Chapter 6

Regulation of LRRK2 activation, localization and dimerization

Laura M. Nederveen-Schippers*, Susanne Terheyden*, Panagiotis S. Athanasopoulos, and Arjan Kortholt

*equal contribution

This chapter is an invited review in Biological Chemistry, under review.

LN wrote the paragraphs about LRRK2 function, the membrane association and the oligomeric state. ST wrote the paragraphs about PD mutations, regulation and function of the G-domain, the crosstalk between G-domain and kinase domain and regulation of LRRK2 dimerization. AP wrote the paragraph about the regulation of LRRK2 localization. All authors wrote the conclusion and discussion, read and edited the manuscript.
Regulation of LRRK2 activation, localization and dimerization

Laura M. Nederveen-Schippers*, Susanne Terheyden*, Panagiotis S. Athanasopoulos and Arjan Kortholt

From the Department of Cell Biochemistry, University of Groningen, Nijenborgh 7, 9747 AG Groningen, The Netherlands.
*equal contributed to this work

Running title: LRRK2 regulation and its effect in PD pathology

Abstract

Leucine-rich-repeat kinase 2 (LRRK2) is a large multi domain protein with two enzymatic functions: kinase and GTPase activity. Mutations in LRRK2 are accounting for the majority of genetically inherited Parkinson’s Disease. Many LRRK2 mediated pathways and interaction partners have been identified, however the cellular functions of LRRK2 and LRRK2 mediated progression of PD are still not completely understood. PD-linked mutations in LRRK2 are found in nearly every domain. Importantly, most of these mutations have been linked to increased kinase and/or reduced GTPase activity. Accumulating data suggest that LRRK2 has a complex activation mechanism, involving multiple layers of regulation. Here we highlight the recent data available on LRRK2 intra-molecular signaling, dimerization and protein-protein interactions and discuss how these processes together regulate LRRK2 signaling and affect LRRK2-mediated PD.

Keywords: GTPase activity, kinase activity, Leucine Rich Repeat Kinase 2, oligomerization, Parkinson’s Disease, Roco proteins
Introduction

Parkinson’s disease (PD) is a progressive motor disorder, caused by the degeneration of dopaminergic neurons in the midbrain. The prevalence of PD increases with age, with 2% of individuals over the age of 80 being affected worldwide (Pringsheim et al., 2014). Key features of PD are tremor, stiffness and postural instability, as well as the formation of Lewy bodies, which are protein aggregates consisting of α-synuclein, Leucine Rich Repeat Kinase 2 (LRRK2) and other proteins (Spillantini et al., 1997; Zhu et al., 2006). PD is mostly sporadic, without a clear cause, but genome wide association studies have shown that at least 10% of the cases can be explained by Mendelian heredity (Lesage and Brice, 2012). PD-associated mutations have been found in several genes, including SNCA/α-synuclein, PINK1, and DJ-1 (Clarimón and Kulisevsky, 2013; Spatola and Wider, 2014). The most frequently mutated gene is LRRK2, which is causing autosomal dominant forms of PD (Paisán-Ruíz et al., 2004; Zimprich et al., 2004; Nalls et al., 2011; Sharma et al., 2012; Sundal et al., 2012). Interestingly, the symptoms of LRRK2-related PD and sporadic PD are very similar (Healy et al., 2008; Alcalay et al., 2013) and therefore insight into LRRK2 function might help understanding Parkinson’s disease in general.

LRRK2 is a large, ~280 kD, multi-domain protein that belongs to the Roco family of proteins (Bosgraaf and Van Haastert, 2003). The catalytic core region of LRRK2 consists of a Ras-of-complex proteins (Roc) G-domain, a C-terminal-of-Roc (COR) dimerization domain, and a kinase domain (Figure 1). This region is flanked by protein interaction domains: armadillo repeats (ARM), ankyrin repeats (ANK) and leucine rich repeats (LRR) at the N-terminus, and a WD40 repeat domain at the C-terminus (Mills et al., 2014). In almost all LRRK2 domains PD-related mutations have been identified, among which the most common are R1441C/G/H, Y1699C, I2012T, G2019S and I2020T, as well as the risk factors R1628P and G2385R (Figure 1 + 2) (Paisán-Ruíz, 2009; Dächsel and Farrer, 2010; Paisán-Ruíz et al., 2013).

LRRK2 has been linked to a wide range of biological processes (Boon et al., 2014; Wallings et al., 2015; Roosen and Cookson, 2016; Rosenbusch and Kortholt, 2016; Tang, 2016a). Importantly, Rab proteins were recently identified as the first physiological substrates of LRRK2 and accumulating evidence suggests that this LRRK2/Rab pathway functions at the interface of vesicular trafficking and autophagy (Cookson, 2016; Steger et al., 2016; Tang, 2017). At the molecular level, it has been found that several PD mutations cause increased LRRK2 kinase activity and/or decreased GTPase activity (West et al.,
2005, 2007; Greggio et al., 2006; Jaleel et al., 2007; Luzón-Toro et al., 2007; Aasly et al., 2010; Sheng et al., 2012; Ho et al., 2016). LRRK2 activation is regulated at multiple levels, involving both intra- and inter-molecular interactions (Greggio et al., 2006, 2009; Pelech, 2006; West et al., 2007; Ito et al., 2007; Sen et al., 2009; Taymans et al., 2011; Webber et al., 2011; Liu et al., 2011a, 2016; Taymans, 2012; Biosa et al., 2013; Waschbüsch et al., 2014; Schapansky et al., 2014; Athanasopoulos et al., 2016; Shu et al., 2016). Furthermore, LRRK2 dimerization and localization at membranes are key for proper LRRK2 functioning (Greggio et al., 2008; Klein et al., 2009; Sen et al., 2009; Berger et al., 2010; Nichols et al., 2010). In this review we highlight the latest insights into the complex regulatory mechanism of LRRK2 activity and discuss how the different levels of regulation impact LRRK2-mediated PD.

**Figure 1:** Schematic LRRK2 domain topology. PD mutations (black) and risk factors (red) are indicated, as well as pathways influenced by LRRK2 via Rab proteins.

**Cellular function of LRRK2**
Over the last decade, LRRK2 has been implicated in the regulation of a pleiotropy of cellular processes, including apoptosis, synthesis and trafficking of vesicles, cytoskeleton dynamics, mitochondrial function, oxidative stress, protein translation and degradation, autophagy, and inflammation (Daniëls et al., 2011a; Tsika and Moore, 2013; Dzamko et al., 2014; Esteves et al., 2014). More recent data strongly suggests that LRRK2 regulates the balance in vesicular dynamics, which involves endocytic vesicular trafficking and autophagy (Roosen and Cookson, 2016; Tang, 2016a). One of the functions of
autophagy is the clearance of protein aggregates such as α-synuclein and tau aggregates (Wang et al., 2010), which may be important for protection against PD. LRRK2 localizes to autophagic vacuoles (Plowey et al., 2008; Alegre-Abarrategui et al., 2009) and overexpression of LRRK2 resulted in accumulation of autophagic vacuoles and induction of autophagy (Plowey et al., 2008; Ramonet et al., 2011; Gómez-Suaga et al., 2012; Bravo-San Pedro et al., 2013). Consistently, knock-down of LRRK2 also stimulated autophagy (Manzoni et al., 2013), indicating that fine-tuning of LRRK2 expression and activity is essential. LRRK2 may regulate autophagy via interaction with the AMPK pathway and the MAPK/ERK cascade (Plowey et al., 2008; Hsu et al., 2010a, 2010b; Gómez-Suaga et al., 2012; Bravo-San Pedro et al., 2013; Waschbüsch et al., 2014). Although LRRK2 only weakly activates the MAPK cascade, it nevertheless produces a strong MAPK-dependent effect in C. elegans (Hsu et al., 2010a).

LRRK2 localizes to endosomes and it interacts with several Rab proteins that are involved in the regulation of exocytic and endocytic vesicular membrane trafficking (Stenmark, 2009; Diekmann et al., 2011; Dodson et al., 2012; Gómez-Suaga et al., 2014; Schreij et al., 2015). The LRRK2 G2019S mutation has been shown to disrupt late endosomal trafficking in a Rab7-dependent manner (Gómez-Suaga et al., 2014). A recent paper by Maekawa et al. described an additional link between LRRK2 and endosomal clearance of α-synuclein in microglial cells: disruption of LRRK2 resulted in enhanced α-synuclein uptake and clearance by microglia, possibly due to an increase of Rab5-positive endosomes (but not Rab7-positive endosomes) (Maekawa et al., 2016). Importantly, Steger et al. (Steger et al., 2016, 2017) recently identified a subset of 14 Rab proteins, including Rab5, Rab7L1, Rab8, Rab10 and Rab12, as the first bona-fide LRRK2 substrates. LRRK2 phosphorylates these Rabs on a conserved threonine residue in the switch II region, which alters the interactions with several Rab effectors and regulators. They also showed that increased phosphorylation of a subset of Rabs by the R1441G PD mutation results in impaired cilia formation (Steger et al., 2017). LRRK2-induced pathogenesis might thus partly be due to the deregulation of ciliogenesis and Rab-mediated protein transport. Rab7L1, also known as Rab29, is genetically linked to PD and phosphorylated by LRRK2 when localized to the trans-Golgi network (Fujimoto et al., 2017). PD-mutated LRRK2 showed increased Rab7L1 phosphorylation and subsequently to changes in the morphology of the Golgi network. Recently it was shown that Rab7L1 not only acts downstream of...
LRRK2, but in turn also stimulates LRRK2 kinase activity and promotes recruitment of LRRK2 to the Golgi (Liu et al., 2017; Purlyte et al., 2017).

LRRK2 has also been reported to localize to mitochondria, where it has been linked to the regulation of mitochondrial fission and clearance (West et al., 2005; Biskup et al., 2006; Niu et al., 2012). Mitochondrial dysfunction has been observed in LRRK2-mediated PD (Biskup et al., 2006). In carriers of the G2019S mutation, mitochondria were elongated and showed a higher interconnectivity, decreased mitochondrial membrane potential and decreased intracellular ATP levels, as well as a general uncoupling of the oxidative phosphorylation (Schapira et al., 1990; Biskup et al., 2006; Mortiboys et al., 2010).

LRRK2 is highly expressed in immune cells and several studies have linked LRRK2 signaling to immune responses and inflammation. LRRK2 mediated its function during inflammation most likely via negative regulation of the transcription factor NFAT (Liu et al., 2011b). Stimulation of inflammation resulted in phosphorylation of LRRK2 via TLR signaling and the MYD88 pathway (Dzamko et al., 2012).

Finally, LRRK2 has been clearly linked to neural development. In primary neurons and neural stem cells, over-expression of mutant LRRK2 reduced neurite development, outgrowth and branching (Greggio et al., 2006; MacLeod et al., 2006; Plowey et al., 2008; Dächsel et al., 2010; Winner et al., 2011; Liu et al., 2012; Bahnassawy et al., 2013; Sepulveda et al., 2013), while knock-down of LRRK2 enhanced neurite development (Schulz et al., 2011; Bahnassawy et al., 2013; Paus et al., 2013). Moreover, transfection of mutant LRRK2 in primary neurons and in neurons derived from iPS cells accelerated neurodegeneration and cell death in a LRRK2 dose-dependent way (Smith et al., 2005; Chan et al., 2011; Skibinski et al., 2014). The molecular mechanisms linking LRRK2 to neuronal development and maintenance are still to be unraveled, but may include polarized transport along actin filaments and microtubules, a process that is partly mediated by Rab proteins, as well as recycling of vesicles at synapses, which is generally required for neuronal maintenance (Saheki and De Camilli, 2012). It has indeed been shown that LRRK2 is involved in cytoskeletal dynamics, including actin cytoskeleton remodeling and trafficking of proteins via microtubule (Chan et al., 2011; Caesar et al., 2013; Law et al., 2014). PKA and Wnt signaling have both been linked to LRRK2 and are directly involved in neural development (Sancho et al., 2009). PKA influences dopamine signaling and synaptogenesis (Parisiadou et al., 2014), while Wnt signaling is essential for several steps in neural development (Berwick and Harvey, 2012).
Together these data show that LRRK2 has been linked to a wide range of functions, which are partly tissue-specific, and that Rab family members are key players in LRRK2 mediated signaling (Figure 1).

**Common effect of PD mutations implies a complex LRRK2 activation mechanism**

Although it is generally accepted that the kinase activity of LRRK2 mediates neuronal toxicity (Greggio et al., 2006; Smith et al., 2006), the effect and impact of the various PD mutations on LRRK2 kinase activity has been the subject of debate for many years. Initially increased kinase activity only had been consistently shown for the G2019S mutation (Figure 2A), whereas for the other mutations, no effect or even a decreased kinase activity has been reported (Reviewed by Greggio and Cookson (Greggio and Cookson, 2009)). The main reason for these conflicting data was the lack of a physiological substrate. After the identification of the physiological LRRK2 substrates, it became clear that all common mutations in the kinase, Roc, COR and WD40 domains result in increased kinase activity (Steger et al., 2016). Importantly, this also implies that the RocCOR tandem and WD40 domain are part of the mechanism that regulates kinase activity. Vice versa autophosphorylation of LRRK2 is considered as one of the major factors in the intramolecular regulation of LRRK2, influencing dimerization, localization and GTPase activity (Pelech, 2006; Greggio et al., 2009; Sen et al., 2009; Liu et al., 2011a, 2016; Taymans et al., 2011; Webber et al., 2011; Taymans, 2012; Biosa et al., 2013; Schapansky et al., 2014). We will discuss this complex LRRK2 activation mechanism in detail below.

**Regulation and function of the LRRK2 G-domain**

Both the Roc G-domain and the kinase domain are essential for LRRK2 functioning (Ito et al., 2007; West et al., 2007; Taymans et al., 2011). Classical G-proteins, such as Ras, are molecular switches that cycle between a GDP-bound off state and a GTP-bound on state. They are involved in the regulation of numerous and diverse cellular processes, ranging from differentiation and growth, to transport mechanisms. For the activation of G-proteins GDP needs to be exchanged to GTP, whereas for deactivation GTP is hydrolysed to GDP. Classical G-proteins need auxiliary proteins, namely guanine nucleotide exchange factors (GEFs) and GTPase activating proteins (GAPs) to go through a cycle of activation (Vetter and Wittinghofer, 2001; Goitre et al., 2014). GEFs reduce the nucleotide affinity from the
pico-molar to the micro-molar range to enable the protein to efficiently exchange the nucleotide and switch from the inactive GDP state to the active GTP state. In order to switch off the G-protein, the catalytic machinery is complemented by GAP proteins which stimulate the hydrolysis reaction from GTP to GDP and $P_i$ by many folds (Vetter and Wittinghofer, 2001; Wittinghofer and Vetter, 2011).

The G-domain cycle of LRRK2 and related Roco proteins appears to be completely different compared to that of classical G-proteins. Several studies have shown that the affinity of LRRK2 and Roco proteins to G-nucleotides is extremely low, which strongly suggests a high off rate and the ability to exchange the nucleotide without the need of GEFs (Gotthardt et al., 2008; Civiero et al., 2012; Liao et al., 2014; Rudi et al., 2015a; Terheyden et al., 2015). For LRRK2 and other Roco proteins it has been suggested that they also do not require GAPs, but are able to hydrolyse GTP in a RocCOR homodimer (Gotthardt et al., 2008; Rudi et al., 2015a; Terheyden et al., 2015). Initially, it was shown for two prokaryotic Roco proteins that dimerization via COR as well as an arginine residue in the Roc domain are important in the hydrolysis process (Gotthardt et al., 2008; Terheyden et al., 2015). We hypothesized that the COR domain forms a stable dimer while Roc dimerization enables the hydrolysis of GTP through the arginine residue in trans. Similar to the data obtained with prokaryotic Roco proteins (Gotthardt et al., 2008; Terheyden et al., 2015), dimeric LRRK2 has a hydrolysis rate that is significantly faster than that of the monomeric Roc domain (Rudi et al., 2015a). Based on these data, Roco proteins including LRRK2 have been classified into the GAD family of proteins (G-proteins activated by nucleotide dependent dimerization) that do not strictly depend on GEF or GAP proteins (Gasper et al., 2009; Terheyden et al., 2016). There are a few reports describing GAPs and GEFs for LRRK2, including ARHGEF7, ArfGAP1 and RGS2. However none of these putative regulators directly bind to the Roc domain, therefore these proteins most likely don’t function as classical GAPs or GEFs, but rather indirectly regulate the G-protein cycle (Haebig et al., 2010; Xiong et al., 2012; Biosa et al., 2013; Dusonchet et al., 2014).

Structural studies revealed that three of the PD mutation sites – N1437H, R1441H/G/C and Y1699C, all lie in the same hydrophobic interface between the Roc and COR domain ((Gotthardt et al., 2008), Figure 2C). In addition to increased kinase activity, several studies have reported a decreased GTPase activity for the R1441H/G/C and Y1699C mutations (Guo et al., 2007; Lewis et al., 2007; Li et al., 2007; Daniëls et al., 2008).
Rudi et al. postulated that these PD mutations impair the hydrolysis by disturbing the RocCOR interface and thereby change the dynamics of the Roc domains (Rudi et al., 2015a).

**Figure 2:** Structural model of LRRK2 in surface representation highlighting the different domains. The model provided by Guaitoli et al. (Guaitoli et al., 2016). (A) The G2019S mutation (here in *Dictyostelium* Roco 4 kinase (PDB: 4F1M (Gilsbach et al., 2012)) the G2019S mutation forms an additional hydrogen bond to Q1919 (here R1077 in Roco4), presumably leading to the stabilization of the active conformation. (B) The phosphorylation cluster S910/S935/S955/S973 at the N-terminus of the LRR domain (light pink). (C) The in PD mutated residues N1437 (here H554), R1441 (here Y558) and Y1699 (here Y804) lie in a hydrophobic interphase between the Roc (light cyan) and the COR domain (turquoise) close to the GTP-binding pocket (P-loop, green) and switch II (yellow).
Cross-talk between the G-domain and kinase domain

There is accumulating evidence that the GTPase and kinase activities influence each other. Nucleotide binding to the Roc domain is important for kinase activity (Ito et al., 2007; West et al., 2007; Taymans et al., 2011), while autophosphorylation of the Roc domains influences the G-protein cycle (Greggio et al., 2009; Webber et al., 2011; Taymans, 2012). In a "classical" way G-domains can adapt distinct active (GTP bound) and inactive (GDP bound) conformations. Consistent with such a mechanism, Liao et al., showed that the GTP bound form of Roc domain is the active conformation that can stimulate LRRK2 kinase activity (Liao et al., 2014). In contrast, several other studies showed that LRRK2 kinase activity did not change upon addition of GDP, GTP, or non-hydrolysable GTP analogues (Liu et al., 2011a; Taymans et al., 2011), while Biosa et al. reported that hydrolysis itself has an activating effect on the kinase domain. The later would imply that LRRK2 needs to go through a full cycle to be able to activate the kinase (Biosa et al., 2013). However, clearly more work is needed to fully understand the molecular mechanism by which the Roc domain regulates kinase activity.

Several mutations in the kinase domain were used to study the effect of either increased (usually G2019S) or decreased (K1994A or K1906M) kinase activity on the G-protein cycle (Greggio et al., 2006). Although the results from these studies are not completely conclusive (reviewed in (Terheyden et al., 2016)), it is clear that the Roc G-protein cycle is at least in part regulated by autophosphorylation of several residues within the Roc domain (Greggio et al., 2009; Liu et al., 2011a, 2016; Taymans et al., 2011; Webber et al., 2011; Taymans, 2012). Several studies have reported that autophosphorylation does not affect GTP binding (West et al., 2005; Greggio et al., 2006; Jaleel et al., 2007; Anand et al., 2009), but that mutants with increased kinase activity have reduced GTPase activity (Liu et al., 2011a) and mutants with reduced kinase activity have increased GTPase activity (Greggio et al., 2009). In contrast, Liu et al., suggested that the phosphorylation of Roc elevates its GTP hydrolysis and supports the formation of Roc dimers (Liu et al., 2016). Two other studies showed that LRRK2 auto-phosphorylation of residues within the Roc domain increases the GTP-binding and thereby the activity of the
Roc domain, suggesting that Roc functions downstream of the kinase domain (Taymans et al., 2011; Webber et al., 2011).

It remains to be determined how changes within the G-domain are transduced to the kinase domain, however recent data suggest this may involve the N-terminal and C-terminal protein-protein interaction domains. An integrative modelling approach revealed that LRRK2 folds in a very compact manner ((Guaitoli et al., 2016), Figure 2). The ANK-repeats as well as LRRs lie in close proximity to the kinase domain and therefore can contribute to its regulation. Moreover the WD40 domain might interact with the ANK domain (Guaitoli et al., 2016). It is likely that binding of additional proteins to these domains changes their conformation, thereby influencing the kinase activity, thus adding another layer of complexity to the LRRK2 activation mechanism.

Membrane association and oligomeric state of LRRK2

Both the localization and oligomeric state of LRRK2 have been a hot topic of discussion during the last couple of years. Various studies have reported that in vitro LRRK2 is mainly dimeric (Greggio et al., 2008; Klein et al., 2009; Terheyden et al., 2014; Nixon-Abell et al., 2016), whereas others have suggested that the protein is mainly monomeric (Ito and Iwatsubo, 2012). Recent data suggest that the oligomeric state of LRRK2 in vivo seems to be dependent on membrane localization. In the cytosol LRRK2 is mainly present as a monomer, while it obtains a dimeric form at the plasma membrane (Berger et al., 2010; James et al., 2012). Overexpressed LRRK2-GFP in Human Embryonic Kidney cells localizes in the cytosol, as well as on inner cellular structures (West et al., 2005; Gloeckner et al., 2006). Subsequent analysis in neuronal cells of endogenous LRRK2 confirmed its colocalization with the plasma membrane and intracellular organelles, including Golgi, early endosomes, lysosomes, transport vesicles, microtubules, and mitochondria (Biskup et al., 2006; Hatano et al., 2007; Schreij et al., 2015). Cell fractionation experiments revealed that a small fraction of LRRK2 is localized as a dimer in the membrane fraction, while the majority of LRRK2 is cytosolic and monomeric (Berger et al., 2010). In addition to this in vitro data, the presence of LRRK2 dimers (or larger oligomers) in the plasma membrane was confirmed in living CHO-K1 cells (James et al., 2012). Interestingly, a transition of LRRK2 from the cytosol to the membrane was observed in monocytic immune cells (Schapansky et al., 2014). These cells express high endogenous LRRK2, and stimulation of the immune response increases the expression and activation of LRRK2 (Thévenet et al.,
In unstimulated cells LRRK2 was predominantly monomeric and cytosolic, while upon immune stimulation the total amount of LRRK2 in the membrane fraction increased and became predominantly dimeric (Schapansky et al., 2014). Importantly, dimeric LRRK2 displayed higher kinase activity compared to the monomer, as shown by several assays with purified protein and cell lysates (Greggio et al., 2008; Klein et al., 2009; Sen et al., 2009; Berger et al., 2010; Nichols et al., 2010). Therefore membrane translocation and dimerization are key for the activation of LRRK2.

Although Berger et al. suggested that the distribution of LRRK2 monomer in the cytosolic fraction and dimer in the membrane fraction was not altered for the PD mutations R1441C, Y1699C and G2019S compared to wild type LRRK2 (Berger et al., 2010), other studies have shown that the R1441C/G and Y1699C mutations weakens the dimerization of the RocCOR domain (Daniëls et al., 2011b). In contrast, the I2020T and I1122V mutations have increased proportions of LRRK2 dimers compared to WT LRRK2 (Sen et al., 2009). Thus altered monomer-dimer equilibria may contribute to PD pathology.

**Regulation of LRRK2 dimerization**

Although, several domains can interact with each other (Guaitoli et al., 2016), structural and biochemical studies with prokaryotic Roco proteins have revealed that the COR domain functions as the main dimerization device in Roco proteins ((Gotthardt et al., 2008; Terheyden et al., 2015), Figure 2). Importantly, the recently published structural model of full-length LRRK2 (Guaitoli et al., 2016) and pull-down experiments with various truncated LRRK2 constructs (Terheyden et al., 2015) confirmed that also in LRRK2 the C-terminal part of COR is essential for dimerization.

Deng et al. solved the structure of the Roc domain as a swapped dimer, suggesting the Roc domain also contributes to dimerization (Deng et al., 2008). Although, this swapped dimer most likely represents a crystallization artifact (Gotthardt et al., 2008; Gilsbach and Kortholt, 2014), it does not exclude that Roc is able to dimerize. It has been shown that GTP binding and hydrolysis in the Roc domain influence dimer formation and translocation (Biosa et al., 2013). In contrast to what had been suggested previously (Civiero et al., 2012), Biosa et al. found that GTP-binding, but not hydrolysis, was required for dimer formation. Initially we hypothesized that RocCOR dimerization was nucleotide independent (Terheyden et al., 2015), but recently we have shown that the Roco protein from *Chlorobium tepidum* (*Ct*) cycles between monomer and dimer in a concentration and
nucleotide dependent manner (Deyaert et al., 2017a). We estimated that the affinity of the two monomers is reduced to around 30 µM when bound to GppNHp, which is much lower than the affinity when bound to GDP. This means that the dimerization of Roco proteins might be dependent on the bound G-nucleotide as well as on the protein concentration. Interestingly, the monomer-dimer cycle was within the timeframe of GTP-hydrolysis, which means that the monomer-dimer transition most likely is part of the GTPase cycle.

The kinase activity of LRRK2 also plays a role in the regulation of both its dimerization and localization (Pelech, 2006). Mutations that inactivate the kinase domain result in impaired dimerization (Sen et al., 2009; Biosa et al., 2013), while LRRK2 phosphorylation most likely proceeds dimer formation (Schapansky et al., 2014). This thus suggest that kinase activity is required for dimerization. However, Berger et al. (Berger et al., 2010), using an organic fluorophore that binds the phosphate moiety of protein complexes, in contrast discovered that dimeric LRRK2 at the membrane is in general less phosphorylated compared to the monomeric LRRK2 which was located in the cytosol. These studies also report different correlation between LRRK2 kinase activity and LRRK2 translocation. Sen et al. suggested that the subcellular distribution of LRRK2 is independent of its kinase activity, while Schapansky et al. showed more recently that LRRK2 phosphorylation precedes the membrane translocation (Sen et al., 2009; Schapansky et al., 2014). To solve this discrepancy in literature further investigation is clearly needed.

**Regulation of LRRK2 localization**

Both the N-terminal and C-terminal domains play an important role in regulating the cellular localization of LRRK2 (Jorgensen et al., 2009; Waschbüsch et al., 2014; Schreij et al., 2015; Zhang et al., 2015). LRRK2 lacking the WD40 domain has abolished dimer formation and does not localize at membranes (Jorgensen et al., 2009). It is unclear how the WD40 domain mediates LRRK2 localization to membranes, however WD40 repeats consist of several hydrophilic surfaces and have a high positive net charge, and therefore often have direct interaction with membranes and/or negatively charged proteins (Mata et al., 2006).

Several proteins, including Rabs, 14-3-3 and Clathrin Light Chain (CLC), regulate LRRK2 localization via binding to the N-terminal domains (Waschbüsch et al., 2014; Schreij et al., 2015; Zhang et al., 2015). In *Drosophila* CLC is able to recruit LRRK2 to
clathrin-coated early endosomes (Schreij et al., 2015), however it remains to be determined if a similar mechanism occurs in human cells. Several studies have shown the importance of Rab binding for proper LRRK2 localization (Waschbüsch et al., 2014; Zhang et al., 2015). Waschbüsch et al. showed that Rab32 binds to LRRK2 and that overexpression of constitutively active Rab32 results in an increase of LRRK2 localization at perinuclear late endosomes (Waschbüsch et al., 2014). Also Rab2a and Rab10 interact with LRRK2. In Paneth cells, Rab2a co-localizes with LRRK2 at dense core vesicles and both proteins inhibit lysozyme degradation through lysosomes (Zhang et al., 2015). Recently, several landmarking studies have shown that Rab proteins act both upstream and downstream of LRRK2 (Liu et al., 2017; Purlyte et al., 2017). LRRK2 pathogenic mutations show both increased Rab7L1 phosphorylation and increased Rab7L1-mediated LRRK2 transport to the trans-Golgi network (Liu et al., 2017). Interestingly, LRRK2 specifically phosphorylates membrane- and GTP-bound Rab7L1, Rab8 and Rab10 (Liu et al., 2017; Purlyte et al., 2017). Rab7L1 phosphorylation subsequently results in the activation of LRRK2, as monitored by autophosphorylation of Serine1292, which is important for LRRK2 toxicity (Liu et al., 2017). Autophosphorylation at the N-terminal phosphosites S910, S935, S955 and S973 was also stimulated by Rab7L1-mediated recruitment to the Golgi (Purlyte et al., 2017), which may influence the binding of 14-3-3 and other N-terminal binding proteins.

14-3-3 proteins stabilize the cytosolic localization of LRRK2 (Rudenko and Cookson, 2010; Doggett et al., 2012). Interaction of 14-3-3 with LRRK2 is dependent on the phosphorylation status of a stretch of serine residues (S910, S935, S955 and S973) situated at the N-terminal part of LRRK2 anterior to the LRR domain ((Dzamko et al., 2010; Nichols et al., 2010; Doggett et al., 2012; Lobbestael et al., 2013) (Figure 2B, D)). Muda et al. identified LRRK2 S1444 within the Roc domain as an additional phosphosite for 14-3-3 binding (Muda et al., 2014). Since several LRRK2 kinase inhibitors block 14-3-3 binding, it was initially proposed that phosphorylation of the N-terminal stretch of serines and 14-3-3 binding is regulated by autophosphorylation. However, recently it was shown that the interaction of the γ and η isoforms of 14-3-3 with LRRK2 is dependent on PKA mediated phosphorylation of S935 and S 1444 (Doggett et al., 2012; Muda et al., 2014). Disrupted phosphorylation of S935 results in strong defects in LRRK2 signaling; the protein is delocalized and accumulates in fibrillar-like structures associated with microtubules, instead of being transported to membranes (Blanka Ramirez et al. 2017, 132
Dzamko et al. 2010). In urinary exosomes purified from kidney epithelial cells, disruption of the 14-3-3/LRRK2 complex by using either a 14-3-3 or LRRK2 kinase inhibitor, blocks LRRK2 release from the exosomes (Fraser et al., 2013). Interestingly, for some LRRK2 PD mutations the phosphorylation levels of S910 and S935 were reduced, as was the binding of 14-3-3 protein to LRRK2, suggesting a direct link between PD mutated LRRK2 and 14-3-3 signaling (Nichols et al., 2010; Doggett et al., 2012).

Because of the important role of N-terminal LRRK2 phosphorylation, several studies have tried to identify phosphatases that dephosphorylate these sites. PP1 is regulating the S910/935/955 and S973 phosphorylation status in vitro and in cell culture (Lobbestael et al., 2013). Excess binding of PP1 to LRRK2 results in dephosphorylation of these four sites, impaired 14-3-3 binding to LRRK2 and accumulation in skein-like structures. Interestingly, PD-mutated LRRK2, including N1437H, R1441G and Y1699C, bind PP1 stronger than wild-type LRRK2 (Lobbestael et al., 2013), which due to the stronger dephosphorylation and impaired 14-3-3 binding, may result in more membrane association and higher LRRK2 activity.
Conclusion and Discussion

LRRK2 is a very complex protein with two enzymatic activities involved in various cellular pathways. The data discussed in this review show that LRRK2 activity and functioning is regulated on multiple levels (Figure 3). Considering the nucleotide affinities, exchange from the GTP to GDP (and vice versa) state is fast, but since the GTP concentration is usually 10 times higher than GDP, in the cytosol LRRK2 is majorly GTP-bound. Moreover it has been demonstrated that the protein exists as a monomer in the cytosol and as a membrane bound dimer which is the more (kinase) active fraction. It is unclear during which step dimerization occurs, however it is crucial for hydrolysis. The GTPase and kinase activity are clearly linked, however it remains to be determined what is the active conformation of LRRK2 at the membrane. 14-3-3 and Rab proteins both bind to the N-terminus of LRRK2 and are key regulators of localization. Phosphorylation dependent binding of 14-3-3 stabilizes LRRK2 in the cytosol, while vice versa Rab proteins recruits LRRK2 to intracellular. The different levels of regulation are all essential for proper LRRK2 functioning and thus can be used for therapeutict targeting. Strategies to tackle LRRK2 neurotoxicity could thus include targeting LRRK2 localization, dimerization, or allosteric modulation of the kinase domain.
**Figure 3**: Schematic model of the intramolecular activation cycle of LRRK2. In the cytosol LRRK2 is majorly GTP-bound and monomeric, a state that is stabilized by 14-3-3 binding. Upon Rab-mediated membrane translocation LRRK2 dimerizes, possibly due to increased local concentration. In this representation dimerization follows translocation, but vice versa may also occur. Dimerization completes the active site in the Roc domain, which stimulates GTP hydrolysis. Most likely one of the transition states during the GTPase cycle, represent the kinase active conformation. After hydrolysis, GDP is rapidly exchanged for GTP due to low nucleotide affinities and high GTP concentrations in the cell, resulting in monomerization and dissociation from the membrane.
References


Liu, Z., Lee, J., Krummey, S., Lu, W., Cai, H., and Lenardo, M.J. (2011b). The kinase LRRK2 is a regulator of the transcription factor NFAT that modulates the severity of


Smith, W.W., Pei, Z., Jiang, H., Moore, D.J., Liang, Y., West, A.B., Dawson, V.L.,


Thévenet, J., Pescini Gobert, R., Hooft van Huijsduijnen, R., Wiessner, C., Sagot, Y.J.,
LRRK2 expression points to a functional role in human monocyte maturation. PLoS One
6, e21519.

Tsika, E., and Moore, D.J. (2013). Contribution of GTPase activity to LRRK2-associated

dimensions. Science 294, 1299–1304.

with LRRK2 function and dysfunction. FEBS J. 282, 2806–2826.

aggregates and clearance by autophagy in an inducible cell model of tauopathy.

Waschbüsch, D., Michels, H., Strassheim, S., Ossendorf, E., Kessler, D., Gloeckner, C.J.,
and Barnekow, A. (2014). LRRK2 transport is regulated by its novel interacting partner

Autophosphorylation in the leucine-rich repeat kinase 2 (LRRK2) GTPase domain
modifies kinase and GTP-binding activities. J. Mol. Biol. 412, 94–110.

West, A.B., Moore, D.J., Biskup, S., Bugayenko, A., Smith, W.W., Ross, C.A., Dawson,

West, A.B., Moore, D.J., Choi, C., Andrabi, S.A., Li, X., Dikeman, D., Biskup, S., Zhang,
LRRK2 link enhanced GTP-binding and kinase activities to neuronal toxicity. Hum. Mol.

Winner, B., Melrose, H.L., Zhao, C., Hinkle, K.M., Yue, M., Kent, C., Braithwaite, A.T.,

a canonical switch motif. Annu. Rev. Biochem. 80, 943–971.

Xiong, Y., Yuan, C., Chen, R., Dawson, T.M., and Dawson, V.L. (2012). ArfGAP1 is a
GTPase activating protein for LRRK2: reciprocal regulation of ArfGAP1 by LRRK2. J.
Neurosci. 32, 3877–3886.

Zhang, Q., Pan, Y., Yan, R., Zeng, B., Wang, H., Zhang, X., Li, W., Wei, H., and Liu, Z.
Immunol. 16, 918–926.

Zhu, X., Babar, A., Siedlak, S.L., Yang, Q., Ito, G., Iwatsubo, T., Smith, M.A., Perry, G.,