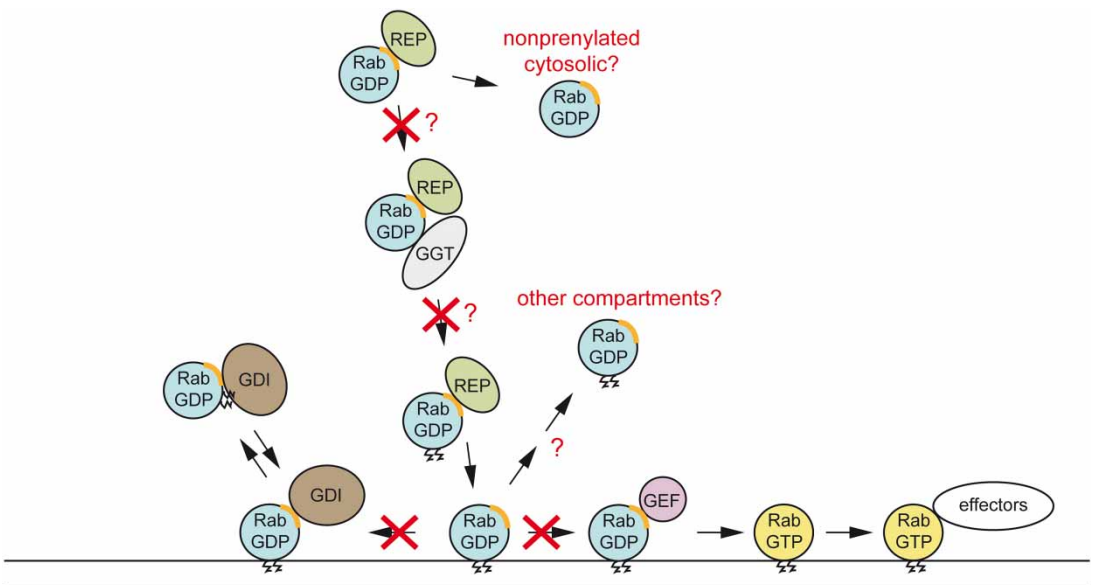

Figure 2. Possible fates for Rab proteins upon LRRK2-mediated phosphorylation.

Upon synthesis, inactive Rab proteins are bound to REP via interactions involving the switch II region (orange). This complex is recognized by GGT that transfers usually two prenyl groups. GGT then dissociates, and the prenylated Rab–REP complex is delivered to membranes. GEF can bind via interactions involving the switch II region and convert the Rab protein into its active, GTP-bound form capable of recruiting effector proteins. If not activated by GEF, inactive Rab proteins bind to GDI1/2 via interactions involving the switch II regions and are reversibly extracted from membranes. LRRK2-mediated phosphorylation may interfere with the Rab–REP interaction, causing accumulation of nonprenylated Rab proteins in the cytosol. Alternatively, loose membrane association, especially of monoprenylated Rab proteins, may cause additional recruitment of the inactive Rab protein to other membraneous and/or nonmembraneous compartments to interfere with their proper functioning. For simplicity, the actions of GDF and GAP are not depicted. For further details see text.

Biochemical Society Transactions (2017) 45 147–154
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proper target membranes, as well as possible accumulation in nontarget compartments (Figure 2). The current hypothesis poses that LRRK2-mediated Rab phosphorylation leads to a loss of function of the specific Rab protein with downstream effects on the membrane trafficking pathway it controls, but aberrant accumulation of phosphorylated inactive Rab proteins in other locations may cause additional toxic gain-of-function phenotypes due to such mistargeting and the resulting effects on other cellular pathway(s). Therefore, it will be important to determine the precise subcellular localizations of the individual phosphorylated Rab proteins in future studies.

Without discarding the existence of other physiologically relevant LRRK2 kinase substrates, the observations that LRRK2 can bind, phosphorylate and alter the activity of a subset of Rab proteins certainly make a strong case for a causal link between increased LRRK2 kinase activity and defective membrane trafficking pathways due to aberrant Rab protein function. Towards understanding pathomechanisms underlying PD, determination of the pathologically most relevant substrates will be crucial. This is not an easy task, as substrates may be phosphorylated to similar degrees also in tissues not affected by the disease process, but pathogenesis may only manifest itself in cells which most heavily rely on the type of trafficking pathway impaired by LRRK2-mediated Rab phosphorylation and inactivation. This is nicely illustrated in the case of autophagy, a process clearly affected in PD and which is especially important for neuronal survival [39]. Thus, it will be important to determine which intracellular membrane trafficking pathways are most relevant to pathomechanisms and which Rab proteins contribute to the proper functioning of those pathways by using RNAi or overexpression approaches, respectively.

Maybe related to this, it remains unknown how many Rab proteins need to be phosphorylated by LRRK2 to cause a PD phenotype. Given the highly interconnected nature of intracellular membrane trafficking pathways, only a minimal amount of substrates may be necessary to cause the observed phenotypes. This is exemplified in the case of Rab7 or Rab9, deficits in which can cause impairments in endosome–lysosome trafficking, autophagosome–lysosome trafficking and retromer-mediated trafficking between the late endosome and Golgi complex, respectively [3,24,39]. In addition, the existence of so-called Rab cascades predicts that dysfunction of one Rab protein can cause deficits in the vectorial nature of entire trafficking cascades. Such cascades arise, for example, when an upstream acting Rab protein recruits the GEF for the later acting Rab protein [40]. In this context, it will be important to determine whether the distinct LRRK2-phosphorylated Rab proteins differentially interact with GEFs with consequences for the respective downstream vesicular trafficking events.

LRRK2-linked PD: another disease of Rab proteins?

Rab protein dysfunction has been linked to a variety of human diseases. For example, mutations in Rab18 cause Warburg Micro Syndrome [41], a developmental disorder characterized by brain, eye and endocrine abnormalities, whereas mutations in Rab23 cause Carpenter Syndrome characterized by various congenital malformations [42]. Mutations in Rab7a are associated with Charcot-Marie-Tooth type 2B, a disease causing neuropathy [43], and mutations in Rab27a cause Griscelli Syndrome type 2 characterized by hypopigmentation and immune system abnormalities [44]. Finally, mutations in GDI1 are known to cause a rare form of nonspecific X-linked intellectual disability [45], whereas mutations in REP1 cause choroideremia (CHM), an X-linked chorioretinal degeneration characterized by blindness [46].

Given the universal importance of Rab proteins in intracellular membrane trafficking, the causal link between Rab protein dysfunction and disease is not surprising. What is striking though are the vastly different and even tissue-specific phenotypes. In some cases, such specificity may be overestimated, as severe clinical phenotypes manifesting early in life are incompatible with determination of the possible importance of a given Rab protein for other tissue types at later time points. For example, deficiency in REP1, which causes Rab hypoprenylation, is associated with an eye-specific phenotype. This is likely due to partial compensation by REP2, leaving only a subset of Rab proteins unprenylated and thus nonfunctional. One of those is Rab27a, highly expressed in the same layers of the eye which degenerate in CHM patients. While Rab27a is also pronouncedly expressed in other tissues that are unaffected in CHM patients, the action of REP2, or indeed other Rab proteins, may compensate for Rab27a function in those tissues [34]. In contrast, patients with Griscelli syndrome type 2 due to mutations in Rab27a may not display a retinal degeneration phenotype due to short life expectancy.

By analogy, the LRRK2-mediated phosphorylation and concomitant inactivation of a subset of Rab proteins may manifest as a PD-specific phenotype, with compensation by redundant and functionally overlapping Rab proteins in other tissues. In this context, the implications of other Rab proteins for PD may be particularly revealing. For example, both Rab7L1 and Rab39b have been genetically linked to PD [14,15,47], and several Rab proteins, including Rab1, Rab3a, Rab8a and Rab11, are known to modulate α-synuclein-mediated aggregation

and toxicity in cellular or animal models of PD [48–50]. Thus, determining the mutual cross-talk between those Rab proteins with respect to membrane trafficking deficits specifically affected in PD will be crucial.

Concluding remarks

The major intracellular role of LRRK2 seems related to vesicular membrane trafficking, especially those related to endocytosis and autophagic clearance of defunct organelles and/or protein aggregates by macroautophagy. It has become clear that LRRK2 can bind to various Rab proteins with possible alterations in its localization, and that it can interfere with Rab activity in some cases mediated by direct phosphorylation. Dissecting out the precise membrane trafficking steps whose impairments underlie cellular demise, in combination with identifying the subset of Rab proteins whose phosphorylation and inactivation contribute to cellular pathogenicitiy, may allow for detailed molecular insights into the disease process, and thus for the safe translation of LRRK2 kinase inhibitors into clinical trials.

Abbreviations

CHM, choroideremia; EGFR, epidermal growth factor receptor; ER, endoplasmic reticulum; GAP, GTPase-activating protein; GDF, guanine nucleotide exchange factor; GDI, GDP dissociation inhibitor; GEF, guanine nucleotide exchange factor; GGT, geranylgeranyl transferase; LRRK2, leucine-rich repeat kinase 2; PD, Parkinson's disease; PINK1, PTEN-induced putative kinase 1; PTEN, phosphatase and tensin homolog; REP, Rab escort protein.

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Competing Interests

The Authors declare that there are no competing interests associated with the manuscript.

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Biochemical Society Transactions (2017) **45** 147–154
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