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Dictyostelium discoideum: A Model System to Study LRRK2-Mediated Parkinson Disease

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1. Introduction

Parkinson disease (PD) is a neurodegenerative disease that affects more than 5 million people worldwide and one in hundred people over the age of 60. PD is both a chronic and degenerative disorder that is characterized by loss of dopaminergic neurons in the substantia nigra, associated with the formation of fibrillar aggregates composed of α-synuclein and other proteins (Lees et al., 2009). PD is clinically characterized by tremor, bradykinesia, rigidity and postural instability. Initially PD was considered to have no genetic cause, however many patients have one or more family member with the disease and genome-wide association studies identified a number of genetic factors segregating with PD (Satake et al., 2009; Simon-Sanchez et al., 2009). Therefore, it is now general believed that PD is caused by a combination of genetic and environmental factors. Recently, missense mutations in LRRK2 have been linked to autosomal-dominant, late-onset PD (Zimprich et al., 2004;Paisan-Ruiz et al., 2004). LRRK2 is a member of the novel Roco family of complex Ras-like GTPases that have an unique domain architecture (Fig. 1) (Bosgraaf and van Haastert, 2003). Roco proteins are characterized by the presence of a Ras-like Guanine nucleotide binding domain, called Roc (Ras of complex proteins), followed by a conserved stretch of 300-400 amino-acids with no significant homology to other described protein domains called the COR domain (C-terminal of Roc; Fig. 1). The Roc and COR domains always occurs as a pair, and so far no proteins have been identified containing either the Roc or COR domain alone, suggesting that these two domains function as one inseparable unit. Roco proteins were first identified in the social amoeba *Dictyostelium discoideum* and are found in prokaryotes, plants and metazoa, but not in *Plasmodium* and yeast (Bosgraaf et al., 2003). Besides a Roc and COR domain, all Roco proteins contain an N-terminal stretch of leucine-rich repeats (LRR), which are supposed to be involved in protein-protein interaction. A large group of Roco proteins, which is only present in *Dictyostelium* and metazoan, contains an additional C-terminal kinase domain of the MAPKKK subfamily of kinases. Next to this general domain composition, individual Roco proteins are found to be combined with a diversity of additional domains such as Guanine nucleotide exchange factor (GEF) and Regulator of G-protein Signalling (RGS) domains, implicating a link between traditional G-protein signalling pathways and Roco proteins (Bosgraaf et al., 2003).
The identification of missense mutations in LRRK2 has redefined the role of genetic variation in PD susceptibility. LRRK2 mutations initiate a penetrant phenotype with

complete clinical and neurochemical overlap with idiopathic disease (Khan et al., 2005;Hernandez et al., 2005;Aasly et al., 2005). The various mutations that have been identified in PD are concentrated in the central region of the protein: one amino acid change within the LRR domain, one amino acid change in the Roc domain, one in the COR domain that can have multiple mutations and two amino acids change in the kinase domain (Fig. 1A, (Cookson, 2010). Identified mutations outside of these domains do not segregate in a Mendelian fashion with PD. The mutations are found in 5-6 % of patients with familial PD, and importantly also have been implicated with sporadic PD with unprecedented 1-2 % prevalence (Gilks et al., 2005). Although much progress has been made during the last few years, the exact pathogenic role and associated biochemical pathways responsible for LRRK2-linked disease are slowly emerging. However, recent evidence suggests that these pathways involve other proteins that have been linked to PD, especially α-synuclein and tau (Cookson and Bandmann, 2010;Cookson, 2010). The considerable number of described disease-linked LRRK2 mutations represent an unique opportunity to biochemically explore the pathogenicity of LRRK2 and identify therapeutic targets for related neurodegenerative disorders. Importantly, all known pathogenic mutations in LRRK2 result in decreased GTPase activity and enhanced kinase activity, suggesting a possible PD-related gain of abnormal/toxic function (West et al., 2005;Greggio et al., 2006;Guo et al., 2007;Ito et al., 2007;Luzon-Toro et al., 2007;Lewis et al., 2007;Li et al., 2007;West et al., 2007). Since LRRK2 kinase activity is critically linked to clinical effects, it presents a viable target for therapeutic modulation.

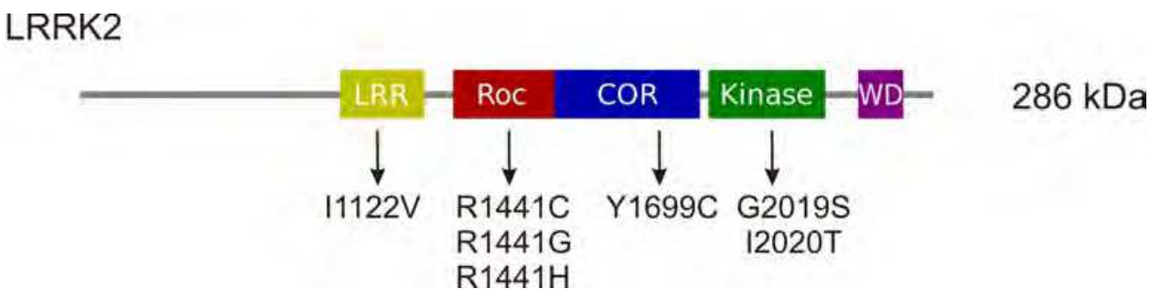

Fig. 1. Domain structure and mutations of LRRK2. The most clearly definined pathogenetic mutations are shown below the diagram.

Attempts to purify mammalian LRRK2 have failed so far in many laboratories. Therefore, the detailed biochemical and structural understanding of LRRK2 is very limited. We have used related proteins, which can serve as models to understand the complex structure and regulatory mechanism of LRRK2. Previously the structure of the Roco protein from the cyanobacterium *Chlorobium tepidum* was elucidated, which revealed that COR is a constitutive dimerization device and that Roco proteins belong to the GAD class of molecular switches (G proteins activated by nucleotide dependent dimerization) (Gotthardt et al., 2008;Gasper et al., 2009). This class also includes proteins such as signal recognition particle, dynamin and septins (Gasper et al., 2009). It is proposed that the juxtaposition of the G domains of two monomers in the complex across the GTP-binding sites activates the GTPase reaction and thereby regulate the biological function of these proteins. The *Chlorobium* Roco structure revealed that the PD-analogous mutations of the Roc and COR domain are in close proximity to each other, and are present in a region of the protein that is strongly conserved between bacteria and man. PD mutations in *Chlorobium*, like that of

LRRK2, decrease the GTPase reaction. Based on the structure and the observed effects of PD-mutations in LRRK2 it is thought that interaction with other proteins modify the dimer interactions resulting in decreased GTPases and enhanced kinase activity (Gotthardt et al., 2008;Gasper et al., 2009). This shows that mechanistic insight can even gained from very distantly related proteins

2. *Dictyostelium discoideum* as model sytem to resolve the function of Roco proteins

This chapter concentrates on *Dictyostelium discoideum* Roco proteins, which are excellent models for LRRK2 and can thus be used to answer key questions for the intramolecular regulation of LRRK2 and give insight in the function of the LRR, the mechanism by which the Roc domain regulates kinase activity, the role that COR plays in this process and how the PD-linked missense mutations alter the interactions between the different domains.

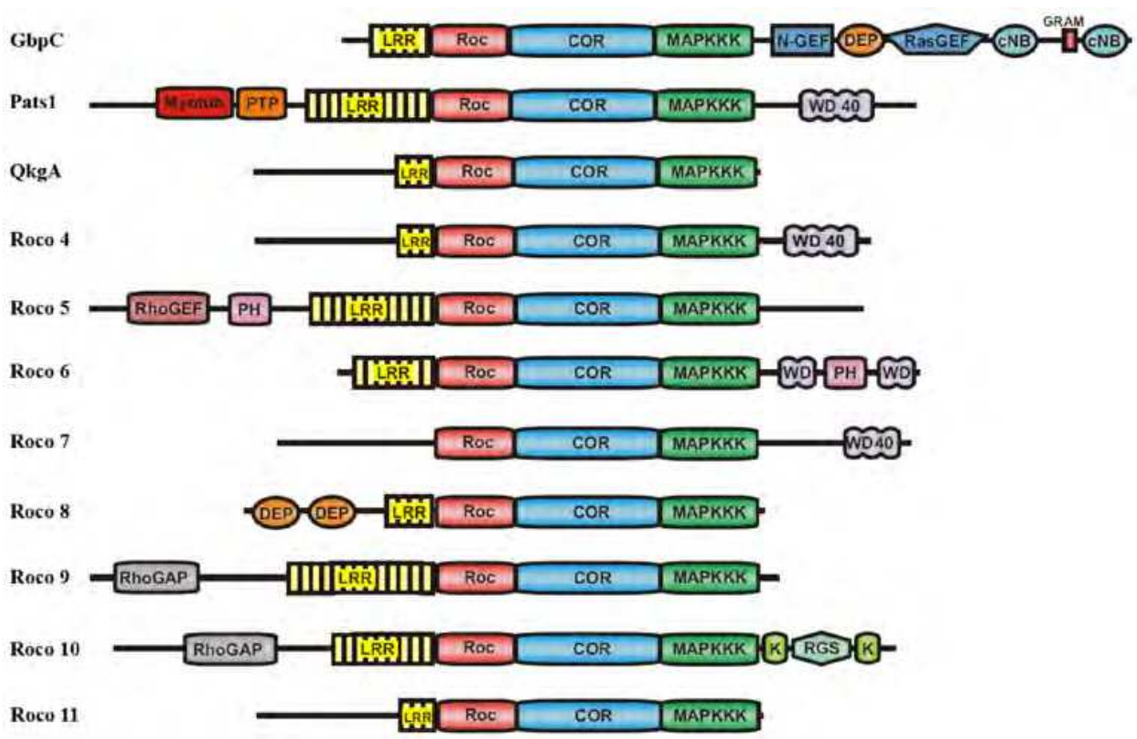

Fig. 2. Domain architecture of the *Dictyostelium* Roco proteins. All proteins contain LRR, the Roc, COR and the kinase domain. Additional a variety of domains are found in specific Roco proteins, such as RasGEFs ,RhoGEFs, RhoGAPs, Regulator of G protein signaling (RGS), and Pleckstrin homology domains (PH).

2.1 Dictyostelium discoideum

Dictyostelium discoideum is a free living soil amoeba. In nature, single *Dictyostelium* cells are feeding on bacteria. They chase bacteria by chemotaxing towards folic acid, which is secreted by the bacteria. Upon starvation cells enter the developmental stage (Kessin, 2000). During development single cells undergo a drastic change in gene expression and start to

secrete cAMP. Neighbouring cells respond by migrating toward the chemoattractant cAMP and by secreting cAMP themselves. Thus a cAMP gradient is created around the initiation point. After six hours of starvation, the chemotaxing cells have formed an aggregation centre at the intiation point, which consists of up to 100.000 cells. Differentiation and morphogenesis culminate in the formation of a fruiting body, or stalks of vacuolated dead cells with a spore head on top. These spores can survive long periods without food, high temperatures and drought. Recently the assembly of the *Dictyostelium* genome was completed (Eichinger *et al.*, 2005). The thirty-four Mb genome contains many genes that are homologous to those in higher eukaryotes and are missing in other model system. Due to the availability of the genome sequence, the well established molecular cloning and imaging techniques, *Dictyostelium* provides a well-established model to study the basic aspects of directed cell movement and development (Devreotes and Zigmond, 1988;Van Haastert and Devreotes, 2004). Chemotaxis or directional movement towards a chemical compound is an essential property of many cells and is fundamentally important for processes as diverse as the sourcing of nutrients by prokaryotes, the organisation of the embryo in metazoa, the formation of multicellular structures in protazoa and the migration of lymphocytes during immune response (Baggiolini, 1998;Campbell and Butcher, 2000;Iijima *et al.*, 2002;Crone and Lee, 2002). Chemotaxis is also linked to the development and progression of many diseases including asthma, arthritis, atherosclerosis, and cancers (Trusolino and Comoglio, 2002;Charo and Taubman, 2004;Eccles, 2005). Since the key signalling pathways underlying chemotaxis are essentially similar to those of mammalian cells, *Dictyostelium* has been used to study cell-motility related pathologies, including deficiencies in the immune system and neurological disorders (Carnell and Insall, 2011;Escalante, 2011;Meyer *et al.*, 2011). *Dictyostelium* also has been used as model in pharmacogenomics and to characterize the molecular basis of human diseases associated with the endocytic and secretory pathway (Williams *et al.*, 2006;Van *et al.*, 2007;Francione *et al.*, 2011;Maniak, 2011;Alexander and Alexander, 2011).

2.2 *Dictyostelium discoideum* and the Roco family of proteins

Four Roco proteins are detected in vertebrates, called LRRK1, LRRK2, DAPK1 and MFHAS1. Remarkably, in *Dictyostelium* eleven Roco family members were identified, that all share the characteristic Roc, Cor and kinase domains and most also have LRR (Fig. 2, (Bosgraaf *et al.*, 2003)). *Dictyostelium* Roco proteins are structurally more varied than the Roco proteins found in all the other species together; various domains are additionally fused to the conserved region. Most likely all the *Dictyostelium* Roco genes have evolved quit recently by gene duplication (Marin, 2006). From a functional point of view, the *Dictyostelium* Rocos have provided the most significant data (van Egmond and van Haastert, 2010).

2.2.1 Functions for *Dictyostelium* GbpC in chemotaxis, streaming and osmotic stress

GbpC, also called Roco1, was originally identified in a bioinformatical screen for molecular targets of the second messenger cGMP and is the founding member of the Roco family of proteins (Bosgraaf *et al.*, 2002;Bosgraaf *et al.*, 2003). Besides the conserved Roco region, GbpC has a unique regulatory C-terminal region, consisting of a Ras Exchange Motif (REM), DEP, CDC25, and two cyclic nucleotide binding (cNB) domains with a GRAM domain inserted in between (Fig. 3,(Goldberg *et al.*, 2002)). In the contrary to LRRK2, the cellular function of GbpC has been characterized in detail. GbpC is the only cGMP-signal transducing protein in

Dictyostelium, it binds to cGMP with high affinity to its cNB domains (Bosgraaf *et al.*, 2002). cGMP mediated GbpC activation is essential for the proper regulation of myosin II during chemotaxis, cell streaming and osmotic-stress (Fig.3, (Kuwayama *et al.*, 1996;Bosgraaf *et al.*, 2002;Goldberg *et al.*, 2002;Veltman and van Haastert, 2008;Araki *et al.*, 2010)). Myosin II is an essential regulator of the cytoskeleton at the rear of moving cells. The establishment of a cellular gradient during chemotaxis leads to major changes in the cytoskeleton; actin polymerization occurs at the leading edge of the cell, while acto-myosin filaments are formed at the rear of the cell. The formed myosin-II filaments are preventing the formation of lateral pseudopods and providing the power to retract the uropod (Levi *et al.*, 2002). In *Dictyostelium*, myosin assembly seems to be strictly dependent on the phosphorylation state of the myosin heavy chain (MHC) (Bosgraaf and van Haastert, 2006). Phosphorylation by MHCKs inhibits filament formation (Cote and Bukiejko, 1987;Kolman *et al.*, 1996), whereas dephosphorylation by protein phosphatase 2A is essential for myosin disassembly (Murphy *et al.*, 1996). Phosphorylation of the myosin light chain (MLC) by MLCKs, promotes myosin motor activity, which is important for supplying contractile force to retract the rear of the cell (De la Roche and Cote, 2001;De la Roche *et al.*, 2002). Cells lacking cGMP formation or GbpC have an impaired recruitment of myosin II to the cytoskeleton and impaired chemotaxis. Cells with elevated levels of cGMP have increased activation of myosin-light-chain kinase A (MLCKa) and subsequently an increased myosin motor activity (Bosgraaf *et al.*, 2002). The role of GbpC, becomes even more evident in longer developed cells, which begin to secrete cAMP, neighbouring cells move towards the cAMP and relay the signal. Due to the resulting wave of cAMP through the population, cells become polarized, connect to each other in a head-to-tail fashion, and form streams of cells. Cells lacking cGMP or GbpC have a severe streaming defect; these cells show extensive stream break up due to reduced cell elongation and the inability to maintain stable head-to-tail cell contacts (Veltman *et al.*, 2008). Together these results show that cGMP and GbpC are important for the formation of stably polarized and elongated cells by regulating myosin filament formation in the posterior of the cell, which is important for both chemotaxis and cell streaming.

The cGMP pathway is not only activated in response to cAMP, but also by folic acid and osmotic stress (Hadwiger *et al.*, 1994;Kuwayama *et al.*, 1996;Kuwayama and van Haastert, 1998). *Dictyostelium* can bind folic acid, secreted by bacteria, to the so far unidentified folic acid receptor, resulting in activation of Gα4 and subsequently activation of the cGMP pathway (Hadwiger *et al.*, 1994). In the contrary, cGMP production in response to osmoshock is independent of heterotrimeric proteins (Kuwayama *et al.*, 1998). Also the kinetics of the cGMP responses are completely different, cGMP production occurs in minutes after osmoshock and in seconds after stimulation with cAMP or folic acid (Kuwayama and van Haastert, 1996). The transcription factor StatC and the protein kinase SAPKα show osmotic stressed-induced phosphorylation (Sun *et al.*, 2003;Araki *et al.*, 2003;Araki *et al.*, 2010). Phosphorylated StatC subsequently translocates to the nucleus to bind its transcriptional targets. Activation of both SAPKα and StatC occurs downstream of cGMP and GbpC; SAPKα and StatC are rapidly phosphorylated after treatment with 8-bromo-cGMP and *gbpC*-null cells are lacking the osmotic-stress-induced StatC translocation (Araki *et al.*, 2010). Although the phosphorylation state of Myosin Light Chain Kinase, the protein kinase SAPKα and transcription factor StatC are cGMP-dependent (Sun *et al.*, 2003;Bosgraaf *et al.*, 2006;Araki *et al.*, 2010), no direct binding of GbpC to these proteins could be detected. To completely understand the function of GbpC in *vivo*, it will be important to identify its direct substrates.

2.2.2 Biological role of QKGA and PATS1

Initially two proteins similar to GbpC were found in *Dictyostelium*, Qkga (now also called Roco3) and Pats1 (now also called Roco2). Qkga (Quick growth factor a) only consist of the central Roco region (Fig. 2), and was first described in a study for a new method to create gene disruptions in *Dictyostelium* (Abe et al., 2003). Cells lacking *qkgA* grow faster suggesting a role in cell proliferation. Consistently, Qkga overexpressed in *qkgA* null cells results in slower groth, indicating that higher amounts of QkgA lead to slower cell proliferation, thus confirming a role for QkgA in this process (van Egmond *et al*., 2010).

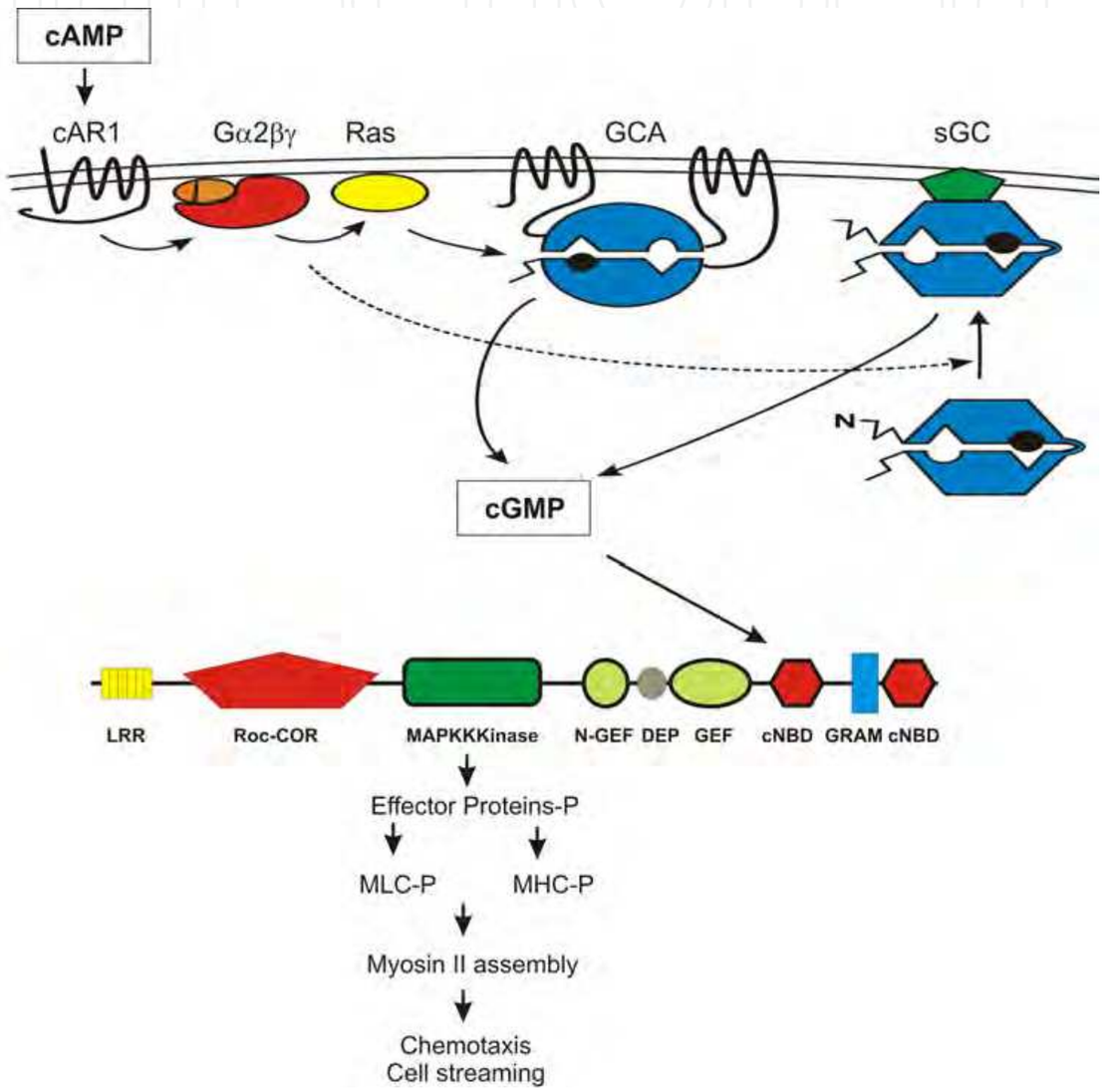

Fig. 3. The cGMP/ GbpC pathway in *Dictyostelium discoideum*. Extracellular cAMP binds to a G-protein coupled receptor cAR1 that stimulates a specific G-protein and Ras. cGMP is synthesised by two guanylyl cyclases, GCA and sGC that are unrelated to mammalian guanylyl cyclases, but are homologs of respectively mammalian membrane and soluble adenylyl cyclase. GbpC, the only target of cGMP, modulates the phosphorylation and assembly of conventional myosin into filaments.

Pats1 was identified in a screen for proteins involved in cytokinesis (Abysalh et al., 2003) and consist, like LRRK2, of LRRs, a ROC, COR, MAPKKK domain and WD40 repeats, and has additionally an N-terminal myotub-related and PTP (Protein Tyrosine Phosphatases) domain (Fig. 2). In a first study, *pats1* was disrupted in DH1 cells, resulting in large multinuclear cells in shaking culture, but these cells divide normally when grown on plate (Abysalh et al., 2003). DH1/*pats1*-null cells show improper localization of MHC to the cleavage furrow and an interaction between the WD40 repeats and the actomyosin was found, suggesting a role for Pats1 in regulating myosin II formation during cytokinesis (Abysalh et al., 2003). In a second study by van Egmond et al., where *pats1* was disrupted in an AX3 background, cells showed large multinuclear cells when grown on plate, but not in shaking culture, which is opposite to *pats1*-null cells that were created in DH1 background (van Egmond *et al.*, 2010). Furthermore, re-expression of the Pats1 kinase domain in *pats1*/DH1 cells, led to rescue of the phenotype and overexpression in DH1 resulted in large multinucleated cells again (Abysalh et al., 2003), whereas no rescue or overexpression effect was observed in the AX3 background (van Egmond *et al.*, 2010). Together these results show that Pats1 has an important role in cytokinesis, but the division-mechanism that it is involved in might vary among different wild-type strains.

2.2.3 Developmental role for Roco4

The complete Roco protein family was identified by Bosgraaf and van Haastert (2003) in a bioinformatic search with the Roc and COR domain of GbpC. Phylogenetic analysis showed that *roco4*, *qkga* and *roco11* are higly similar and are resulting from an ancestor *roco4* gene that was duplicated late in evolution (later than 300 million years ago) (van Egmond *et al.*, 2010). Interestingly, Roco4 has the same domain architecture as LRRK2 (Fig. 1 + 2). The expression of many *Dictyostelium* genes is strictly regulated during the life cycle. RT-PCR experiments showed that *roco4* expression is also developmentally regulated, with a strong elevated expression levels during the slug stage, suggesting a role for Roco4 in late development (van Egmond *et al.*, 2010). To study the function during development, the *roco4* gene was disrupted, and *roco4*-null cells were subjected to starvation on nutrient-free agar plates. During the first hours of development, no difference between *roco4*-null and wild-type could be observed. Cells start to aggregate and form characteristic streams after 6 hours starvation. After 9 hours, aggregation is complete and both cell strains have formed mounds. Developmental defects of *roco4*-null cells become visible after 12 hours of starvation, when wild-type cells are at the onset of forming slugs and form first fingers, while in *roco4*-null this process is first observed after 16 hours of starvation. After 24 hours, wild-type cells culminate in the formation of a fruiting body, while *roco4*-null slugs migrate for many hours before making multiple attempts to culminate, a process that sometimes takes up to 72 hours after the onset of starvation. Eventually, this aberrant culmination results in fruiting bodies consisting of sporeheads that are located on the agar surface, because a proper stalk is not present to lift the sporehead into the air (Fig. 4, (van Egmond *et al.*, 2010)). Re-expression of Roco4 completely rescues the phenotype of *roco4* disruption. Consistent with the developmental defects, Roco4 expression is highly enriched in the prestalk cell and *roco4*-null cells have severely reduced cellulose levels. Cellulose is known in *Dictyostelium* to be the cement of stalking cells, necessary for stability (van Egmond *et al.*, 2010). Together these results show that Roco4 is a prestalk-specific protein involved in the proper production of cellulose.

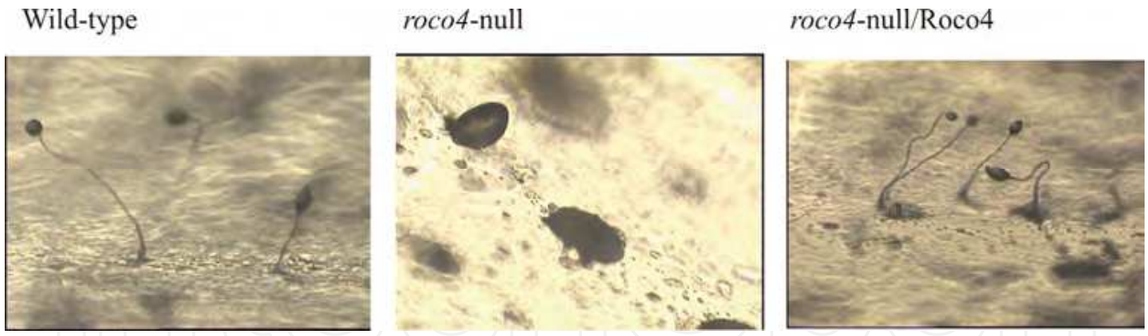

Fig. 4. Phenotype of *roco4*-null cells. Wild-type, *roco4*-null cells, and *roco4*-null cells re-expressing Roco4, were allowed to develop on nutrient-free agar. *roco4*-null cells fail to make a normal fruiting body due to defective synthesis of cellulose.

2.2.4 Function of other *Dictyostelium* Roco proteins

To further investigate the role of Roco proteins during the *Dictyostelium* life cycle, van Egmond et al., (2010) knocked out the 8 remaining *roco* genes and analysed their developmental phenotypes. *Dictyostelium* Roco proteins have distinct expression patterns during development; no major differences in expression were found for Roco5, Roco8 and Roco10 during development. In contrast, Roco6 and Roco11 show, like Pats1, QkgA and Roco4, elevated expression levels during the slug phase. Roco7 and Roco9 are expressed mostly during aggregation, similar to GbpC. Although *roco5*-null cells were previously identified in a large screen for mutants with defects in the developmental cycle (Sawai *et al.*, 2007), they did not show any recognizable developmental phenotype. In the contrary *roco11*-null cells show mild developmental defects: these cells develop significantly larger fruiting bodies; in particular, the multicellular structures have longer stalks compared to wild-type cells, re-expression of Roco11 in *roco11*-null cells rescues this defect. All other *roco*-null mutants did not show any phenotype in development and it will be interesting to see which biological function these proteins have in *Dictyostelium*.

2.3 Activation mechanism of Roco proteins

Pathogenic mutations in LRRK2 result in decreased GTPase activity (West *et al.*, 2005;Greggio *et al.*, 2006;Guo *et al.*, 2007;Ito *et al.*, 2007;Luzon-Toro *et al.*, 2007;Lewis *et al.*, 2007;Li *et al.*, 2007;West *et al.*, 2007). Furthermore, it has been shown that activity of the Roc domain is required to modulate downstream kinase activity, but kinase activity does not have a significant effect on GTP-binding of the Roc domain (Luzon-Toro *et al.*, 2007;West *et al.*, 2007). These results lead to the unifying model that the pathogenetic gain-of-function of LRRK2 relates to increased kinase activity, either directly through mutation of residues in the kinase domain, or indirectly through mutations in the GTPase domain or predicted protein binding domains. However, detailed information about the activation mechanism is missing; it is for example still unclear by which mechanism the Roc domain regulates kinase activity, the role that COR plays in this process and importantly how the PD-linked missense mutations alter the interactions between the different domains. The strong and diverse phenotypes of the *Dictyostelium* Roco disruption mutants provide a strong tool to investigate the activation mechanisms of Roco proteins.

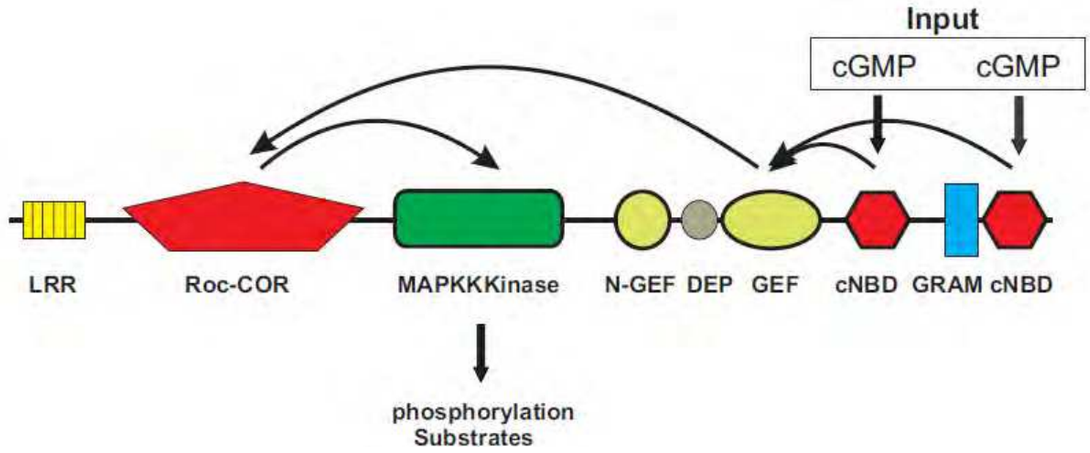

Fig. 5. GbpC: An intramolecular signalling cascade on one protein.

2.3.1 GbpC an intramolecular signaling cascade on one protein

The biochemical properties of GbpC were investigated by rescue analysis of the chemotactic defects of *gbpC*-null cells (van Egmond *et al.*, 2008). Whereas, re-expression of GbpC completely rescued the phenotype, mutants that lack a functional GEF, Roc or kinase domain are inactive. G-proteins function as molecular switches; they cycle between an active GTP- and inactive GDP-bound state. Consistently, in GbpC and LRRK2 the Roc domain is also activated upon GDP/GTP exchange, which subsequently increases kinase activity. The conventional Ras cycle is strictly regulated by guanine nucleotide exchange factors (GEFs) that catalyze the exchange of GDP for GTP, thereby activating the Ras protein. GTPase activating proteins (GAPs) stimulate an otherwise low intrinsic GTPase activity by many orders of magnitude, reverting the conformation back to the inactive GDP-bound form (Bourne *et al.*, 1991). So far it is unclear whether the GDP/GTP cycle of Roco proteins is regulated by GEFs and GAPs, and which structural consequences the GDP and GTP binding has. GbpC differs from the other Roco family members, in the sense that it already contains its own putative GEF domain (Bosgraaf *et al.*, 2003). In vitro nucleotide exchange assays showed that the RasGEF of GbpC specifically activates its own Roc domain. Furthermore, cGMP-binding to GbpC strongly stimulates binding of GbpC to GTP-agarose. Together these results suggest that GbpC contains a complete intramolecular signal transduction pathway; cGMP-binding to the cNB domains causes activation of the GEF domains, the subsequent GDP/GTP exchange of the Roc-COR domain, leading to the activation of the MAPKKK domain and phosphorylating downstream targets (Fig. 5, (van Egmond *et al.*, 2008).

2.3.2 Roc and kinase activities

Dictyostelium Roco4 has the same domain architecture as LRRK2, but in contrast to LRRK2, Roco4 is biochemically and structurally more tractable. The strong developmental phenotype of *roco4*-null cells was used to determine essential structural elements in the protein. Furthermore, high yields of Roco4 and combinations of its domains can be produced in *E. coli*. Similar to LRRK2, a functional Roco4 Roc domain is essential for kinase activity, the COR domain functions as dimerization device and disruption of Roc or the

kinase domain by a single point mutation leads to the complete inactivation of the protein, which was also found for all other biochemically studied Roco proteins so far. Also, kinase inactivation does not lead to loss of GTP-binding, thus suggesting that Roc activation occurs upstream of kinase activity. These results indicate that Roco4 has properties very much resembling those described for LRRK2, indicating that Roco4 protein can serve as a valid model to understand the complex structure and regulatory mechanism of LRRK2. As to the relevance for understanding Parkinson we have demonstrated that all Roco4 PD-related mutants show a decreased GTPase and increased kinase activity, except the Roco4 L1180T mutant (LRRK2 I2020T) which shows a large decrease in kinase activity. Strikingly, also for LRRK2 I2020T a reduced kinase activity has been reported, and it has been postulated that the higher neurotoxity of this mutant might be due to a higher susceptibility of the mutant to intracellular degradation (Jaleel et al., 2007;Ohta et al., 2010).

2.3.3 Different roles for the WD40 repeats in Roco4 and LRRK2

For LRRK2 it was found that deletion of the WD40 repeats leads to lower kinase activity in vitro, which could be restored by introduction of one of the PD-mutations (Iaccarino et al., 2007). Surprisingly, we found that deletion of the Roco4 WD40 repeats does not lead to effects on Roco4 activity *in vivo* (van Egmond et al., 2010). This suggests that in the contrary to LRRK2, the WD40 repeats of Roco4 are apparently not needed for full activation of the kinase domain. A possible explanation for this discrepancy comes from phylogenetic data; Roco4, QkgA and Roco11 have a common ancestor that was duplicated only relatively recently in evolution. QkgA and Roco11 do not have the WD40 repeats that are present in all Roco4 proteins, suggesting that during or after duplication, *qkgA* and *roco11* have lost the WD40 repeats. Apparently, the WD40 repeats were not important enough for the regulation of Roco proteins, that they had to be maintained during evolution (van Egmond *et al.*, 2010).

2.3.4 The LRR are essential for biological activity

The LRR of LRRK2, and Dictyostelium GbpC and Roco4 are not involved in Roc or kinase activation in vitro, but are absolutely essential for activity of the protein in vivo (Iaccarino et al., 2007;van Egmond et al., 2008). Recent data suggest that the LRR are directly involved in determining input/output specificity of the roco proteins, most likely by binding upstream proteins that activate specifically the Roco protein and/or by selectively binding of the substrate (unpublished data).

2.3.5 Subcellular localization of Roco proteins important for activity and function

Recent data suggest that also the subcellular localization of LRRK2 is important for the activity and function. LRRK2 is present both in the cytosol and at the membrane, and the membrane-associated LRRK2 dimer most likely represents the physiologically active form of the protein (Berger *et al.*, 2010). The regulation of membrane association is not well understood, but probably includes dimerization, post-translational modifications and protein-protein interactions (Sen *et al.*, 2009;Berger *et al.*, 2010;Nichols *et al.*, 2010). To better understand the distribution of Roco proteins in the cell, we studied the localization of GbpC (ms in preparation). In resting cells, the protein is present uniformly in the cytosol, but during stream formation and osmotic stress the protein localizes to the membrane. Also

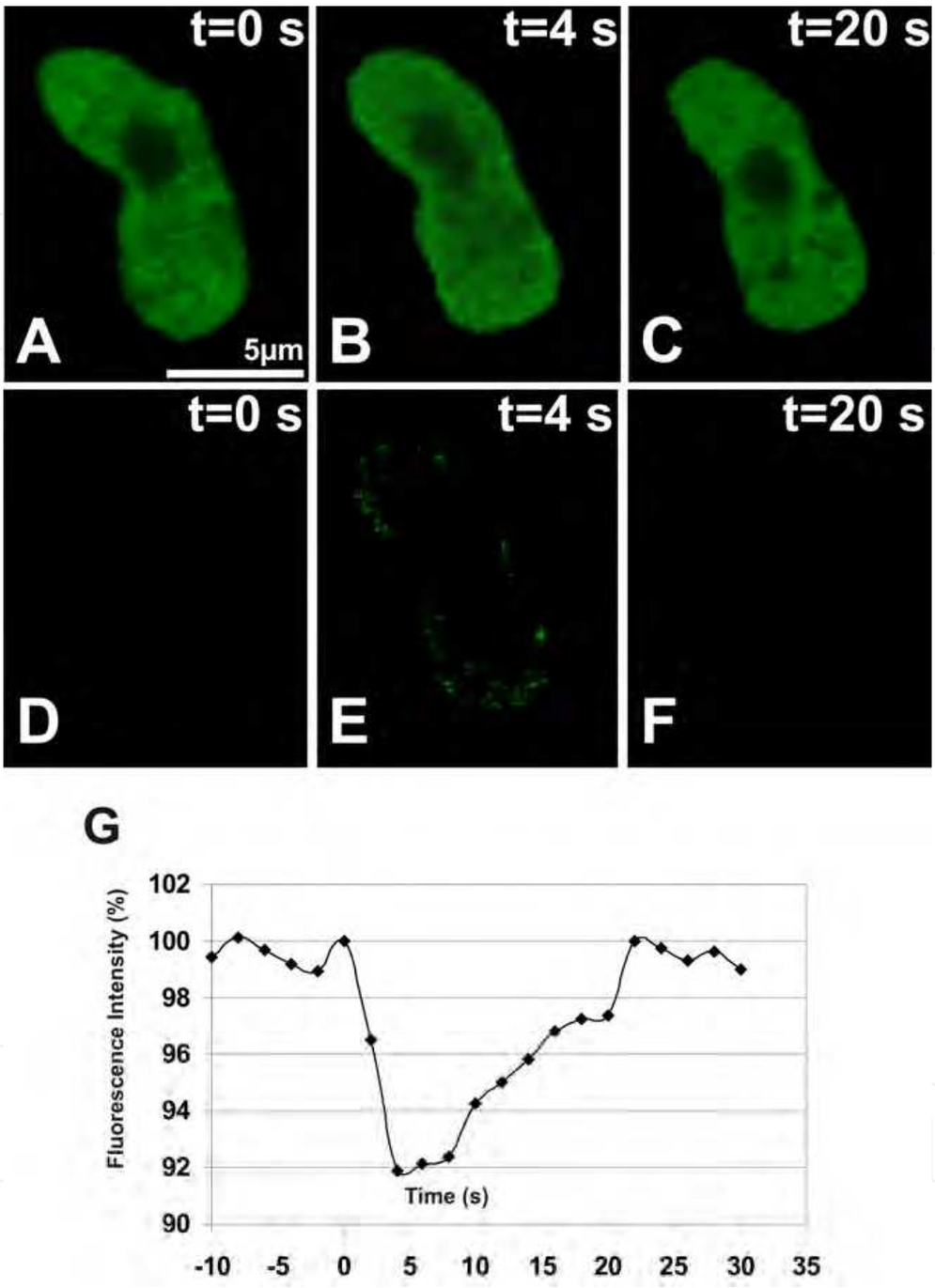

Fig. 6. GbpC translocates to the membrane upon cAMP-stimulation. Starved *gbpC*-null cells expressing GbpC-GFP were stimulated with 10^{-6} M cAMP and movies were recorded with time frames of one or two seconds. Shown are confocal images of three frames, at the point of cAMP-stimulation (A; t = 0 s), 4 seconds after stimulation (B), and 20 seconds after stimulation (C). To highlight membrane localization, D-F show equivalents of A-C after subtraction of the average cytosolic fluorescence intensity. Panel (G) shows the decrease of the fluorescence intensity of the cytosol averaged over 8 cells, which was analyzed using ImageJ

stimulation with the chemoattractant cAMP induces a rapid translocation to the cell membrane (Fig 6.). This translocation occurs independent of cGMP and the below described intramolecular signaling cascade in GbpC (van Egmond et al., 2008); GbpC still translocates in mutants that lack cGMP production or a functional GEF, Roc or kinase domain. In the contrary, mutations in the GRAM domain of GbpC lead to disturbed membrane association upon cAMP-stimulation; furthermore, the GRAM domain itself associates with cellular membranes and binds various phospholipids *in vitro*. Furthermore, mutants in the GRAM domain cause inactivation of GbpC *in vivo*. Together, the results show that GbpC receives multiple input signals: cAMP-stimulation induces a cGMP-dependent signaling cascade leading to kinase activity, and independently GRAM-dependent translocation of GbpC to the membrane is needed for proper functional activity.

2.4 Model for the activation of Roco proteins

Together these data show that although there is a high variation of additional regulatory domains among the Roco proteins, the Roco core itself functions in a similar way in all proteins. We have translated our biochemical, genetic and structural data into a model for the regulatory mechanism of LRRK2 (Fig 7). LRRK2 is a constitutive dimer by interaction of the COR domains. In the GDP-bound inactive state the G-domains are flexible, but in the active form the G-domains come in close proximity to each other. This conformational change is transmitted to the kinase domains to allow the activation loops of the two kinase protomers to be autophosphorylated and activated. The GTPase reaction is also dependent on dimerization, because efficient catalytic machinery is formed by complementation of the active site of one protomer with that of the other protomer. In this way the GTPase reaction functions as a timing device for the activation of the kinase and the biological function of the protein. Consistently, PD-related mutations have reduced GTPase activity and enhance kinase activity (unpublished data, (Cookson *et al.*, 2010)). Since the GTPase reaction is regulated by homodimerization and Roco proteins have a low nucleotide affinity (in the μM range), regulation by GEFs and GAPs is not necessary (Gotthardt *et al.*, 2008). However in some transient responses, as shown for GbpC, additional stimulation of the already high intrinsic exchange rate by GEF protein might be required. To completely understand the mechanism it will be important to know how the GDP-GTP cycle changes the RocCOR tandem and how it might influences the output of other parts of the protein. Therefore it will be important to solve structures of wild-type and/or PD-analogous mutants of Roco proteins in the different nucleotide states. The N-terminal segment, including the LRRs, is determining the input/output specificity of the proteins, but the exact mechanism is not clear. We propose two non-exclusive mechanisms: the N-terminal segment may selectively bind its substrates, brings it in close proximity of the Roco kinase domain and is subsequently phosphorylated. Alternatively, the N-terminal segment is binding upstream protein that activates specifically the Roco protein. In the context of LRRK2, 14-3-3 might be one of these upstream regulators: 14-3-3 binds in a phosphorylation dependent way to the N-terminal segment of LRRK2, thereby regulating its subcellular localization (Sen *et al.*, 2009;Nichols *et al.*, 2010).

3. Conclusion

Together, our results show that *Dictyostelium* provides an excellent model to study the function and activation mechanism of LRRK2. Roco proteins are the result of recent gene duplications, and are very homologous to mammalian LRRK2. Disruption of *Dictyostelium*

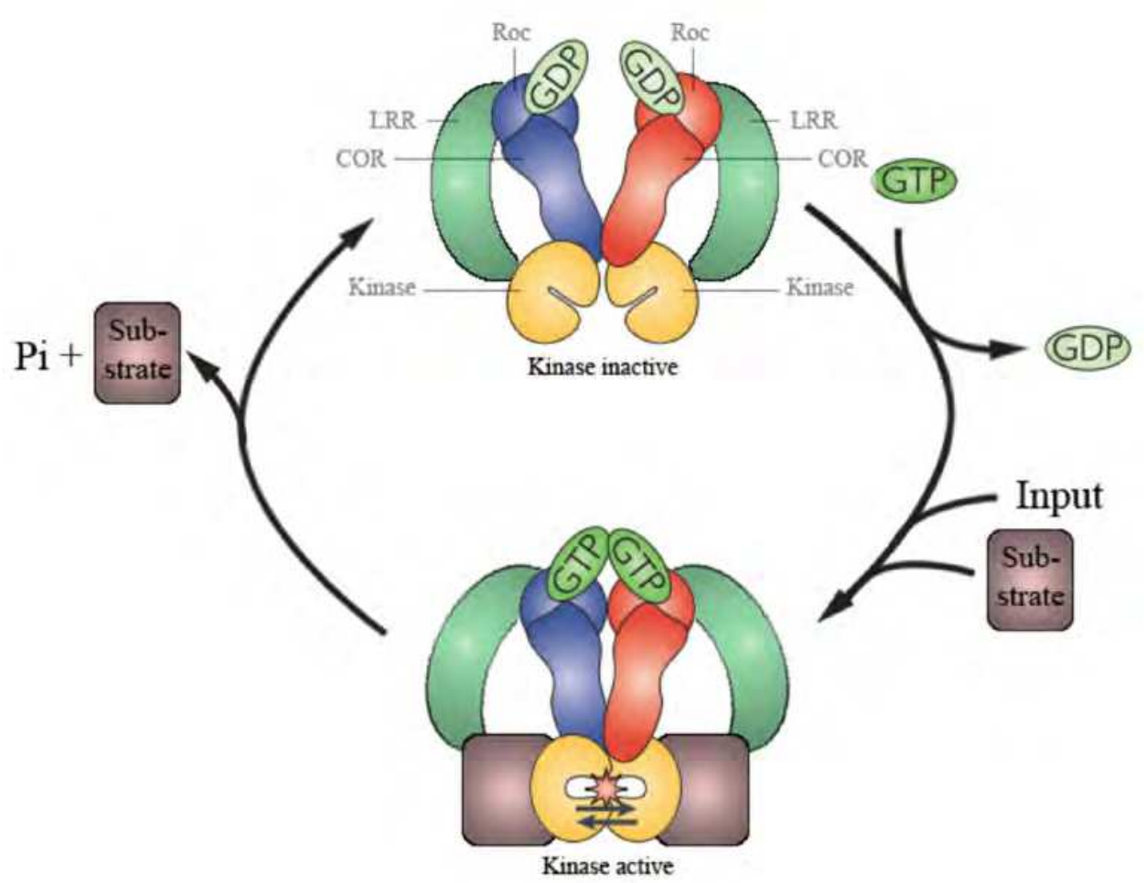

Fig. 7. Proposed model for the function and mechanism of LRRK2. GTP binding to the Roc domain results in dimerization of the Roc domain and subsequently activation of the kinase domain. The GTPase reaction is also dependent on dimerization; the efficient catalytic machinery is formed by complementation of the active site of one protomer with that of the other protomer. The LRR are directly involved in determining input/output specificity of the Roco proteins, most likely by binding upstream proteins that activate specifically the Roco protein and/or by selectively binding of the substrate.

Roco genes leads to very different phenotypes, indicating that they are involved in multiple cellular processes: they participate in cell division, osmotic-stress-response and development (van Egmond et al., 2010). The strong and diverse deletion phenotypes provide a unique opportunity to study PD-related mutations in living cells. These *roco2*- or *roco4*-null cells also provide a tool to express chimera proteins of Roco2, Roco4 and LRRK2 (full length proteins with domains derived from different sources). In contrast to LRRK2, many large parts of Roco4 can be expressed in *E.coli* to high levels in a stable and active form. Sufficient Roco4 protein and combinations of its domains could be purified for biochemical studies and crystallization. All Roco4 constructs both of wild-type and PD-related mutants show properties very much resembling those described for LRRK2. We have translated our results in a model, which can serve as a framework for the basic understanding for the complex regulatory mechanism of LRRK2, and provides a new starting point to answer major questions in the Parkinson field (Cookson, 2010): i) what are

the upstream activators of Roco proteins, ii) what is the 3D structure of Roco proteins and how are they activated, iii) what are the output substrates of activated kinase, and iv) can we identify small molecule inhibitors of the activated kinase to revert the activity of the PD-associated mutations. Our studies in *Dictyostelium* might be instrumental in this enterprise and can give important insights in the molecular mechanism of LRRK2 activation, and how mutations of LRRK2 result in neuronal toxicity. In this way we hope to contribute to the understanding of the biochemical pathways responsible for LRRK2-linked PD and help to identify therapeutic targets for PD and related neurodegenerative disorders.

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Mechanisms in Parkinson's Disease - Models and Treatments
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Parkinson's disease (PD) results primarily from the death of dopaminergic neurons in the substantia nigra. Current PD medications treat symptoms; none halt or retard dopaminergic neuron degeneration. The main obstacle to developing neuroprotective therapies is a limited understanding of the key molecular mechanisms that provoke neurodegeneration. The discovery of PD genes has led to the hypothesis that misfolding of proteins and dysfunction of the ubiquitin-proteasome pathway are pivotal to PD pathogenesis. Previously implicated culprits in PD neurodegeneration, mitochondrial dysfunction, and oxidative stress may also act in part by causing the accumulation of misfolded proteins, in addition to producing other deleterious events in dopaminergic neurons. Neurotoxin-based models have been important in elucidating the molecular cascade of cell death in dopaminergic neurons. PD models based on the manipulation of PD genes should prove valuable in elucidating important aspects of the disease, such as selective vulnerability of substantia nigra dopaminergic neurons to the degenerative process.

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