ANNUAL REVIEWS

Annual Review of Cell and Developmental Biology

Parkinson's: A Disease of Aberrant Vesicle Trafficking

Pawan Kishor Singh and Miratul M.K. Muqit

MRC Protein Phosphorylation and Ubiquitylation Unit, Sir James Black Centre, School of Life Sciences, University of Dundee, Dundee DD1 5EH, United Kingdom; email: m.muqit@dundee.ac.uk

Annu. Rev. Cell Dev. Biol. 2020. 36:237–64

First published as a Review in Advance on August 4, 2020

The *Annual Review of Cell and Developmental Biology* is online at cellbio.annualreviews.org

https://doi.org/10.1146/annurev-cellbio-100818-125512

Keywords

Parkinson's disease, α-synuclein, LRRK2, PINK1, Parkin, Rab, SNARE, exosome, vesicle trafficking, mitophagy

Abstract

Parkinson's disease (PD) is a leading cause of neurodegeneration that is defined by the selective loss of dopaminergic neurons and the accumulation of protein aggregates called Lewy bodies (LBs). The unequivocal identification of Mendelian inherited mutations in 13 genes in PD has provided transforming insights into the pathogenesis of this disease. The mechanistic analysis of several PD genes, including α-synuclein (α-syn), leucine-rich repeat kinase 2 (LRRK2), PTEN-induced kinase 1 (PINK1), and Parkin, has revealed central roles for protein aggregation, mitochondrial damage, and defects in endolysosomal trafficking in PD neurodegeneration. In this review, we outline recent advances in our understanding of these gene pathways with a focus on the emergent role of Rab (Ras analog in brain) GTPases and vesicular trafficking as a common mechanism that underpins how mutations in PD genes lead to neuronal loss. These advances have led to previously distinct genes such as vacuolar protein–sorting-associated protein 35 (VPS35) and LRRK2 being implicated in a common signaling pathway. A greater understanding of these common nodes of vesicular trafficking will be crucial for linking other PD genes and improving patient stratification in clinical trials underway against α-syn and LRRK2 targets.

Contents

INTRODUCTION

Parkinson's disease (PD) was first defined as a clinical entity just over two centuries ago, and it now represents the second most common neurodegenerative disorder, affecting from 2% to 3% of the population greater than 65 years age (Poewe et al. 2017). The hallmark motor symptoms of PD include resting tremor, postural instability, bradykinesia, and rigidity. However, PD also involves nonmotor symptoms that contribute to patients' disability, including anosmia, cognitive dysfunction, sleep–awake dysregulation, dysautonomia, depression, and constipation (Poewe et al. 2017). PD pathology is characterized by the loss of pigmented dopaminergic neurons in the substantia nigra pars compacta (SNpc) midbrain region, and PD motor symptoms are thought to occur with a 60–70% loss of dopaminergic neurons (Lang & Lozano 1998). In association with neuronal loss is the widespread accumulation of α-synuclein (α-syn)-containing aggregates in surviving neurons, termed Lewy bodies (LBs) (Spillantini & Goedert 2018). Although the common cause of PD, termed sporadic or idiopathic, is unknown, multiple risk factors including aging and environmental factors are believed to contribute to disease pathogenesis (Poewe et al. 2017). These risk factors are thought to interact with genetic susceptibility, and over the last decade, great advances have been made in uncovering the genetic architecture of PD (Bandres-Ciga et al. 2020). Our understanding of mechanisms and pathways underlying PD has been transformed by the discovery of gene mutations in rare familial forms of PD that account for from 5% to 10% of all cases. The

mechanistic analysis of several genes, namely α-syn, PTEN-induced kinase 1 (PINK1), Parkin, and leucine-rich repeat kinase 2 (LRRK2), has broadly highlighted major pathways including protein aggregation, mitochondrial dysfunction, and defects in endolysosomal trafficking; this has thereby implicated Rab (Ras analog in brain) GTPases as playing a central role in the pathogenesis of PD.

Rab GTPases are the key regulators of intracellular trafficking in eukaryotic cells, with more than 60 members in mammals and 11 homologs in budding yeast (Muller & Goody 2018). Rabs exist within cells in two conformations: an active GTP-bound form, which associates with intracellular membranes, and an inactive GDP-bound conformation, which is present in the cytosol. Rabs are prenylated at their C-terminal cysteine residues with the help of geranylgeranyl transferases (GGTs) and Rab escort proteins (REPs); this process is required for their association with the membrane. In their membrane-bound form, Rabs are activated by the exchange of GDP to GTP, which is catalyzed by guanine nucleotide exchange factors (GEFs). In their active state, Rabs interact with various effector proteins to regulate myriad functions including cargo selection, vesicle budding, vesicle transport along the cytoskeletal track, and tethering and fusion of the vesicle with the target membrane compartments. There Rabs can hydrolyze their GTP into GDP with the aid of GTPase-activating proteins (GAPs) and thereby switch into an inactive state. In the GDP-bound inactive state, Rabs are retrieved and sequestered in the cytosol by GDP dissociation-inhibitor (GDI) proteins (Bonet-Ponce & Cookson 2019, Stenmark 2009). Whether the deregulation of Rab function and vesicular trafficking is a primary cause of PD neurodegeneration, as well as the relevance of Rab biology to the more common general form of PD, is an outstanding question that is being intensively studied.

PARKINSON'S DISEASE GENETICS AND TRAFFICKING

The Genetic Landscape of Parkinson's Disease

The most recent meta-analysis of genome-wide association studies (GWAS) of sporadic PD has identified 90 independent gene risk variants that overall account for 16% to 36% of the heritable risk of PD (Nalls et al. 2019). The functional analysis of these genes has revealed links to lysosomal biology, mitochondrial homeostasis, autophagy, and vesicular trafficking as well as protein aggregation (Nalls et al. 2019). However, whether the gene assignment of risk variants based on proximity is appropriate for all cases is unclear. Of the 23 PD genes and loci (designated *PARK*) that have been identified in rare families with monogenic forms of PD, clear-cut mutations have been confirmed in 13 genes (Lunati et al. 2018). These genes are classified into autosomal dominant genes, namely *SNCA/PARK1/PARK4* (α-syn), *LRRK2/PARK8* (LRRK2), and *VPS35/PARK17* (vacuolar protein–sorting-associated protein 35; VPS35); X-linked gene mutations in *RAB39B* (Rab39B); autosomal recessive genes, including *PRKN/PARK2* (Parkin), *PINK1/PARK6* (PINK1), and *PARK7* (DJ-1); and finally autosomal recessive genes associated with complex Parkinsonian phenotypes, including *ATP13A2/PARK9* (ATPase 13A2; ATP13A2), *PLA2G6/PARK14* (phospholipase A2 group VI; PLA2G6), *FBX07/PARK15* (F-box only protein 7; FBX07), *DNAJC6/PARK19* [DnaJ heat shock protein family member C6 (DNAJC6); auxilin], *SYNJ1/PARK20* (Synaptojanin 1; SYNJ1), and *VPS13C/PARK23* (vacuolar protein–sorting 13 homolog C; VPS13C) (Lunati et al. 2018). A further four loci/genes have been confirmed as major risk factors for late-onset PD including *PARK10*, *MAPT* (microtubule-associated protein tau), *GBA* (glucocerebrosidase; GBA) and *PARK16*, whose locus contains the gene *RAB29* (Rab29) (Martin et al. 2011). The identification of a PD-causing mutation in the α-syn gene and the subsequent discovery that α-syn (**Figure 1*a***) is the major protein component of LBs have shown that protein misfolding and aggregation is a central pathway in PD pathogenesis. In parallel, the discovery of the physiological

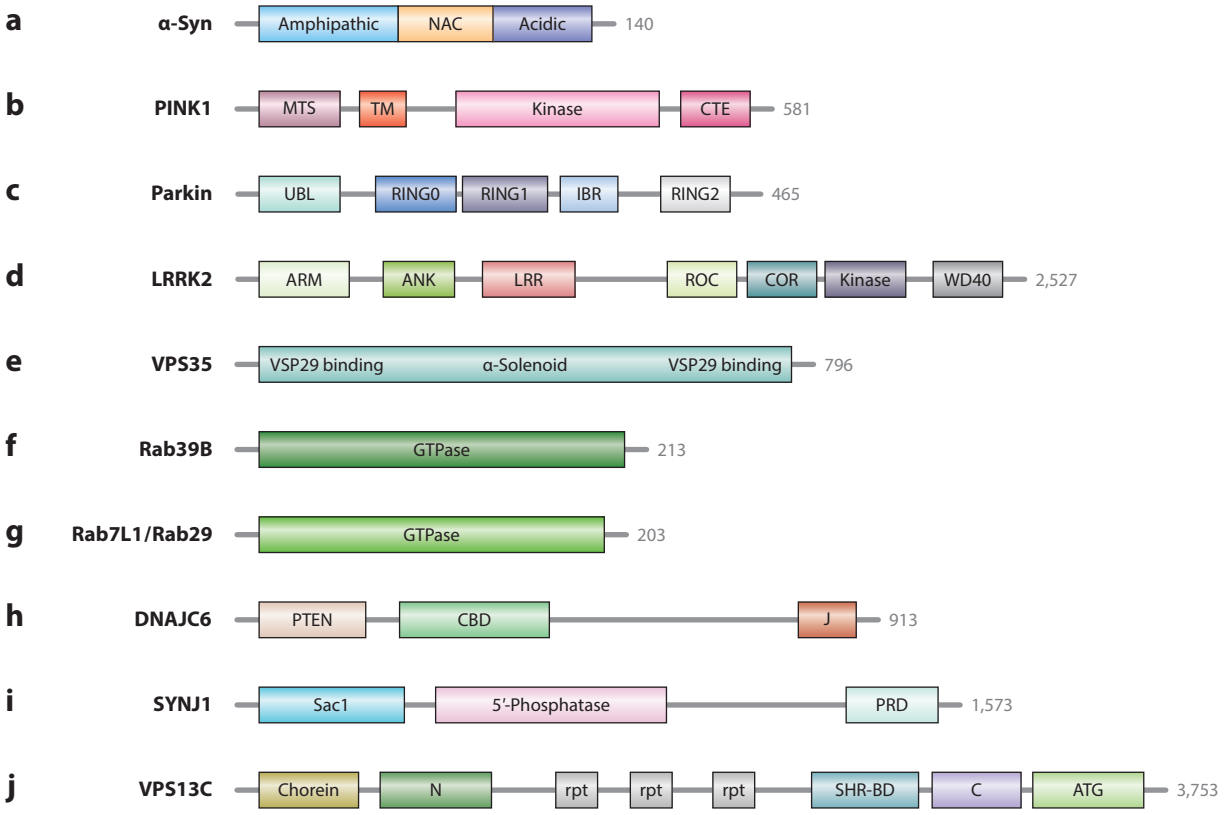


Figure 1

Domain architecture of PD proteins linked to membrane trafficking. Protein domains of human (*a*) α-syn (140 aa), (*b*) PINK1 (581 aa), (*c*) Parkin (465 aa), (*d*) LRRK2 (2,527 aa), (*e*) VPS35 (796 aa), (*f*) Rab39B (213 aa), (*g*) Rab7L1/Rab29 (203 aa), (*h*) DNAJC6/auxilin (913 aa), (*i*) SYNJ1/Synaptojanin (1,573 aa), and (*j*) VPS13C (3,573 aa). Abbreviations: α-syn, α-synuclein; aa, amino acids; ANK, ankyrin; ARM, armadillo; ATG, autophagy-related protein; C, C-terminal domain; CBD, clathrin-binding domain; COR, C-terminal of ROC; CTE, C-terminal extension; DNAJC6, DnaJ heat shock protein family member C6; GTP, guanosine triphosphate; IBR, in-between RING; J, J-domain; LRRK2, leucine-rich repeat kinase 2; MTS, mitochondrial targeting sequence; N, N-terminal domain; NAC, nonamyloid-β component; PD, Parkinson's disease; PINK1, PTEN-induced kinase 1; PRD, proline-rich domain; PTEN, phosphatase and tensin homolog; Rab, Ras analog in brain; RING, really interesting new gene; ROC, Ras of complex; rpt, repeat; SHR-BD, SHORT ROOT–binding domain; SYNJ1, Synaptojanin 1; TM, transmembrane; UBL, ubiquitin-like; VPS, vacuolar protein–sorting protein.

role that α-syn plays in SNARE (soluble N-ethylmaleimide-sensitive factor attachment protein receptor) complex assembly and synaptic vesicle trafficking (see the section titled Protein Aggregation Pathways and Vesicular Trafficking) and how this is disrupted by α-syn mutations have also been of major interest. Mutations in α-syn are rare, accounting for only from 1% to 2% of familial PD. These comprise gene duplications, triplications, or missense mutations ([Ala30Pro], [Glu46Lys], [Gly51Asp], [Ala53Glu], and [Ala53Thr]). In general, the phenotype of these patients does not resemble that of patients with typical PD, instead having a high prevalence of dementia, neuropsychiatric signs, and a moderate response to L-Dopa. Pathologically there is a high burden of LBs, Lewy neurites (LNs), and neuronal loss in the SNpc (Lunati et al. 2018).

The discovery of mutations in the PINK1 and Parkin genes has spearheaded understanding of the roles of these genes in mitochondrial quality control pathways linked to PD

neurodegeneration (Harper et al. 2018). Autosomal recessive mutations in Parkin were first discovered in 1998 and are the most common cause of early-onset PD, accounting for nearly 40% of all cases (Kitada et al. 1998). Parkin encodes a multidomain ubiquitin (Ub) E3 ligase containing an N-terminal Ub-like (Ubl) domain and RING0, RING1, in-between RING (IBR), and RING2 domains (**Figure 1*c***). The catalytic cysteine (Cys431) lies within the RING2 domain (Wenzel et al. 2011). Numerous PD-causing mutations have been identified including rearrangements that drastically destabilize the protein and missense mutations across the whole protein that disrupt critical regulatory amino acid residues, e.g., [Cys431Phe] within RING2 that disrupts the Ub thioester formation required for Ub transfer to substrates (Wenzel et al. 2011) and [Ser65Asn] that prevents phosphorylation and activation by PINK1 (McWilliams et al. 2018). Autosomal recessive mutations in PINK1 that encode a mitochondria-localized protein kinase represent the second leading cause of early-onset PD. Over 30 missense and nonsense mutations have been reported, mainly localized within the kinase domain (**Figure 1*b***), that lead to loss of function through catalytic inactivation or truncations, respectively (Valente et al. 2004). Structural and biochemical studies have elaborated the mechanism by which PINK1 phosphorylates and activates Parkin, and cell biological studies have demonstrated that these enzymes function in a common signaling pathway to stimulate the removal of damaged mitochondria by mitophagy (see the section titled Mitochondrial Quality Control and Vesicular Trafficking) (Harper et al. 2018, McWilliams & Muqit 2017). Consistent with this, patients harboring PINK1 or Parkin mutations exhibit identical phenotypes with early-onset disease, an excellent response to L-Dopa, and a relatively high frequency of dystonia and dyskinesias (Lunati et al. 2018). Pathologically, PINK1 and Parkin patient brains exhibit relatively restricted neurodegeneration within the substantia nigra (SN) and a paucity of LBs, although variability in LB burden has been reported in select cases for each gene (Kunath et al. 2019).

LRRK2 encodes a multidomain protein comprising core kinase and GTPase [Ras of complex (Roc)-C-terminal of Roc (COR)] domains (**Figure 1*d***) together with several protein-interaction domains; mutations in this gene represent the most common cause of PD (Taylor & Alessi 2020). To date nine missense mutations have been confirmed as pathogenic that are mainly localized within the kinase or Roc-COR domains, including [Asn1437His], [Arg1441Gly/Cys/His], [Arg1628Pro], [Tyr1699Cys], [Gly2019Ser], [Ile2020Thr], and [Gly2385Arg] (Lunati et al. 2018). The [Gly2019Ser] mutation is the most frequent, varying from 1% to 5% of PD cases in Europe and up to 40% of cases in North Africa; however, the penetrance is incomplete and increases with every decade of patients' lives to greater than 90% by the ninth decade (Lunati et al. 2018). The phenotype resembles typical PD with asymmetrical onset, nonmotor symptoms, and good L-Dopa response that is indistinguishable from typical PD (Lunati et al. 2018). However, the mean age of onset is lower at 58 years and the progression slower than that of typical PD cases (Lunati et al. 2018). Interestingly, there does not appear to be any difference in severity between heterozygous and homozygous [Gly2019Ser] patients (Lunati et al. 2018). Furthermore, the neuropathology of LRRK2 is variable, with LBs found in [Gly2019Ser] cases but few LBs and a predominance of tau inclusions found in the other mutations (Lunati et al. 2018). All pathogenic mutations lead to hyperactivation of the kinase activity of LRRK2, and Type I kinase inhibitors are currently undergoing clinical trials (Alessi & Sammler 2018).

Rabs: *RAB39B* Mutations and *PARK16* (*RAB29*) Risk Variants in Parkinson's Disease

The most compelling evidence for the deregulation of Rabs as a primary cause of PD has been the discovery of direct Rab mutations in familial PD cases. X-linked mutations in *RAB39B* were

first reported in 2014, including a ~45 kb loss-of-function deletion mutation in an Australian family with intellectual disability and early-onset PD and a missense [Thr168Lys] mutation in a Wisconsin family with a similar phenotype (Wilson et al. 2014). These mutations led to complete loss of protein expression, and patients exhibited a typical PD phenotype that evolved later in life (usually before age 45) and had a good response to L-Dopa. Women were reported to exhibit a milder phenotype with a later age of onset of PD and less intellectual disability (Wilson et al. 2014). Pathological analysis of a male patient revealed typical PD pathology with extensive loss of dopaminergic neurons from the SN and classic LBs and LNs (Wilson et al. 2014). A loss-of-function [Trp108Stop], null-truncating mutation was reported in a 39-year-old man with typical early-onset PD and mild intellectual disability (Lesage et al. 2015). A missense [Gly192Arg] mutation has been reported in families with L-Dopa-responsive, early-onset PD without any intellectual disability and affecting both males and females (albeit with reduced penetrance), suggesting that some mutations may be able to exert a dominant-negative effect (Mata et al. 2015). This mutation lies within the C-terminal hypervariable domain of Rab39B and is predicted to disrupt intracellular localization and interaction with effectors (**Figure 1*f***). Rab39B is highly expressed in the brain and in situ hybridization studies indicate this occurs exclusively in neurons (Giannandrea et al. 2010). The subcellular localization of Rab39B is predominantly within the Golgi apparatus, and *RAB39B* knockdown studies reveal effects on vesicle trafficking and a reduction in synapse formation of neurons in culture (Giannandrea et al. 2010).

Genetic studies have identified and confirmed a locus, *PARK16*, as a susceptibility factor for sporadic PD (Nalls et al. 2014, Satake et al. 2009). Of 5 genes within this region, *RAB29* (*RAB7L1*) has emerged as the leading candidate (**Figure 1*g***), although the mechanism of how the risk variant leads to PD is unclear; in one study using protein–protein interaction arrays, analysis suggested that Rab29 is a binding partner of LRRK2 and promotes the removal of Golgi-derived vesicles through the autophagy-lysosome pathway (ALP) (Beilina et al. 2014). Other genetic studies of PD patient cohorts have found common variants within the *LRRK2* and *RAB29* genes that function coordinately to increase PD risk nonadditively (Pihlstrøm et al. 2015). Perhaps the most compelling evidence for *RAB29* has been functional studies showing that the Rab29 protein functions as a critical upstream regulator of LRRK2 leading to increased LRRK2 activity and phosphorylation of Rabs (Liu et al. 2018, Purlyte et al. 2018). Rab29 is closely related to Rab32 and Rab38, and Rab32 has been found to interact with LRRK2 via its N-terminal armadillo (ARM) domain (**Figure 1*d***); similar to Rab29, however, there is no evidence for *RAB32* mutations in familial PD cases (McGrath et al. 2019).

VPS35 and *VPS13C* Mutations in Parkinson's Disease

Autosomal dominant mutations in *VPS35* were identified in 2011 in patients with late-onset PD (Vilariño-Güell et al. 2011, Zimprich et al. 2011). *VPS35* encodes the cargo-binding subunit of the trimeric retromer complex (also containing VPS29 and VPS26) whose function is to transport specific endosomal cargoes into vesicles and tubules, delivering these to either the trans-Golgi network or the plasma membrane (Williams et al. 2017). To date, the only definite pathogenic mutation identified is an [Asp620Asn] (*D620N*) mutation that structural analysis indicates is located at the surface of the convex face of an α-helical solenoid domain (**Figure 1*e***), suggesting that the *D620N* mutation may impact interaction with an as-yet-unknown effector (Williams et al. 2017). The symptoms of these patients generally resemble those of patients with typical PD but with a mean age of onset of 50 years; however, several cases have been reported with earlier age of onset in the 30s and 40s (Lunati et al. 2018). Disease progression tends to be slow, cognitive decline rare, and response to L-Dopa good (Lunati et al. 2018). The discovery that the *D620N* mutation

significantly enhances LRRK2 activation, as assessed by autophosphorylation and Rab phosphorylation, places VPS35 upstream of LRRK2 in a common pathway, although the mechanism by which it activates LRRK2 is unknown (Mir et al. 2018). Cell biological studies have shown that the *D620N* mutation also disrupts the association of VPS35 with the WASH complex via impaired binding to FAM21, which leads to trafficking defects (McGough et al. 2014, Zavodszky et al. 2014); further studies are required to assess whether this is linked to LRRK2 hyperactivation.

Autosomal recessive mutations in *VPS13C* have been identified in patients with early-onset PD presenting between 25 and 45 years of age (Lesage et al. 2016). *VPS13C* encodes a large 3,753–amino acid protein that belongs to the VPS13 (A-D) family of proteins. VPS13C and VPS13A function as lipid transporters at organelle contact sites (Kumar et al. 2018). VPS13C is localized at sites bridging the endoplasmic reticulum (ER) with an endolysosomal system, specifically vesicles containing the lysosomal marker LAMP1 and Rab7. VPS13C is anchored to the ER via its N-terminal domain (**Figure 1*j***) through a conserved FFAT motif that binds both the ER vesicle-associated membrane protein (VAMP)-associated protein (VAP) and lipid droplets via its C-terminus (Kumar et al. 2018). Interestingly, the analysis of cells expressing PD-associated *VPS13C* mutants found mitochondrial defects suggesting that this may be an indirect consequence of defects in the endolysosomal system (Lesage et al. 2016). The clinical phenotype is atypical for PD, with a moderate response to L-Dopa and a rapid and severe disease progression with early cognitive decline. Pathological analysis of a single case has revealed severe pathological changes that resemble diffuse LB disease (Lesage et al. 2016).

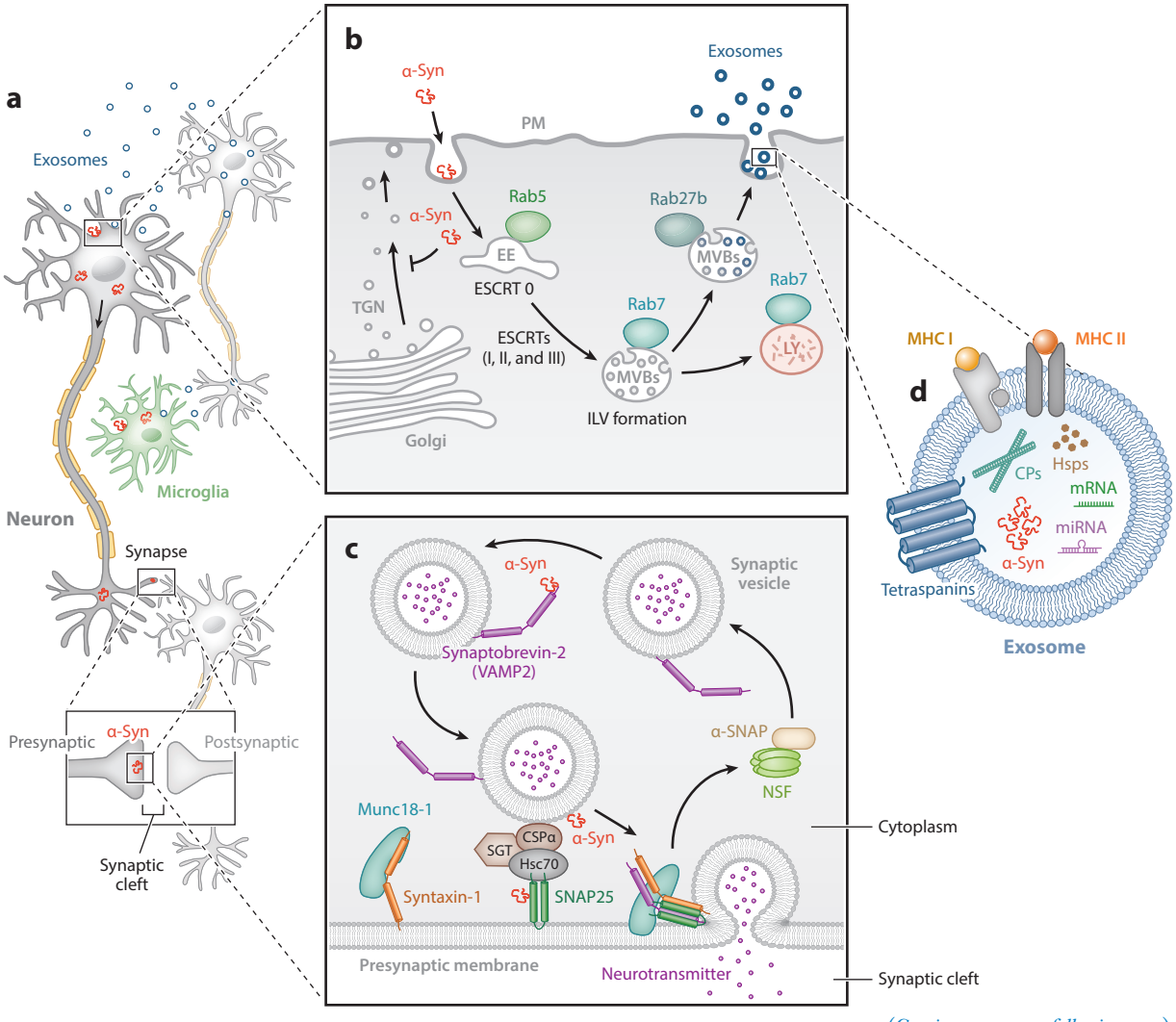
Synaptic Vesicle Trafficking: *DNAJC6* and *SYNJ1* Mutations in Parkinson's Disease

Synaptic transmission at dopaminergic synapses is critically dependent on the recycling of dopamine receptors and other proteins via synaptic vesicle endocytosis followed by trafficking and sorting within the endosomal pathway. Interestingly, mutations in two genes encoding components of this process have been identified in autosomal recessive early-onset complex PD patients. Mutations in *DNAJC6* encoding auxilin (**Figure 1*b***) cause juvenile-onset PD (Edvardson et al. 2012) and are loss-of-function with exonic deletions and splice-defective mutants. Auxilin is part of the conserved DNAJ/Hsp40 family that stimulates ATPase activity of heat shock cognate 70 (Hsc70) to mediate uncoating of clathrin-coated vesicles (CCVs) following endocytosis (Edvardson et al. 2012). Without the removal of clathrin, vesicles are unable to fuse and be taken up into the endosomal pathway for cargo sorting. Loss-of-function mutations in Synaptojanin-1 (*SYNJ1*), which encodes a lipid 5′ phosphatidylinositide phosphatase, lead to early-onset PD (Drouet & Lesage 2014, Fasano et al. 2018). SYNJ1 dephosphorylates phosphatidylinositide 4,5-bisphosphate to trigger the release of adaptor proteins from CCVs that enables auxilin-mediated clathrin uncoating (Drouet & Lesage 2014). Mutations including [Arg258Gln] and [Arg459Pro] have been identified within the Sac1 domain (**Figure 1*i***) and have been shown to reduce phosphatase activity (Hardies et al. 2016, Vanhauwaert et al. 2017). Patients harboring *DNAJC6* or *SYNJ1* mutations have a similar phenotype, with typical onset between 20 and 40 years of age and complex deficits including Parkinsonism with poor response to L-Dopa and epilepsy and cognitive decline (Lunati et al. 2018). Consistent with this observation, *SYNJ1* or *DNAJC6*-knockout mice exhibit the same phenotype with neurological deficits and accumulate CCVs at the synaptic terminal (Cao et al. 2017). *SYNJ1* and *DNAJC6* mutations highlight the critical role of synaptic vesicle endocytosis and the endosomal pathway in the maintenance of dopaminergic striatal terminals to prevent PD; whether these factors interact with other PD gene components remains unknown.

PROTEIN AGGREGATION PATHWAYS AND VESICULAR TRAFFICKING

α-Synuclein and Rab GTPases

Multiple studies have established the role of Rab GTPases in α-syn aggregation–induced PD neurodegeneration in different model systems. An initial study reported the role of Rab5a, an early-endosomal Rab GTPase, in the endocytosis of α-syn leading to neuronal cell death, whereas the expression of GTPase-deficient Rab5a rescued the phenotype due to perturbed endocytosis (Sung et al. 2001). The role of Rab5b, another isoform of Rab5, was also linked to the α-syn-induced endocytosis of the NMDA receptor (a glutamate and ion channel protein receptor) by upregulating its expression (Cheng et al. 2011). The role of endocytic Rabs contributing to α-syn trafficking was further established following the discovery of Rab7 in the autolysosomal clearance of α-syn aggregates (**Figure 2*b***). When a pathogenic *A53T* mutant of α-syn was expressed in HEK293



(*Caption appears on following page*)

Figure 2 (*Figure appears on preceding page*)

Vesicular trafficking mechanisms of α-syn aggregation–mediated neurotoxicity. (*a*) α-Syn aggregates can be transmitted to other neurons through endocytic mechanisms or exosomes, or they can block neurotransmitter release at synapses. (*b*) Under normal conditions, α-syn aggregates can enter the cell through an endocytic mechanism, first reaching Rab5-positive EEs, where they can be recycled back through a recycling mechanism (not shown) or sorted within the MVBs to form ILVs through the involvement of ESCRT machinery (complexes 0, I, II, and III). When MVBs fuse with the plasma membrane, exosomes are secreted into the extracellular milieu. Rab27b has been suggested to play a role in the exosome-mediated secretion of α-syn aggregates, or these aggregates can be degraded within lysosomes through Rab7-mediated endolysosomal fusion. The perturbation of these processes in PD causes the accumulation of pathogenic α-syn aggregates within the cell, leading to toxicity. (*c*) Under physiological conditions, α-syn functions as a SNARE chaperone to regulate the exocytic release of neurotransmitter at the presynaptic terminal. In PD, α-syn aggregates block neurotransmitter release by engaging VAMP2 and SNAP25, leading to neurodegeneration. Panel *c* is adapted from Rizo & Sudhof (2012). (*d*) Exosomes containing α-syn aggregates may facilitate propagation to other cells. Other constituents of exosomes include proteins such as tetraspanins, MHCs I and II, CPs, Hsps, miRNA, and mRNA. Abbreviations: α-SNAP, α-soluble NSF-attachment protein; α-syn, α-synuclein; CP, cytoskeletal protein; CSPα, cysteine string protein α; EE, early endosome; ESCRT, endosomal sorting complex required for transport; Hsc70, heat shock cognate 70; Hsp, heat shock protein; ILV, intraluminal vesicle; LE, late endosome; LY, lysosome; MHC, major histocompatibility complex; miRNA, microRNA; mRNA, messenger RNA; Munc18-1, mammalian uncoordinated 18-1; MVB, multivesicular body; NSF, N-ethylmaleimide-sensitive factor; PM, plasma membrane; Rab, Ras analog in brain; SGT, small glutamine-rich tetratricopeptide repeat; SNAP25, synaptosomal-associated protein 25; SNARE, soluble N-ethylmaleimide-sensitive factor attachment protein receptors; TGN, trans-Golgi network; VAMP2, vesicle-associated membrane protein 2.

cells and *Drosophila melanogaster*, Rab7 overexpression was found to lead to the clearance of α-syn aggregates, reduced cell death, and rescue of the locomotor deficit. These effects were specific to Rab7, as other Rab GTPases such as Rab5, Rab9, or Rab23 failed to rescue the pathogenic phenotype (Dinter et al. 2016). Increased levels of GTP-bound Rab5 and Rab7 were also found upon α-syn overexpression that interacted with the motor protein dynein in a transgenic mouse model of PD (Fang et al. 2017). Rab11A, which regulates the trafficking of recycling endosomes, also regulates the secretion of extracellular α-syn through its interaction with Hsp90 (Liu et al. 2009). In *Drosophila* models of PD, Rab11 rescues the α-syn-induced increased synaptic vesicle size, the degeneration of dopaminergic neurons, and reduced life span in both larval and adult flies (Breda et al. 2015).

Secretory pathway Rabs have also been shown to work in concert with endocytic Rabs to regulate α-syn trafficking. Pull-down studies (Dalfó et al. 2004b) have identified the interaction of secretory pathway Rabs such as Rab3A and Rab8 as well as endocytic Rab5 with mutant A30P human α-syn in a transgenic mouse model. Immunoprecipitation studies (Dalfó et al. 2004a) have also showed an increased interaction of Rab3A with α-syn aggregates and a decreased affinity for its effector Rabphilin in LB disease (LBD) cases. Rab3A was also immunoprecipitated with α-syn from the SN but not cerebral cortex extracts from the postmortem brains of patients with PD (Dalfó & Ferrer 2005). In PC12 cells, soluble α-syn interacts with Rab3A to enhance exocytosis through vesicle priming and fusion by releasing calcium (Ca^{2+}) from thapsigargin (an inhibitor of ER Ca^{2+} ATPase)-sensitive Ca^{2+} pools (Huang et al. 2018). In an RNA interference (RNAi)-based microscopic screen of α-syn aggregation in human neuroglial cells, Rab8B, Rab11A, and Rab13 knockdown were found to rescue α-syn-induced cytotoxicity, whereas Rab11A and Rab13 expression induced α-syn endocytic recycling and secretion (Gonçalves et al. 2016).

α-Synuclein also blocks trafficking between the ER and Golgi compartments in yeast cells and increased Rab1 (yeast homolog Ypt1) expression protects against dopaminergic neuronal loss in PD animal models (Cooper et al. 2006). Similarly, in a neuronal model of PD, post–Golgi vesicle–associated Rab8A also inhibited α-syn toxicity (Gitler et al. 2008). In a cellular model of PD, Rab1, Rab2, and Rab8 regulated Golgi fragmentation, leading to the aggregation of α-syn as well as the impairment of anterograde and retrograde transport between the ER and the Golgi apparatus

(Rendón et al. 2013). Taken together, Rab GTPases can regulate α-syn aggregation and secretion as well as its clearance in the various model systems of PD.

α-Synuclein and SNAREs

SNAREs are a family of proteins that associates with intracellular membranes and regulates the docking and fusion of donor intracellular vesicles with target compartment membranes. SNAREs can be functionally categorized into v-SNAREs, which associate with donor vesicles, and t-SNAREs, which are associated with target compartments (Jahn & Scheller 2006). The specific interaction of v-SNAREs with t-SNAREs leads to the formation of a quaternary helix complex that facilitates membrane fusion. These complexes are further recycled with the help of two soluble proteins: NSF (N-ethylmaleimide-sensitive factor), an AAA (ATPases associated with diverse cellular activities)-type ATPase, and α-SNAP (α soluble NSF-attachment protein), which maintains SNARE availability for subsequent fusion events (Chen & Scheller 2001). Similar to Rab GTPases, SNAREs also define a layer of specificity by localizing to specific membrane compartments, but their role is considered more promiscuous than that of Rabs. Synaptic vesicle fusion and neurotransmission are regulated by the neuronal SNAREs syntaxin-1, synaptobrevin/VAMP1, and synaptosomal-associated protein 25 (SNAP25) (Rizo & Sudhof 2012). Therefore, any perturbation in SNARE proteins per se or in regulatory proteins such as α-syn leads to the neurotransmission defects underpinning many neurological diseases including PD (Chua & Tang 2011).

Our understanding of the role of SNARE proteins involved in PD came from an earlier metabolic labeling study in the late nineties (Jensen et al. 1999), when SNAP25, a predominantly neuronal SNARE, was found to be colocalized with and involved in α-syn axonal transport in rat retinal ganglion cells. α-Syn was later discovered to cooperate with cysteine string protein α (CSPα) in mediating chaperone activity toward neuronal SNARE complexes (**Figure 2*c***) and to prevent neurodegeneration in mice. This modulation was achieved through the phospholipid binding of wild-type α-syn but not of the A30P mutant, which failed to attach to the membrane and could not rescue the phenotype (Chandra et al. 2005). A subsequent study (Darios et al. 2010) suggested that α-syn modulated SNARE complex formation and exocytosis through sequestering arachidonic acid, a common component of phospholipids. A transgenic mouse model of PD expressing truncated human α-syn (1–120) showed an age-dependent reduction of dopamine release accompanied by a redistribution of the SNARE proteins SNAP25, syntaxin-1, and synaptobrevin-2 in the striatum (Garcia-Reitböck et al. 2010). However, another study (Anwar et al. 2011) suggested no difference in the distribution of synaptic SNAREs in the dorsal striatum of triple-synuclein knockout mice. The first evidence of the direct interaction of α-syn with SNAREs came through a study (Burré et al. 2010) suggesting its binding with synaptobrevin-2 (VAMP2) through its C-terminus to maintain the presynaptic SNARE complex for efficient neurotransmitter release. Remarkably, a follow-up study by the same group (Sharma et al. 2011) also discovered that CSPα, which forms complexes with Hsc70 and the small glutamine-rich tetratricopeptide-repeat protein (SGT), directly interacts with SNAP25 to promote SNARE-complex formation (**Figure 2*c***) by preventing its aggregation and ubiquitylation-dependent proteasomal degradation. Analysis of the CSPα-knockout mouse that leads to neurodegeneration revealed that the neurodegenerative phenotype was mainly due to low levels of SNAP-25 leading to aberrant synaptic SNARE-complex assembly (Sharma et al. 2012). While soluble α-syn monomers promote the assembly of such complexes, how α-syn oligomers cause neurotoxicity has also been a major question. One report (Choi et al. 2013) suggested that α-syn oligomers bind to the N-terminal domain of VAMP2 and inhibit SNARE-mediated lipid mixing by preventing SNARE-complex formation. Interestingly, another study (DeWitt & Rhoades 2013) also

suggested that α-syn inhibits SNARE-dependent vesicle fusion by binding to the lipid membrane without direct interaction with SNAREs. α-Syn aggregates have also been shown to directly sequester SNARE proteins such as VAMP2 and SNAP25 (**Figure 2*c***), and this interaction, which is resistant to SDS treatment, leads to neurotoxicity. Furthermore, this sequestration is partially blocked by a VAMP2 peptide, resulting in reduced neurotoxicity (Choi et al. 2018).

Proteins regulating SNARE functions have also been found to be involved in modulating α-syn activity. Munc18-1 (mammalian uncoordinated 18-1), which is important for regulating the SNARE-dependent fusion of synaptic vesicles and thereby neurotransmitter release, regulates α-syn aggregation by acting as a molecular chaperone (Chai et al. 2016). In a mouse model of lysosomal storage disorders, α-syn aggregation and proteasomal degradation of CSPα have been reported, resulting in inhibition of SNARE-complex assembly and reduced recycling of synaptic vesicles and leading to neurodegeneration (Sambri et al. 2017). In conclusion, α-syn interacts with SNAREs to regulate neurotransmitter release, but this process is disrupted by oligomeric α-syn; conversely, SNAREs can, in turn, regulate α-syn function.

α-Synuclein and Exosomes

Exosomes are a type of extracellular vesicle measuring 30–100 nm in diameter that are secreted into the extracellular medium by many cell types in both normal and pathological conditions. Exosomes are composed of a lipid bilayer and are found to contain various proteins, lipids, RNAs, and other substances released by the secreting cell (Mathivanan et al. 2010). Exosomes are formed through inward budding in multivesicular bodies (MVBs) that generates intraluminal vesicles (ILVs) (**Figure 2*d***), which are then secreted extracellularly by the fusion of MVBs with the plasma membrane. ESCRT (endosomal sorting complex required for transport) machinery, which includes ESCRT complexes 0, I, II, and III, are involved in cargo sorting, budding, and fission of exosomes within MVBs. Several families of proteins such as tetraspanins, Hsps, cytoskeletal proteins, and trafficking proteins as well as various lipids, miRNAs, mRNAs, immune regulatory molecules such as major histocompatibility complexes (MHCs) I and II, and pathological components including viral proteins are all found in exosomes (Gupta & Pulliam 2014) (**Figure 2*d***). Initial hints about the role of exosomes in α-syn trafficking came through its detection in human plasma and cerebrospinal fluid and reports of the secretion of its monomeric and oligomeric forms from primary cortical neurons as well as from human neuroblastoma cells (Borghi et al. 2000; El-Agnaf et al. 2003, 2006; Lee et al. 2005; Tokuda et al. 2010). Later it was reported that α-syn is secreted through exosomes in a Ca^{2+}-dependent manner and affects the survival of neighboring neurons (Emmanouilidou et al. 2010). Another study (Alvarez-Erviti et al. 2011) in SH-SY5Y cells reported that lysosomal dysfunction, which has been reported in PD, can lead to the increased exosome-mediated secretion of α-syn and to its transmission to neighboring cells. In transgenic mice expressing human phosphorylation mimic S129D α-syn, which aggregates, exosomes loaded with small interfering RNA (siRNA) against α-syn and expressing neuron-specific rabies viral glycoprotein were found to be effective in reducing intraneuronal aggregates (Cooper et al. 2014). Exosomes have also been suggested to provide a catalytic environment for nucleation that promotes α-syn aggregation (Grey et al. 2015).

Microglia have been suggested to play an important role in the transmission of α-syn-containing exosomes through the uptake of pathogenic exosomes (**Figure 2*a***). This uptake results in inhibition of the autophagy and scavenging activities of microglia, which leads to further enhanced secretion of pathogenic exosomes and transmission to other cells (Xia et al. 2019). The reduced phagocytosis of exosomes containing α-syn with increased tumor necrosis factor (TNF)-α secretion was shown in mice microglia as well as in human monocytes derived from aged donors.

This might lead to an age-dependent susceptibility to misfolded proteins and infections in neurodegenerative diseases such as PD (Bliederhaeuser et al. 2016). Exosomes derived from the serum of PD patients have also been shown to be pathogenic to mice due to the presence of an increased abundance of α-syn and inflammatory factors such as interleukin (IL)-1β and TNF-α in comparison to healthy controls. This leads to α-syn, Ub, and p62 aggregation, leading in turn to microglial activation, dopaminergic neuronal cell death, and motor defects in recipient cells (Han et al. 2019). Quantitative proteomic profiling using laser-capture microdissection has identified the enrichment of extracellular exosomal proteins in the SN of human brain samples (Griesser et al. 2020). A recent study also suggested the role of Rab27b, a GTPase involved in the endolysosomal pathway, in α-syn secretion through both exosomal (**Figure 2*b***) and nonexosomal pathways as well as its clearance and toxicity (Underwood et al. 2020). Microglia secreted α-syn through exosomes when they were treated with human α-syn preformed fibrils, which help in the transmission of α-syn pathology and cause aggregation in neuronal cells (Guo et al. 2020). Collectively, α-syn secreted in exosomes may play an important role in PD progression and appears to be a promising biomarker and therapeutic target. However, future studies are required to uncover the fusion mechanism by which exosomes are taken up by recipient cells, and how the RNA or protein contents of exosomes escapes from its membrane coat following engulfment of exosomes to mediate cellular effects.

LRRK2: AT THE NEXUS OF VESICULAR TRAFFICKING IN PARKINSON'S DISEASE

The Landscape of Rab GTPases Regulated by LRRK2

Early studies implicating LRRK2 with Rab GTPases came from localization of the *Drosophila* homolog of LRRK2 (Lrrk) to the membranes of late endosomes and lysosomes and found that it regulates endolysosomal transport and autophagy (Dodson et al. 2012, 2014). Further, LRRK2 was found to interact with Rab7 to inhibit the perinuclear positioning of lysosomes, whereas the G2019S disease mutant promoted perinuclear positioning through microtubules and dynein (Dodson et al. 2012). Mutant LRRK2 was further shown to delay EGFR receptor degradation by decreasing Rab7 activity, which led to the blocking of late endosomal budding (Gómez-Suaga et al. 2014) (**Figure 3**). Another study also suggested that LRRK2 directly interacts with Rab32 and Rab38 to regulate endolysosomal trafficking (Waschbüsch et al. 2014). The interaction of LRRK2 with Rab32 and Rab38 (a related Rab32 subfamily of GTPases) occurred via the LRRK2 N-terminal armadillo domain (**Figure 1*d***) with GTP-bound Rab proteins (McGrath et al. 2019). Interestingly, Rab2a was also identified as an LRRK2-interacting partner that regulated the trafficking of lysozyme-containing dense core vesicles to promote the symbiosis of commensal bacteria in Paneth cells (Zhang et al. 2015). In human pluripotent stem cell–derived macrophages, LRRK2 was also found to be recruited onto LAMP1- and Rab9-positive maturing phagosomes, where it in turn recruits Rab8 and Rab10 and might regulate phagocytic trafficking (Lee et al. 2020). These latter studies suggested the role of LRRK2 in infection and inflammatory bowel disease that could also be linked to PD pathogenesis (Ahmadi Rastegar & Dzamko 2020, Herbst & Gutierrez 2019, Houser & Tansey 2017, Wallings & Tansey 2019). Another study in mice (Kuwahara et al. 2016) suggested that LRRK2 and Rab29 work in a coordinated fashion to regulate axonal morphology and lysosome integrity, as knockout of either gene resulted in a similar phenotype. Furthermore, altered LRRK2 kinase activity is associated with defects in neurite outgrowth, and PD pathogenic mutants of LRRK2, including R1441C, R1441G, Y1699C, and I2020T, have been shown by immunoelectron microscopy and electron tomography studies (Kett et al. 2012) to exhibit enhanced binding to microtubules appearing as filamentous structures in primary neuron cultures. Cryo-electron tomography analysis has recently demonstrated that pathogenic LRRK2

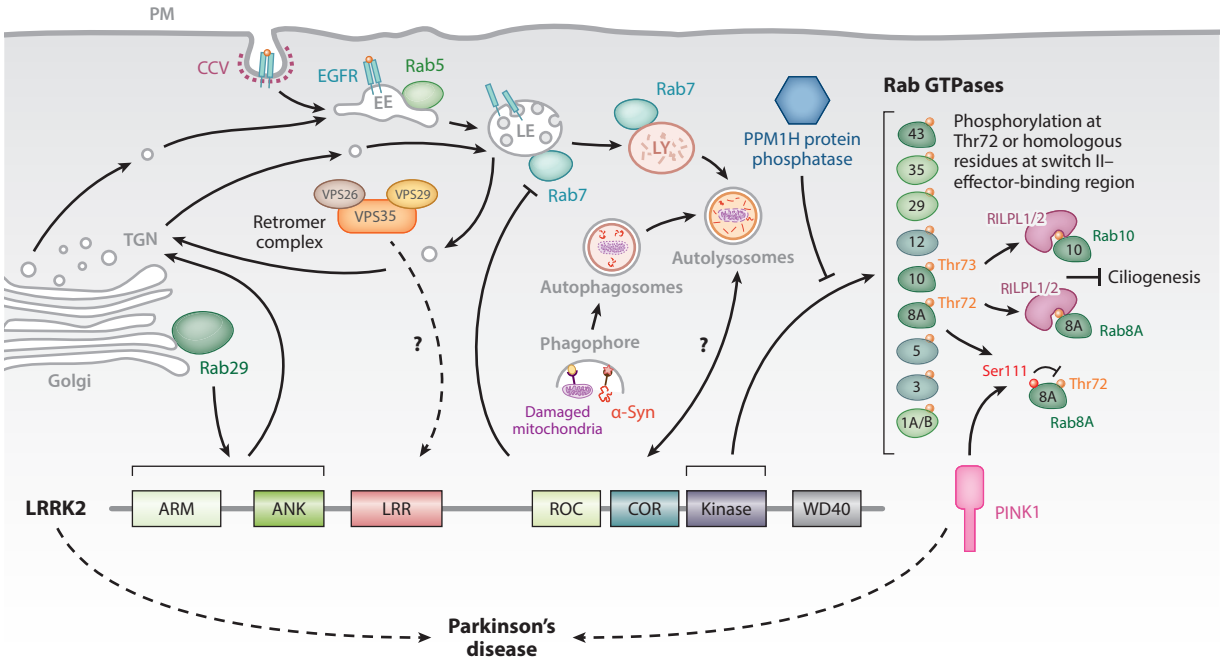


Figure 3

The regulation and downstream function of LRRK2. Pathogenic mutations of LRRK2 have been identified in PD that lead to various trafficking perturbations. Rab29 can activate LRRK2, which leads to its recruitment to the TGN through the ARM and ANK domains. VPS35, a core component of the retromer complex, is mutated in PD (*D620N*) and is associated with the hyperactivation of LRRK2 activity by an unknown mechanism. LRRK2 has been reported to disrupt endocytic pathways (e.g., EGFR trafficking) via the inhibition of Rab7 activity. LRRK2 phosphorylates Thr72 or homologous residues in the switch II–effector-binding region of a subset of Rab GTPases. PPM1H phosphatase reverses the LLRK2-mediated phosphorylation of Rab GTPases. The phosphorylation of Rab10 and Rab8A regulates ciliogenesis through binding to RILPL1 and RILPL2. Rab8A is phosphorylated in response to PINK1 activation at the Ser111 residue, which can modulate phosphorylation by LRRK2. Abbreviations: α-syn, α-synuclein; ANK, ankyrin; ARM, armadillo; CCV, clathrin-coated vesicle; COR, C-terminal of ROC; EE, early endosome; EGFR, epidermal growth factor receptor; LE, late endosome; LRRK2, leucine-rich repeat kinase 2; LY, lysosome; PD, Parkinson's disease; PINK1, PTEN-induced kinase 1; Rab, Ras analog in brain; RILPL, Rab-interacting lysosomal protein-like; ROC, Ras of complex; TGN, trans-Golgi network; VPS, vacuolar protein–sorting.

oligomerizes around microtubules (Watanabe et al. 2019), and a high-resolution cryo-electron microscopy structure has been solved that shows the C-terminal half of the LRRK2 kinase domain bound to tubulin (Deniston et al. 2020). In future work it will be interesting to understand how LRRK2 regulation of Rab GTPase influences vesicle trafficking along microtubules (Horgan & McCaffrey 2011).

However, the most compelling evidence for a link between LRRK2 and Rab biology was the discovery that a subset of Rab GTPases, including Rab1A/B, Rab3, Rab5, Rab8A, Rab10, Rab12, Rab29, Rab35, and Rab43, were substrates of LRRK2 via phosphorylation of a conserved Thr72 or homologous residue within their switch II–effector-binding regions (**Figure 3**). The phosphorylated Rab8A and Rab10 were suggested to regulate cilia formation through their antagonistic roles by interacting with RILPL1 and RILPL2 (Rab-interacting lysosomal protein-like 1/2), respectively (Dhekne et al. 2018; Steger et al. 2016, 2017) (**Figure 3**). Interestingly, Rab phosphorylation by LRRK2 was found to be counteracted by the action of the Golgi-localized PPM1H phosphatase, which dephosphorylates Rab proteins (**Figure 3**)

to regulate primary cilia formation (Berndsen et al. 2019). The LRRK2 activation and subsequent phosphorylation of various Rab GTPases have been suggested to depend on the LRRK2 association at the membrane of different intracellular compartments (Gomez et al. 2019). Rab8A phosphorylation by LRRK2 was also suggested to promote lipid storage, which might be indirectly linked to PD pathogenesis (Yu et al. 2018). In iPSC (induced pluripotent stem cell)-derived dopaminergic neurons carrying the LRRK2 G2019S mutation, the use of the integrated transcriptomic and proteomic method led to the observation of endocytic dysregulation, which might be due to changes in the expression of several Rab proteins, including Rab5B, Rab7A, and Rab10, along with other endocytic proteins (Connor-Robson et al. 2019). Interestingly, Rab29 was suggested to be an activator of LRRK2 that recruits it to the trans-Golgi network (TGN) to stimulate its kinase activity (Liu et al. 2018, Purlyte et al. 2018) (**Figure 3**). A major question has been which Rabs are relevant to neurodegeneration in PD. One study (Jeong et al. 2018) using in vitro kinase assays and tandem mass spectrometry suggested that the dysregulation of Rab35 phosphorylation was associated with neurodegeneration. In a transgenic mouse model expressing mutant LRRK2, Rab35 protein expression was found to be increased in the SN and was suggested as a potential biomarker (Chiu et al. 2016). In an in vivo electroretinogram screen in selected *Drosophila* dopaminergic neurons, Rab10 was found to be the strongest interactor and Rab3 the weakest with the PD-associated LRRK2-G2019S mutant form (Petridi et al. 2020).

Autophagy and LRRK2

A role for LRRK2 in regulating autophagy has been suggested by many studies using knockout or expressing wild-type or PD-associated mutants in many model systems. That said, whether LRRK2 positively or negatively regulates autophagy remains unclear (**Figure 3**). In differentiated SH-SY5Y cells, the expression of the LRRK2 PD-associated mutant G2019S led to an increase in autophagic vacuoles (AVs) marked by LC3 and neurite shortening (Plowey et al. 2008). This phenotype was similar to that seen in the deletion of *Atg7* (an essential autophagy gene) in Purkinje cells of conditional knockout mutant mice and also leads to the presynaptic accumulation of LRRK2 (Friedman et al. 2012, Komatsu et al. 2007). Moreover, the localization of LRRK2 R1441C to ILVs of MVBs as well as in caveolae neck and filopodia in the endosomal-autophagic pathway has been reported. Here also LRRK2 was reported to cause autophagic impairment leading to an increase in AVs with partly digested cargoes, the accumulation of MVBs, and increased levels of the p62 autophagy receptor (Alegre-Abarrategui et al. 2009). In *Saccharomyces cerevisiae*, the GTPase domain of LRRK2 (**Figure 3**) has been shown to be important for modulating endocytic and autophagic activity (Xiong et al. 2010). In mice studies (Tong et al. 2010, 2012), knockout of LRRK2 showed age-dependent biphasic alterations in autophagy pathways in kidneys leading to altered LC3-II and p62 levels with an accumulation of lipofuscin granules. Additionally, transgenic mice expressing LRRK2 G2019S showed autophagic impairment in the aged brain (Ramonet et al. 2011). Interestingly, the ALP was found to be impaired in the brains of double-knockout mice of both LRRK2 and LRRK1 (a functional homolog of LRRK2, which is not mutated in PD) but not in the single knockout of either gene (Giaime et al. 2017). Using LRRK2 kinase inhibitors in SH-SY5Y cells, LRRK2 activates a protective autophagic process through the disruption of dynamin-related protein-1 (DRP-1)-dependent mitochondrial dynamics and mitochondrial-derived reactive oxygen species signaling (Saez-Atienzar et al. 2014). Overall, LRRK2 likely modulates autophagic activity, but in future work it will be important to address whether these are direct or indirect effects of LRRK2. With the knowledge that LRRK2 phosphorylates Rab GTPase, future studies investigating autophagy should be undertaken within the prism of potential Rab-dependent mechanisms.

Golgi Trafficking Mediated by LRRK2 and Rab29

The association of LRRK2 and Rab29 (also known as Rab7L1), a TGN-associated GTPase that regulates the retrograde trafficking of the mannose-6-phosphate receptor, came from both genetic and protein interaction studies, further suggesting the involvement of aberrant trafficking defects in PD (Feng et al. 2018, Wang et al. 2014). In primary rodent neurons as well as a fly model of PD, Rab29 was found to interact with LRRK2 and to rescue a pathogenic degeneration defect associated with the expression of familial PD–associated LRRK2 G2019S or R1441C mutants (MacLeod et al. 2013). Later, a large-scale GWAS also identified Rab29 as a risk locus for PD (Nalls et al. 2014). In an unbiased screen, Rab29 was found to be an interacting partner of LRRK2 in both in vitro and in vivo systems and promoted the clearance of Golgi-derived vesicles through the ALP (Beilina et al. 2014). This TGN-associated activity of LRRK2 might involve its constitutive phosphorylation by the CK1α kinase and its interaction with VPS52, a subunit of the Golgi-associated retrograde protein complex that regulates fusion at the TGN (Beilina et al. 2020, Chia et al. 2014). The finding that LRRK2 phosphorylates many members of the Rab GTPase family, including Rab29, opens a new avenue of research regarding trafficking perturbation in PD. LRRK2 was found to phosphorylate Rab29 at both the Thr71 and Ser72 residues, and the N-terminal domain of LRRK2 is important for optimal phosphorylation at Thr71 (Kalogeropulou et al. 2018, Steger et al. 2017). The Ser72 phosphorylation of Rab29 by LRRK2 has been suggested to alter the morphology and distribution of the TGN (Fujimoto et al. 2018). Furthermore, LRRK2 directly phosphorylates Rab29 in its GTP-bound, membrane-associated form, which in turn regulates LRRK2 activity and recruits it to the TGN (Liu et al. 2018, Purlyte et al. 2018). The Rab29-mediated recruitment of wild-type LRRK2 to the Golgi (**Figure 3**) leads to centrosomal cohesion deficits due to aberrant Rab8A phosphorylation (Madero-Pérez et al. 2018a, Madero-Pérez et al. 2018b). This centrosomal cohesion deficit resembles defects associated with pathogenic PD-associated LRRK2 mutants that also showed ciliogenesis defects and depends upon both Rab8A and Rab10 phosphorylation as well as on RILP1 (Lara Ordónez et al. 2019) (**Figure 3**).

The Interplay Between Retromer Activity and LRRK2 Phosphorylation

Our understanding of the dynamic interplay between retromer activity and LRRK2 activity has come from many studies. In a *Drosophila* model of PD, the overexpression of VPS35, a core component of the retromer complex (**Figure 3**), rescued the disease phenotype linked with the expression of mutant LRRK2 associated with PD (Linhart et al. 2014). Loss of function of *lrrk*, the *Drosophila* homolog of LRRK2, causes aberrant autophagy and endolysosomal trafficking that was rescued by the expression of the constitutively active form of Rab9 (a small GTPase that regulates retromer-dependent recycling from late endosomes to the Golgi) (Dodson et al. 2014). Furthermore, LRRK2 and VPS35, along with Rab5 and Rab11, work in cooperation to regulate synaptic vesicle recycling in *Drosophila* (Inoshita et al. 2017). In addition, in brain tissues, reduced LRRK2 expression in LRRK2 mutant carriers also showed a reduced retromer function with a decreased level of VPS35 (Zhao et al. 2018). Moreover, the retromer VPS35 PD-associated mutant *D620N* was found to enhance the kinase activity of LRRK2 as measured by Rab8A, Rab10, and Rab12 phosphorylation (Mir et al. 2018, Vilarino-Guell et al. 2011, Zimprich et al. 2011). The Rab32 interaction with sorting nexin 6 (SNX6) was also suggested to regulate LRRK2-dependent Golgi trafficking (Waschbüsch et al. 2019). This further strengthened the evidence of an interplay between these two genes in trafficking perturbations associated with PD. Overall these studies have firmly placed VPS35 upstream of LRRK2, and the mechanism of how the *D620N* mutant of VPS35 activates LRRK2 remains to be solved in future work.

MITOCHONDRIAL QUALITY CONTROL AND VESICULAR TRAFFICKING

PINK1/Parkin Regulation of Mitophagy and Link to Rabs

PINK1 and Parkin function in a common mitochondrial signaling pathway to generate the Ub-dependent elimination of damaged mitochondria by autophagy (mitophagy) (Harper et al. 2018, McWilliams & Muqit 2017) (**Figure 4**). Under healthy cellular conditions, PINK1 is imported into mitochondria, where it undergoes proteolytic cleavage and then is released back into the cytoplasm where it is degraded via the N-end rule pathway (Yamano & Youle 2013), and Parkin resides in the cytosol in an autoinhibited conformation mediated in part by its N-terminal Ubl domain (**Figure 1*c***) and partly by an autoinhibitory interface between the RING2 and RING0 domains (Chaugule et al. 2011, Trempe et al. 2013, Wauer & Komander 2013). However, in response to mitochondrial damage (specifically mitochondrial depolarization that can be induced by mitochondrial uncouplers), PINK1 accumulates at the outer mitochondrial membrane (OMM), where it becomes activated and autophosphorylated (Kondapalli et al. 2012, Narendra et al. 2010) (**Figure 4**).

The major role of active PINK1 is to phosphorylate and activate the Parkin E3 ligase; it achieves this via a three-step activation process whereby it phosphorylates Ser65 on Ub resident on protein substrates on the OMM to yield Ser65-phosphorylated Ub (phospho-Ub). Phospho-Ub binds with high affinity to Parkin via a phosphate-binding pocket that leads to release of the Ubl domain, thereby enabling PINK1 to phosphorylate Parkin at a conserved Ser65 site within the Ubl domain (equivalent to the Ub residue) (Kane et al. 2014, Kazlauskaite et al. 2014, Kondapalli et al. 2012, Koyano et al. 2014, Ordureau et al. 2014) (**Figure 4**). The formation of the cocomplex of Ser65-phosphorylated Parkin bound to phospho-Ub represents the active Parkin conformation and leads to the ubiquitylation of multiple OMM substrates including mitofusins 1 and 2, voltage-dependent anion-selective channel 1–3 (VDAC1–3), and CDGSH iron–sulfur domain-containing protein 1 (CISD1) (Ordureau et al. 2018, 2020; Sarraf et al. 2013). The assembly of new Ub chains at the OMM of mitochondria leads to further PINK-dependent phosphorylation, Parkin recruitment, and activation, thereby creating a powerful feed-forward amplification step that drives mitophagy (Ordureau et al. 2014) (**Figure 4**). Furthermore, pSer65 poly-Ub chains are resistant to degradation by deubiquitinases, which promotes mitophagic signaling (Wauer et al. 2015). The mechanism of Parkin activation by phosphorylation has been comprehensively studied by structural analysis, revealing the sequential release of autoinhibitory interfaces culminating in the binding of the phospho-Ser65 Ubl domain to the RING0 domain to release its autoinhibition of the catalytic Cys431 within the RING2 domain. This release enables the transthiolation of the Cys431 by Ub from the charged E2 to allow substrate ubiquitylation (Gladkova et al. 2018, Sauvé et al. 2018).

While early cell biology studies used human cell lines and Parkin overexpression, this model of Parkin activation by PINK1 has been validated in both primary mouse neurons and human-induced neurons (McWilliams et al. 2018; Ordureau et al. 2018, 2020). The model has also been demonstrated by showing that mutation of Parkin Ser65 to Ala or Asn inactivates Parkin activity in a mouse knock-in model in vivo and leads to early-onset PD in humans, respectively (McWilliams et al. 2018). The contribution of endogenous PINK1 to stress-evoked mitophagy has recently been demonstrated in human-induced neurons exposed to mitochondrial depolarization using the mitophagy reporter mito-Keima (Ordureau et al. 2020). This conclusion also follows from previous studies using PINK1 and Parkin knockout models that showed that, in response to mitochondrial stress in vivo (induced genetically in a mouse model of mitochondrial stress or acquired through exhaustive exercise regimes), PINK1 and Parkin are critical for

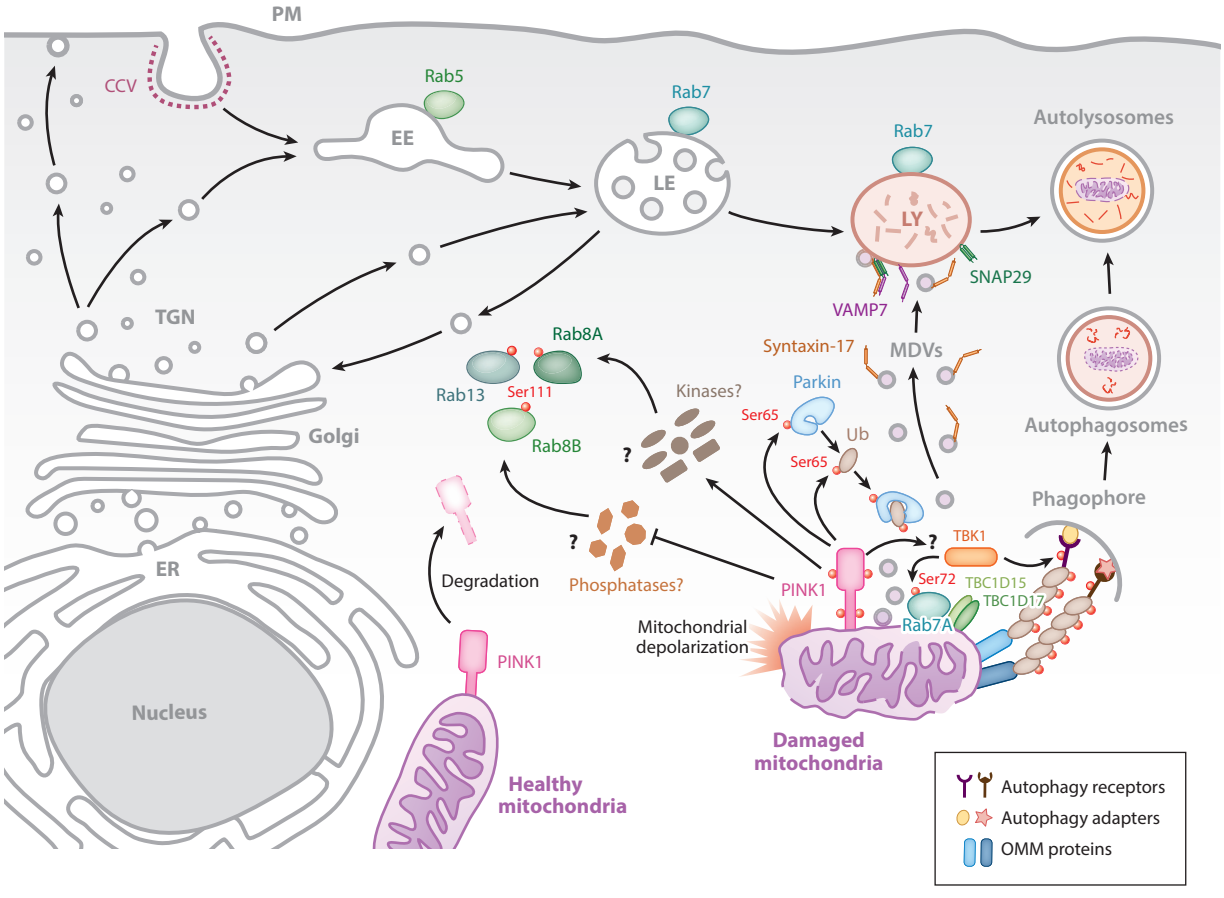


Figure 4

The PINK1-Parkin signaling pathway. Under healthy conditions, PINK1 is degraded in the cytosol after its cleavage following precursor import into mitochondria by the intramembrane protease PARL, which leads to its release into the cytoplasm. Upon mitochondrial depolarization, PINK1 accumulates on the outer mitochondrial membrane through an unknown mechanism and is activated and autophosphorylated (*small red spheres*). PINK1 phosphorylates both Parkin and Ub at the Ser65 residue. Ser65-phosphorylated Ub binds to Parkin, leading to the activation of its E3 Ub ligase activity. Parkin ubiquitylates multiple OMM proteins. Damaged mitochondria also lead to the recruitment of several proteins, including Rab7, Rab7-GAPs, TBC1D15, and TBC1D17, as well as TBK1 kinase, which phosphorylates Rab7 at Ser72. In addition, this damage recruits autophagy receptors that bind ubiquitylated proteins and they in turn recruit autophagy machinery to degrade mitochondria through the selective autophagy known as mitophagy. In response to oxidative stress, mitochondria can also release MDVs that fuse with lysosomes through the recruitment of syntaxin-17 that forms a SNARE complex with SNAP29 and VAMP7. PINK1 activation also leads to the phosphorylation of several Rab GTPases such as Rab8A, Rab8B, and Rab13 at the Ser111 residue. The kinases or phosphatases involved in these phosphorylation events remain unknown. Abbreviations: CCV, clathrin-coated vesicle; EE, early endosome; ER, endoplasmic reticulum; GAP, GTPase-activating protein; LE, late endosome; LY, lysosome; MDV, mitochondria-derived vesicle; NSF, N-ethylmaleimide-sensitive factor; OMM, outer mitochondrial membrane; PARL, presenilins-associated rhomboidlike; PINK1, PTEN-induced kinase 1; PM, plasma membrane; Rab, Ras analog in brain; SNAP, soluble NSF-attachment protein; SNARE, soluble N-ethylmaleimide-sensitive factor attachment protein receptors; TBK1, TANK-binding kinase 1; TGN, trans-Golgi network; Ub, ubiquitin; VAMP, vesicle-associated membrane protein.

maintaining dopaminergic neuronal survival and preventing the development of Parkinsonian features (Pickrell et al. 2015, Sliter et al. 2018).

Vesicular trafficking machinery is required for multiple steps in autophagy including the delivery of membranes to the site of phagophore formation and autophagosome-lysosome fusion (Søreng et al. 2018). The initial evidence for a specific role of Rabs in mitophagy was the report that the Rab7 GTPase-activating protein (Rab7-GAP) TBC1D15 (**Figure 4**) regulates mito-autophagosome biogenesis, and that TBC1D15 knockout cells exhibit excessive LC3-positive autophagosome structures around damaged mitochondria (Yamano et al. 2014). A similar effect was seen with TBC1D17 (**Figure 4**) and was mitigated by knockdown of Rab7, suggesting that overactivation of Rab7 activity underlies the phenotype and highlighting the critical role of Rab7 in the regulation of mito-autophagosome formation (Yamano et al. 2014). Because Rab7 is found primarily on late endosomes and lysosomal membranes, the mechanism was initially not clear. However, Yamano and colleagues (2018) further demonstrated that Rab7 is recruited to damaged mitochondria together with the upstream regulator Rab5. The activation of Rab5 at mitochondria led to the recruitment of its effectors MON1/CCZ1, which in turn recruited GDP-bound Rab7 dependent on Parkin activation (Yamano et al. 2018). An alternative model for the role of Rab7 in mitophagy has been proposed through which Rab7 activity and localization to the mitochondria is regulated by the interaction between the retromer complex and TBC1D5 (retromer-associated Rab7-specific GAP) (Jimenez-Orgaz et al. 2018). Further, mitophagy was suppressed in cells in which VPS29, VPS35, and TBC1D5 were knocked out, and Rab7 localization was shifted to late endosomal-lysosomal membranes where it was hyperactive (Jimenez-Orgaz et al. 2018).

Several Ub-binding autophagy receptors have been identified, including Optineurin (OPTN1), Tax-1 binding protein (TAX1BP1), p62/SQSTM1, and coiled-coil domain-containing protein 2 (CALCOCO2/NDP52), that are recruited to Ub chains assembled at the OMM and are required for mitophagy (Heo et al. 2015, Lazarou et al. 2015, Richter et al. 2016). Akin to other forms of selective autophagy such as xenophagy, PINK1/Parkin–induced recruitment of OPTN1 is associated with the activation of TANK-binding kinase 1 (TBK1), which phosphorylates OPTN1 to increase its affinity for Ub chain binding (Heo et al. 2015, Richter et al. 2016). This promotes further OPTN1 recruitment to damaged mitochondria, in turn increasing further TBK1 activation (**Figure 4**) and creating a feed-forward loop that is critical for mitophagy (Heo et al. 2015, Richter et al. 2016). TBK1 has also been demonstrated to phosphorylate Rab7 at Ser72 (**Figure 4**) that lies within the switch II domain and is the equivalent site on other Rabs targeted by the LRRK2 kinase (Heo et al. 2018). The phosphorylation of Rab7 at Ser72 is critical for mitophagy because cells expressing the nonphosphorylatable Rab7 [Ser72Ala] mutant could no longer undergo mitophagy and were associated with a defect in the recruitment of autophagosome components to sites of damaged mitochondria (Heo et al. 2018). The contribution of Rab Ser72 phosphorylation to mitophagy has been established in HeLa cells overexpressing Parkin, and probing the contribution of Rab 7 phosphorylation in cells more relevant to PD pathogenesis will be interesting. Because TBK1 is also implicated in regulating the cyclic GMP-AMP synthase (cGAS)-stimulator of interferon genes (STING) pathway in innate immune signaling, it will also be intriguing to explore the role of Rab7 in the STING-mediated neuroinflammation that has been found in PINK1/Parkin mouse models in which STING inflammation is increased by the accumulation of mtDNA mutations in the cytosol following exhaustive exercise (Sliter et al. 2018).

PINK1/Parkin and Mitochondrial-Derived Vesicles

In *Drosophila*, while PINK1/Parkin–dependent mitophagy is evident, the half-lives of several mitochondrial proteins dependent on PINK1/Parkin were also found to be independent of ATG7,

suggesting that there are additional autophagy-independent mechanisms of mitochondrial protein turnover (Vincow et al. 2013). This has been further extended in cultured mammalian cell lines in which mitochondria-derived vesicles (MDVs) have been detected that traffic specific mitochondrial protein cargoes to lysosomes for degradation in a strict PINK1/Parkin–dependent manner (McLelland et al. 2014, Soubannier et al. 2012). MDVs occur under basal conditions but are upregulated in response to oxidative stress, which promotes the generation of MDVs containing oxidized cargoes (McLelland et al. 2014). The composition and identity of MDVs are dependent on the source of oxidative stress, with intramitochondrial stressors such as antimycin A (complex III inhibitor) inducing double-membrane MDVs positive for inner membrane and matrix components (e.g., pyruvate dehydrogenase [PDH]) but negative for OMM proteins, whereas more general oxidative stress outside the mitochondria led to the formation of single-membrane MDVs that were positive for OMM components (e.g., Tom20 or VDAC1) but negative for PDH1 (McLelland et al. 2014, 2016). Interestingly, endogenous PINK1 and Parkin have not been detected in MDVs, although this may be due to limitations on detection with current antibody tools. While disease-associated Parkin mutants (e.g., C431F) affect the formation of MDVs under oxidative stress, the Ub-dependent mechanisms of MDV formation remain to be fully elucidated—whether there are differential substrates, Ub linkages, and Ub receptors to those of mitophagy are unknown (McLelland et al. 2014). Furthermore, it remains to be established whether phospho-Ub is required for MDV generation. However, the regulation of PINK1/Parkin–dependent MDVs appears to be distinct from mitophagy, as the former can occur independently of Drp1, which promotes mitochondrial fission (Soubannier et al. 2012). Furthermore, the targeting of MDVs to the late endosome/lysosome compartment is dependent on the SNARE syntaxin-17 that forms a ternary SNARE complex with SNAP29 and VAMP7 to mediate MDV-endolysosome fusion (**Figure 4**), and this SNARE machinery is not required for mitophagy (McLelland et al. 2016). In striking contrast to cultured cell lines, PINK1 and Parkin have been demonstrated to inhibit MDV formation in immune cells, which is required for mitochondrial antigen presentation (MitAP) critical for the expression of MHC I peptides at the cell surface for T cell recognition and activation (Matheoud et al. 2016). The generation of MDVs and MitAP is dependent on the SNX9 required for clathrin-mediated endocytosis and the Rab9 required for late endosome fusion, and SNX9 levels have been observed to be regulated by Parkin (Matheoud et al. 2016). The relevance of this pathway to PD has been strikingly demonstrated in PINK1-knockout mice exposed to intestinal infection by gram-negative bacteria (Matheoud et al. 2019). This led to a MitAP response and the induction of cytotoxic mitochondrial $CD8^+$ T cells in the periphery, which in turn triggered dopaminergic pathological deficits in the brain associated with L-Dopa-responsive motor deficits (Matheoud et al. 2019). This has opened a new area of biological research in which PINK1 and Parkin may have critical roles in adaptive immunity to repress mitochondrial antigens that may provoke the CNS neuroinflammation contributing to PD pathogenesis.

PINK1 Regulation of the Rab SF3 Motif

A subset of Rab GTPases, including Rab8A, Rab8B, Rab13, and Rab1A/1B, have been reported to be phosphorylated at a highly conserved Ser111 residue upon PINK1 activation (Lai et al. 2015) (**Figure 4**). The regulation of Rabs by PINK1 appears to be indirect, and the identity of the upstream Rab kinase remains unknown. The Ser111 residue lies within a C-terminal α3/β5 loop of Rab8A known as the SF3 motif and lies adjacent to the effector-binding switch II domain. Structural heterogeneity within the SF3 motif across diverse Rabs suggests that this region also determines effector binding. Previous studies (Ostermeier & Brunger 1999) have reported a role for the SF3 motif in the selective recruitment of effectors via direct effector-specific contact sites,

e.g., the recruitment of Rabphilin by Rab3A via SF3 motif–binding sites. Clues to how phosphorylation of the SF3 motif alters such effector interactions have recently been obtained by exploiting gene codon expansion technologies to purify preparative amounts of Ser111-phosphorylated Rab8A (Vieweg et al. 2020). Phosphorylation of Rab8A led to impaired interactions with its GEFs, Rabin8, and GAPs. Analysis of the phospho-Ser111 Rab8A crystal structure revealed an intramolecular H bond created by the presence of the phosphate at Ser111 with an Arg79 side chain (Vieweg et al. 2020). Molecular dynamics (MD) simulation studies of the Rab8A:Rabin8 complex structure demonstrate a salt-bridge interaction between residue Asp187 of Rabin8 with Arg79; this structure would be predicted to be weakened or disrupted by the addition of a negative charge by pSer111 that lies opposite the negative surface patch of Rabin8 containing Asp187 (Pourjafar-Dehkordi et al. 2019). The specific effectors for phospho-Ser111 Rab8A remain unknown, a gap that has hampered progress in understanding the downstream biology of phospho-Ser111 Rab8A; however, phospho-Ser111 Rab8A has been shown to impair the ability of the LRRK2 kinase to phosphorylate its Thr72 residue within the switch II domain (Vieweg et al. 2020) (**Figure 3**). Because hyperactive *R1441G* LRRK2-phosphorylated Rab Thr72 recruits RILPL1 and RILPL2 leading to inhibition of primary cilia in the brains of mice (Dhekne et al. 2018), understanding the contribution of PINK1-regulated Rab phosphorylation to this process in vivo would be of interest. The contribution of the Ser111 phosphorylation of Rabs to mitophagy and MDV generation is also unknown, although none of these Rab components were found to be essential in a recent CRISPR genome-wide screen for mitophagy regulators (Heo et al. 2019).

CONCLUDING REMARKS AND CLINICAL APPLICATION

The identification of monogenic forms of PD has led to the elaboration of vesicular trafficking pathways whose disruption are major causal mechanisms in PD. This knowledge has been crucial for the development of therapeutic strategies against PD. Currently, clinical trials are advancing for the evaluation of human monoclonal antibodies that preferentially bind oligomeric α-syn and prevent cell-to-cell transmission and progression, including the PASDENA Phase II trial being undertaken by Roche. Trials for LRRK2 inhibitors are also underway in several companies including Denali Therapeutics, whose LRRK2 inhibitor DNL201 was found to be safe for humans in Phase I studies. In addition, Biogen is currently evaluating an antisense oligonucleotide against LRRK2 in Phase I studies. A major question is whether these strategies will be beneficial for patients beyond those monogenic individuals harboring causal mutations, and this will be dependent on uncovering common overlapping pathways between genes. An exemplar of this has been the discovery that VPS35 [D620N] mutations lead to increased LRRK2-mediated Rab phosphorylation, thereby placing VPS35 upstream of LRRK2 and suggesting that *VPS35* patients may also benefit from LRRK2 inhibitors and should be assessed in ongoing clinical trials (Mir et al. 2018). Our increased understanding of Rab biology and vesicular trafficking mechanisms between distinct PD gene pathways now provides a tractable framework to uncover further knowledge that will expand our ability to stratify PD patients for future disease-modifying therapies.

DISCLOSURE STATEMENT

M.M.K.M. is a member of the Scientific Advisory Board of Mitokinin Inc. and serves as a member of the Wellcome Trust Clinical Interview Committee. M.M.K.M. is funded by a Wellcome Trust Senior Research Fellowship in Clinical Science (210753/Z/18/Z), the Michael J. Fox Foundation for Parkinson's Research, the Medical Research Council, the Rosetrees Trust, Astex Pharmaceuticals Ltd., and an EMBO (European Molecular Biology Organization) Young Investigator Program Award.

ACKNOWLEDGMENTS

We apologize to our colleagues whose work we were unable to discuss or cite due to space limitations.

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