The emerging role of Rab GTPases in the pathogenesis of Parkinson's disease.

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Abstract

The identification of pathogenic mutations in *RAB39B* and *RAB32* that cause Parkinson's disease (PD) has highlighted the emerging role of protein trafficking in disease pathogenesis. Rab GTPases function as master regulators of membrane trafficking, including vesicle formation, movement along cytoskeletal networks and membrane fusion. Recent studies have linked Rab GTPases with alpha-synuclein, Leucine-rich repeat kinase 2 and Vacuolar protein sorting 35, three key proteins in PD pathogenesis. In this review, we will discuss the various RAB GTPases associated with PD, current progress in the research and potential future directions. Investigations into the function of RAB GTPases will likely provide significant insight into the etiology of PD and identify novel therapeutic targets for a currently incurable disease.

Parkinson's disease (PD) is a common neurodegenerative disorder affecting up to 5% of elderly individuals.[1, 2] Further, it is the most common cause of Parkinsonism, a spectrum of clinical disorders that present with a similar range of motor deficits, including bradykinesia, resting tremor, cogwheel rigidity and postural instability.[3] In PD, these symptoms are mediated by the pathological hallmark features, including a loss of dopaminergic neurons in the substantia nigra *pars compacta* (SNpc-a region in the midbrain responsible for the initiation and fine-tuning of voluntary movement) and the presence of intracellular protein inclusions (Lewy bodies) composed primarily of alpha-synuclein (αSN) in surviving dopaminergic neurons.[4] Current therapeutics for PD provide symptomatic relief but do not halt disease initiation or progression and lose efficacy over time, with undesirable side effects such as dyskinesia.[5] Therefore, a greater understanding of the etiology and pathological mechanisms underlying PD is required for the development of more targeted and effective therapies.

A large proportion of affected individuals have idiopathic PD, however a causal mutation can be identified in ~5-10% of patients. Due to the clinical similarities between genetic and idiopathic PD, it is hypothesized that they share common pathological pathways. Therefore, understanding the role of PD-associated genes in disease initiation and progression may elucidate common underlying pathological mechanisms. Currently, mutations in over 20 genes and loci have been shown to be associated with or causative of PD.[6, 7] Functional studies of the encoded proteins has revealed common themes in the mechanisms of PD pathology, including dysfunctions in protein degradation, mitochondria, and apoptotic pathways. More recently, a role has emerged for protein trafficking in the pathogenesis of PD. In particular, the dysregulation of members of a family of proteins essential for all aspects of cellular trafficking,

called Ras Analog in Brain (RAB) GTPases, have been shown to be directly causative or associated with PD. The underlying mechanisms involve RAB regulation of αSN and interactions with a range of other PD associated genes. In this review, we discuss the evidence linking the RAB protein family to PD and consider how functional studies of RAB GTPases can provide insights into disease pathogenesis and potential therapeutics.

RAB GTPase Proteins

RAB proteins are small GTPases that belong to a superfamily of over 60 distinct proteins in humans. They act as molecular switches during vesicular transport and membrane trafficking, by cycling between a GDP-bound inactive and GTP-bound active form. This switching activity is tightly controlled by protein regulators and involve a conformational change in two regions of the protein that interact with effectors, switch I and II.[8] Specifically, the GTP-bound active GTPase can interact with protein effectors or target membranes to alter downstream signaling cascades. The main regulators of GTPases include the GTPase activating protein (GAP), which inactivates GTPases by catalyzing GTP to GDP hydrolysis. The inactive GTPase, predominantly localized to the cytoplasm, is stabilized by a GDP dissociation inhibitor (GDI). GDI binds inactive GTPases with high affinity and prevents its reactivation by Guanine exchange factor (GEF). GEF activates the GTPase by promoting GDP to GTP exchange (Figure 1).[9] A single RAB GTPase can display a multitude of functions through the use of different effector proteins and vice versa. For example, more than 20 proteins have been identified to directly or indirectly interact with GTP-RAB5, a core component of the endosomal docking apparatus.[10] GTPases can also bind to protein cargos enclosed within transport vesicles. For example, RAB27A is a key component of

the vesicular trafficking machinery in melanocytes and is known to transport melanosomes, vesicles in which melanin is synthesized and stored.[11]

Commonly, RAB GTPases are ubiquitously expressed in all cell types and are responsible for the regulation of fundamental aspects of cellular trafficking that are highly conserved in all eukaryotes.[12] They include participation in various steps in trans/cis-Golgi transport, endosomal transport, lysosomal transport, vesicle transport, endocytosis and autophagy. These activities are vital for the regulation of the spatiotemporal localization and thus function of intracellular proteins, the degradation or recycling of various protein components and the establishment of membrane identity. As these functions have previously been comprehensively reviewed they will not be described further.[13-16]

Conversely, RAB GTPases that are enriched in certain tissues, or which regulate tissue-specific effectors, can confer functional specificity. For example, RAB GTPases that are expressed predominantly in the brain are involved in the regulation of neural development, neurite growth, axonal transport and synaptic vesicles.[17] Dysregulation of these specialized RAB proteins can contribute to the pathogenesis of disease.[18] For example, mutations in *RAB27A* cause Griscelli syndrome and mutations in *RAB7* cause Charcot-Marie-Tooth type 2B.[19, 20] Additionally, mutations in RAB regulatory (GAP/GEF) or effector proteins, which dysregulate RAB GTPase localization and/or function, can also result in disease. For example, mutations in *GDI1*, encoding a GDI common to all RAB GTPases (αGDI), have been shown to cause X-linked non-specific mental retardation.[21] Both loss of and reduced function of αGDI leads to altered synaptic vesicle biogenesis and recycling in the hippocampus.[22] Thus, both causative mutations

and more subtle 'secondary' mechanisms are evident in the contributions of RAB proteins to disease pathogenesis.

Mutations in *RAB39B* and *RAB32* cause PD

RAB39B was first identified in 2002 [23] and loss of function mutations were initially linked to X-linked mental retardation (XLID).[24] Subsequently, multiplication of *RAB39B* was associated with XLID, indicating altered *RAB39B* dosage contributes to neurological disease.[25, 26] More recently, *RAB39B* was the first RAB GTPase to be causally linked to the development of PD, with loss of function mutations shown to cause an X-linked recessive form of early onset PD.[27] Two families with loss of function mutations in *RAB39B* were identified. The affected individuals presented with typical PD associated clinical features, including bradykinesia, resting tremors and rigidity. Additional features, including XLID and macrocephaly, matched those previously described in the XLID study.[24] In the post mortem brain, there was neuron loss and the presence of αSN positive Lewy bodies and Lewy neurites.[27] Subsequently, additional studies have confirmed the role of *RAB39B* in PD [28-33] although negative results in several large mutation screens suggest germline mutations in *RAB39B* are a rare in PD.[31, 34-37]

The function of *RAB39B* is currently being investigated. *RAB39B* has been shown to be highly expressed in neurons compared to other cell types [24] but its intracellular localization remains uncertain. Different studies have shown RAB39B to localize to the Golgi, early endosome, recycling endosome, and plasma membrane in various *in vitro* models.[24, 27, 28] These results suggest a function in the trans-Golgi network (TGN), which directs the distribution of newly synthesized proteins from the Golgi to various subcellular compartments, such as endosomes,

or the plasma membrane. Functional studies of RAB39B have indicated a role in the regulation of αSN homeostasis. Downregulation of *Rab39b* in *in vitro* models (P19 cells and primary murine hippocampal neurons) resulted in reduced steady state levels of αSN and reduced density of αSN immunoreactive puncta in dendritic processes.[27] In a second study, knockdown of *RAB39B* in human neuroglial (H4) cells did not affect steady state levels, but did result in increased oligomerization of an αSN reporter.[38] How these *in vitro* observations relate to the observed αSN pathology in RAB39B-mediated PD is an active area of current research, but has been suggested to reflect differences between simple cell models and the complex and prolonged cellular alterations associated with end-stage disease.[27] Indeed, it is currently unknown if there is a direct or indirect interaction between RAB39B and αSN and this warrants further investigation. Further, RAB39B may modulate synaptic plasticity and the regulation of neuronal outgrowth, as both downregulation and overexpression of *Rab39b* in primary neurons results in reduced neuronal branching and synapse formation.[24, 25, 39] Most recently, RAB39B has been linked to autophagy. C9ORF72 was shown to complex with the WD Repeat Domain 41 (WDR41) and Smith-Magenis Syndrome Chromosome Region Candidate 8 (SMCR8) proteins to act as a GEF protein for RAB39B and RAB8A.[40] Repeat expansion affecting *C9ORF72* results in the linked neurodegenerative disease familial amyotrophic lateral sclerosis and fronto-temporal dementia.[41] C9ORF72 is also a known RAB1A effector, and regulates RAB1A dependent trafficking of the ULK1 complex, which is responsible for the initiation of autophagy.[42] It was proposed that C9ORF72 participates in a novel RAB signaling cascade, in which RAB1A recruits the C9ORF72/SMCR8/WDR41 complex to activate RAB39B and RAB8A. Subsequently, these

activated RAB GTPases regulate the maturation of autophagosomes by directly or indirectly interacting with the downstream autophagy pathway.

Mutations in a second RAB gene (*RAB32*) have now been shown to cause late onset familial PD. RAB32 was previously linked to cellular processes including autophagy, mitochondrial dynamics, phagocytosis and inflammation in the brain.[43-46] Our very recent study showed a p.Ser71Arg mutation in *RAB32* segregated with late onset autosomal dominant PD in three unrelated families.[47] This serine/threonine residue is found in the switch II domain of most rabs, and is differentially phosphorylated by LRRK2.[48] Of note a direct interaction between RAB32 and LRRK2 was previously demonstrated and shown to be important for late endosomal transport and sorting.[49] These studies implicate *RAB32*, in association with *LRRK2*, in the pathogenesis of PD and are likely to be an area of intense study in the near term.

Functional linkages between RAB GTPases and αSN

The gene encoding αSN (*SNCA*) was the first gene causally linked to PD in 1997.[50] Subsequently, additional point mutations as well as gene dosage increases were identified, demonstrating that both qualitative and quantitative changes in *SCNA* expression can cause PD.[51-55] Recent genome-wide association studies (GWAS) have indicated variants in the *SNCA* promotor and 3' UTR regions, associated with elevated expression, also correlate with a strong risk of developing sporadic PD.[56-58] Despite its fundamental role in the pathogenesis of PD, the function of αSN in the brain or the periphery remains largely unknown. The protein is abundant in the mammalian brain, comprising up to 1% of total cytoplasmic protein lysate [59] and is enriched in presynaptic

nerve terminals, where it is thought to play a role in the regulation of synaptic vesicle transport.[60, 61]

One of the first studies linking αSN and RAB GTPases utilized a transgenic mouse overexpressing PD associated mutant *p.Ala30Pro αSN*, (NM_000345.3: c.88G>C, p.Ala30Pro), and showed abnormal interaction of p.Ala30Pro αSN with RAB3A, RAB5A and RAB8A by co-immunoprecipitation analysis of brain homogenates.[62] Further, abnormal binding of wildtype (WT) αSN to RAB3A was found in brain homogenates from individuals with idiopathic PD, diffuse Lewy body disease or multiple system atrophy.[63, 64] These interactions suggest that unconventional binding of WT or mutant αSN to RAB proteins could disrupt the intricate control of RAB function and alter the homeostasis of αSN. Interestingly, RAB3A is involved in synaptic vesicle transport and thus the two proteins may possess complementary functions.[17] Indeed, a study demonstrated that active GTP-bound RAB3A directly interacted with and stabilized WT αSN to the membranes of presynaptic vesicles. Concurrently, when RAB3A is removed from these membranes following GTP hydrolysis, it also acts to free αSN during synaptic activity.[65] WT αSN has also been shown to interact with RAB8A and this is enhanced by phosphorylation of αSN at the serine 129 (S129), promoting binding to the switch I and II regions of RAB8A.[66] Given that up to 90% of αSN present in Lewy bodies is phosphorylated at S129,[67] this interaction potentially implicates RAB8A in the formation of αSN aggregates and the clearance of toxic oligomeric αSN species in the cell.

Yeast models have proven to be a powerful platform for elucidating the functional relationships between αSN and RAB proteins. Overexpression of *WT* or *p.Ala53Thr αSN* (NM_000345.3: c.157G>A, p.Ala53Thr) caused an inhibition of ER to Golgi trafficking due to impaired

docking/fusion of vesicles to Golgi membranes. This phenotype could be rescued by overexpression of RAB GTPase *Ypt1p* (Yeast homolog of *Rab1*) which promotes ER to Golgi transport.[68] Further, in both *C. elegans* and rat primary midbrain cultures, overexpression of *Rab1a* was able to rescue dopaminergic neuron loss induced by overexpression of *WT* or *p.Ala53Thr αSN*.[69] Consistent with a potential neuroprotective role for RAB1A, overexpression has also been shown to rescue other aspects of αSN toxicity. For example, some cell and animal models with overexpression of *WT αSN* show impaired macroautophagy, a process that degrades and recycles cellular contents by autophagosome formation and lysosome fusion. This deficit can be rescued by *RAB1A* overexpression.[70] However, autophagy impairment was not observed when *p.Ala53Thr* and *p.Ala30Pro* αSN mutants were overexpressed, suggesting this mechanism is associated with toxicity induced by increased dosage of αSN. Furthermore, the authors demonstrated that increased dosage of αSN impairs autophagy by inhibiting RAB1A activity, culminating in the mislocalization of ATG9, a protein that regulates the formation of autophagosome precursors.[70] In another study, overexpression of human *WT αSN* in a rat model showed dopaminergic neuron loss, Golgi fragmentation in surviving dopaminergic neurons and motor impairments.[71] *RAB1A* overexpression in this model improved motor behavior and rescued Golgi fragmentation, but did not ameliorate neuronal loss. In dopaminergic neurons generated from iPSC with triplication of *αSN*, overexpression of *RAB1A* rescued phenotypes induced by increased *αSN*, including Golgi fragmentation and disruptions in lysosomal hydrolase trafficking, as well as reduced αSN levels.[72] The authors proposed that accumulation of αSN in the cell body resulted in the mislocalization of RAB1A from its normal ER-Golgi localization, thus disrupting its function. In support, a study in yeast has shown that

WT αSN overexpression induces mislocalization and accumulation of many RAB GTPase proteins to αSN positive aggregations or vesicles. These include RAB proteins of various functions, Ypt1p, Ypt6p, Ypt7p Sec4p, Ypt10p and Ypt32p, homologs to RAB1A, RAB6 and RAB7, RAB8A, RAB10 and RAB11 in mammals respectively.[73] The authors therefore proposed that αSN was dysregulating general trafficking events, rather than interacting specifically with each RAB protein, a highly probable hypothesis. For example, RAB7 and RAB11A are crucial effectors of the late and recycling endosomes respectively. In drosophila models overexpressing *p.Ala53Thr αSN*, *RAB7* overexpression has been shown to clear αSN aggregates via autophagy, much like RAB1A, and increase the size and acidity of RAB7 positive vesicles.[74] In mammalian cell models with overexpression of *WT αSN*, *RAB11A* overexpression has been shown to reduce αSN aggregation and toxicity by facilitating the secretion of αSN via exocytosis.[75, 76] *RAB11* overexpression was also shown to rescue dopaminergic neuron loss and abnormal synaptic signaling in a drosophila model overexpressing *WT αSN*, presumably by decreasing insoluble αSN in the cell.[77] Thus, there is considerable evidence that a range of endosomal trafficking events, including autophagy and exocytosis, are implicated in αSN toxicity.

In addition to exocytosis, αSN is also known to be secreted via exosomes in a process regulated by *RAB11* and *RAB27*.[78] Exosomes, or extracellular vesicles, are released into the extracellular environment by many cell types and function in intercellular communication. In a human cell line overexpressing *SNCA*, αSN was shown to be secreted via exosomes in a calcium dependent manner, and this secreted or extracellular αSN propagated its cytotoxicity when taken up by neighboring cells.[79] *RAB11* has been shown to participate in this process by recycling and secreting extracellular αSN via exocytosis.[75] Taken together, this suggests that αSN secretion via

exocytosis and exosomes may facilitate the spreading of αSN from cell to cell in PD, much like in Prion related pathologies. Beyond PD, *RAB11* has been linked to other neurodegenerative diseases characterized by misaggregation of protein, suggesting dysfunctions in protein trafficking and secretion may be a common pathogenic pathway. For example, overexpression of *RAB11* is known to correct defects in synaptic signaling and vesicle formation in a Huntington's disease (HD) drosophila model.[80] RAB11 been shown to interact with Presenilin 1 and 2 in Alzheimer's Disease (AD), suggesting a role in the processing of amyloid precursor protein (APP).[81]

More recently, Gonçalves *et al.* performed an RNAi screen of over 1300 genes involved in trafficking and phosphotransferase activity to identify proteins that modulate αSN oligomerization and aggregation.[38] The screen was performed in human neuroglial cells overexpressing *WT SNCA*, using a biomolecular fluorescence complementation assay. Four of nine genes identified in the screen encoded RAB GTPases (*RAB8B*, *RAB11A, RAB13* and *RAB39B*). In *RAB8B* and *RAB13* KD cells, αSN oligomerization was correlated with a dysregulation in the level of αSN and an increase in cell cytotoxicity. However, αSN oligomerization occurred independent of these phenotypes in *RAB11A* and *RAB39B* KD cells. Moreover, overexpression of *RAB8B*, *RAB11A* and *RAB13* was able to rescue αSN aggregation, possibly by increasing the endocytic recycling of αSN. It is currently unknown whether these RAB GTPases function independently, however they should be considered as potential targets for PD therapeutics.

Functional linkages between RAB GTPases and LRRK2

Mutations in the Leucine-rich repeat kinase 2 gene (*LRRK2*) are the most common genetic cause of late onset autosomal dominant PD. The most frequent mutation (NM_198578.3:c.6055G>A, p.Gly2019Ser) is present in 1% of patients with sporadic PD and 4% of patients with PD showing autosomal dominant inheritance.[4, 56] LRRK2 is a large multidomain protein and there is considerable evidence suggesting the activity of the LRRK2 Ras-like GTPase and kinase domains mediates toxicity.[82] Several *LRRK2* mutations, including p.Gly2019Ser, have been shown to increase LRRK2 kinase activity and are toxic when overexpressed in cellular models.[83, 84] Previously, LRRK2 was shown to be important in the regulation of neurite outgrowth and autophagy.[85, 86] LRRK2 is predominantly localized to the cytoplasm, but is present at the ER, Golgi apparatus, early endosomes, lysosomes and synaptic vesicles, suggesting dysfunctional LRRK2 could disrupt normal axonal protein trafficking.[83] A study in drosophila demonstrated that the LRRK2 homolog Lrrk functionally interacts with RAB7 in the transport of late endosomes.[87] Moreover, in transiently transfected cells, Lrrk was shown to physically interact with Rab7, and WT Lrrk showed a markedly stronger interaction with the inactive form of Rab7 compared to the both wildtype and constitutively active forms. This difference was not observed with Lrrk p.Gly2019Ser. Thus, the authors suggested that Lrrk may play a role in stabilizing the inactive form of Rab7, resulting in negative regulation of Rab7-dependent lysosomal positioning and transport.[87] PD-causing *LRRK2* mutations dysregulate this interaction, thus abrogating the ability of Lrrk to negatively regulate Rab7-induced lysosomal trafficking and clustering. This is consistent with evidence from fibroblasts derived from PD patients (n=5) expressing *LRRK2 p.Gly2019Ser*. A reduction in RAB7 activity was associated with a delay in late endosome to lysosome trafficking of the epidermal growth factor receptor.[88]

A second RAB GTPase shown to be associated with *LRRK2* is *RAB7L1/RAB29.*[89] *RAB7L1* maps to PARK16, a locus of five genes that is a genetic risk factor for idiopathic PD.[90] MacLeod *et al.* showed that overexpression of either WT or constitutively active *RAB7L1* orthologue in a drosophila model rescued dopamine neuron loss and premature mortality induced by LRRK2 p.Gly2019Ser. In addition, overexpression of WT or constitutively active *RAB7L1* in rat primary neuronal cells overexpressing LRRK2 p.Gly2019Ser rescued phenotypes such as lysosomal swelling and reduced neurite process length during outgrowth.[89] In contrast, overexpression of WT Rab7L1 in WT primary mouse cortical cultures caused neurite shortening, perhaps due to dysregulation of the stoichiometric ratio of components of the LRRK2 complex that are required for normal LRRK2 function.[91] In humans, interaction of variants at additional genetic loci that can influence PD susceptibility may explain why *Rab7L1* has been associated with both elevated and reduced risk of PD.[92] In a *C. elegans* model, the protein complex 3 (AP3), a known regulator of endosome to lysosome trafficking, was shown to be a downstream effector of LRRK2 and RAB7L1. Thus, RAB7L1 together with LRRK2 can act to regulate AP3 and its various functions, including lysosomal trafficking and the coordination of axonal morphology.[93] Overall, defects in the LRRK2-RAB7L1 pathway are proposed to alter endo-lysosomal trafficking, with detrimental impacts on protein degradation and abnormal neuron growth.

The close functional relationship between LRRK2 and RAB proteins was further demonstrated by Steger and colleagues, who identified a subset of RAB GTPases as key LRRK2 substrates. LRRK2 was shown to phosphorylate target RAB GTPases at an evolutionarily conserved residue in the switch II domain.[48] It was shown *in vitro* that RAB proteins such as RAB1B, RAB8A and RAB10, which encode a threonine at this particular residue, were more efficiently

phosphorylated than those possessing a serine residue, such as RAB5B, RAB7A, RAB7L1, RAB12 and RAB39B. Further, LRRK2 mutants that display an increase in kinase activity, such as p.Gly2019Ser, were able to increase RAB phosphorylation by up to threefold compared to WT LRRK2. Importantly, the authors found that LRRK2 phosphorylation could potentially compromise RAB protein function. Using RAB8A as an example, LRRK2 phosphorylated RAB8A showed a decreased interaction with GEF protein Rabin8, inhibiting GDP exchange fourfold and resulting in decreased RAB8A activation. Further, phosphomimetics of RAB10 and RAB12 showed an inability to bind GDI proteins, which could potentially disrupt the subcellular distribution and/or activity of the RAB protein. These results are consistent with a model for *LRRK2*-mediated PD, where LRRK2 mutants with increased kinase activity dysregulate RAB protein cycling by altering affinity for cognate regulatory proteins. The predicted outcome would be dysregulated cellular trafficking, such as RAB5 and RAB7 mediated endosomal pathways and RAB7L1-mediated lysosomal trafficking. It will be important to further investigate the mechanisms underlying these LRRK2-RAB interactions, especially for *RAB7L1, RAB32* and *RAB39B*, which have shown independent associations with PD.

Interactions between RAB GTPase and Parkin/PINK1

Biallelic loss of function mutations in *PARK2*, encoding the Parkin protein, cause early onset PD, typically prior to age 40. Loss of function mutations account for up to 15% of early onset patients showing autosomal recessive inheritance, a proportion that decreases significantly with increasing age of onset.[94] Parkin is an E3 ubiquitin ligase that functions in the ubiquitin proteasomal system (UPS) and ubiquitinates proteins to mark them for degradation.[1, 95] Loss of

function mutations in *PARK2* impair the protein's ability to interact with the E2 ligase and its protein substrates, effectively abolishing its ability to target the correct substrates for degradation. After *PARK2*, mutations in the gene encoding PTEN-induced putative kinase 1 (*PINK1*) are the second most common genetic cause of early onset PD. *PINK1* mutations account for up to 8% of early onset PD showing an autosomal recessive inheritance.[96] PINK1 and Parkin play a prominent role in the regulation of mitophagy, a specialized form of autophagy for mitochondria. Early studies suggested that *PARK2* and *PINK1* knockout animals exhibited similar mitochondrial dysfunctions, including increased oxidative stress and reduced mitochondrial respiratory capacity.[97] Later, it was shown in drosophila models that *PINK1* is functionally upstream of Parkin and recruits Parkin to damaged mitochondria, where it promotes mitophagy.[98] *PINK1* encodes a mitochondrial targeting sequence to the outer mitochondrial membrane. On healthy mitochondria, PINK1 levels are very low due to cleavage by voltage-dependent proteolysis. However, when mitochondria are damaged and the membrane potential is reduced, PINK1 begins to accumulate and subsequently recruits and activates Parkin by phosphorylating ubiquitin and the ubiquitin-like domain of Parkin.[99, 100]

Recently, another function for PINK1 was uncovered. Analogous with LRRK2, PINK1-mediated kinase activity regulates RAB GTPases by phosphorylation. A report showed that activation of PINK1 kinase activity was required for downstream phosphorylation of RAB8A, RAB8B and RAB13 at a highly conserved serine residue, Ser111.[101] Unlike LRRK2 phosphorylation of RAB proteins, this was not shown to be a direct kinase-substrate interaction, suggesting intermediate kinases or phosphatases that are regulated by PINK1 are directly targeting these RAB GTPases. Further, this appears to occur independent of Parkin phosphorylation at Ser65,

and thus is proposed to be independent of the PINK1 mitophagy pathways. The functional outcome of Ser111 phosphorylation of RAB8A was impaired interaction with GEF protein Rabin8 in cell models.[101] Given that Ser111 lies within a region of the protein involved with effector binding, interactions with additional proteins are also predicted to be disrupted. Therefore, the loss of PINK1 activity observed in autosomal recessive PD not only prevents the turnover of damaged mitochondria, but may also have a detrimental impact on the activity of RAB GTPases by disrupting the function of intermediary proteins, kinases or phosphatases that directly interact with downstream RAB proteins. The preliminary data suggest additional pathways exist for *PINK1*-mediated PD, and understanding the specific mechanism of these pathways could potentially improve current disease models.

Interactions between RAB GTPase and VPS35.

Vacuolar sorting protein 35 (*VPS35*) is a subunit of the retromer complex, which is made up of the VPS26-VPS29-VPS35 trimer and a sorting nexin (SNX) dimer. The retromer complex plays a vital role in cargo selection in the retrograde transport pathway from endosomes to the Golgi,[102] and the identification of PD-causing mutations in VPS35 strongly implicated these pathways in disease pathogenesis. Currently, the only *VPS35* variant with proven pathogenicity is the p.Asp620Asn mutation (NM_018206.5:c.1858G>A, p.Asp620Asn).[103, 104] The most well studied retromer cargo is the cation-independent mannose-6-phosphate receptor (CI-M6PR), which participates in the sorting of acidic lysosomal hydrolase precursors from the TGN to endosomes and lysosomes. The retromer functions to recycle the receptor back to the TGN and thus ensures proper regulation of lysosomal enzymes.[105] This process is disrupted by the

p.Asp620Asn VPS35 mutation, which is in the region of VPS35 that directly interacts with CI-M6PR.[106] This process can also be impaired by dysregulation of RAB7, a known interactor of the VPS26-29-35 trimer which mediates the recruitment of retromers to late endosomes.[107] Dysfunctions in retromer trafficking could lead to functional consequences in downstream pathways carried out by its cargoes. For example, the CI-M6PR ligand cathepsinD, is a protease that has been shown to degrade αSN.[108, 109] Expression of CathepsinD degrades αSN and is neuroprotective in cells and animals overexpressing *αSN*. This suggests upregulating retromer activity is a potential avenue for PD therapeutics, and indirectly reduces the level of toxic αSN in cells via activity of its protein cargoes. The function of the retromer complex in the endo-lysosomal pathway overlaps with that of LRRK2 and RAB7L1. In cell models with reduced RAB7L1 activity, or cell and animal models overexpressing PD associated mutant forms of LRRK2, there is a significant decrease in the steady state levels of VPS26, VPS29 and VPS35.[89] This implies a deficiency in retromer activity may contribute to the neuropathology underlying LRRK2-mediated PD, and requires further investigation.

In addition to endo-lysosomal trafficking, VPS35 functions in synaptic transmission and recycling, and regulation of the trafficking of neurotransmitter receptors such as the GluA1 subunit of alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptors (AMPARs) at neuron synapses.[110] This function is perturbed in neuron models differentiated from pluripotent stem cells encoding *p.Asp620Asn VPS35*. Rab39b has been reported to regulate trafficking of the AMPAR subunit GluA2 to the cell surface,[39] raising the possibility that VPS35 and RAB39B converge on the same cellular pathways. Beyond PD, the retromer complex has been implicated in neurogenetic disorders such as Alzheimer disease and Down syndrome,

which are characterized by protein aggregation. Indeed upregulation of retromer in cell and animal models has been shown to improve recycling and rescue pathological features associated with these disorders.[111]

Conclusions and Future Directions

RAB GTPase proteins are implicated in multiple pathological mechanisms underlying PD (Table 1), and it is an exciting time in the field with an acceleration of novel discoveries being reported. Many PD associated proteins have a role in trafficking pathways or interact directly with RAB GTPases (Figure 2). Dysregulation of these proteins has been shown to disrupt essential steps in intracellular trafficking, in particular affecting the late endosome, lysosome and autophagy pathways. Other genes associated with Parkinsonism including *ATP13A2*, *VPS13C*, *GBA1*, *MAPT* and *DCTN1*, not discussed in this review due to space constraints, have also been shown to be involved in similar trafficking events,[112-117] highlighting the role of protein trafficking in the development of disease. More broadly, these data demonstrate that it is highly plausible that impaired intracellular trafficking is a key contributor to idiopathic PD. [118] Although it is unclear why certain neuronal populations, such as DA neurons in the ventral tier of the SNpc, are selectively lost by the early symptomatic stage of PD, there are several compelling possibilities. It has been proposed that dopamine might induce oxidative and nitrosative damage of axon terminals through the production of reactive species.[119] In addition, the vulnerability of DA neurons may be impacted by the energy costs required to maintain transport/synaptic activity in the massive, unmyelinated arbors typical of these cells.[120, 121] However, these features cannot entirely account for the selectivity, given other neuronal

populations with similar architecture, such as DA of the ventral tegmental area, are much less affected during disease. Most recently, it has been suggested that susceptible neurons play a key role in neuromodulatory networks, and have distinctive features including reduced calcium buffering capacity and slow, tonic activity which may result in increased reactive oxygen species and dysregulated calcium homeostasis.[122] Notably, all of these cellular processes are intricately connected, and vulnerable to, disruption in protein trafficking pathways. It is possible that relatively minor perturbations or reductions in the efficiency of trafficking pathways may have cumulative effects over the lifetime of a neuron, ultimately reaching a threshold that causes neurodegeneration and cell death.

The recent discoveries that mutations in *RAB39B* or *RAB32* can cause PD provides the most direct functional linkage to RAB proteins. Moreover, given that a substantial genetic contribution to Parkinsonism remains to be identified,[123] the application of modern genomic technologies is likely to identify additional causal genetic variants in RAB GTPases and associated proteins. Studying the function of these RAB proteins will expand our growing knowledge on the effect of trafficking defects in PD, ultimately leading to a better understanding of disease mechanisms and better therapeutics. Indeed, as discussed in this review, manipulating the expression of several RAB GTPases has already been shown to ameliorate disease-associated features in various cell and animal models of PD. There is also the possibility that RAB GTPases could confer neuroprotective effects. In transgenic mouse models overexpressing *αSN*, reduced dopamine release and reuptake could be observed prior to any signs of axonal damage or degeneration of dopaminergic neurons.[124, 125] This was associated with alterations in the distribution and recycling of synaptic vesicles.[125-127] The

potential for RAB proteins to rescue these early trafficking defects and protect neurons from irreversible damage warrants further investigation. Therefore, studies into the RAB GTPase family of proteins, in association with cellular trafficking pathways, hold great potential for the discovery of novel PD therapeutics, to slow or halt the disease process.

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Author Roles

YG: Research project execution and writing the first draft of the manuscript.

GW: Research project execution and review/critique of the manuscript.

SS: Research project execution and review/critique of the manuscript.

KB: Research project execution and review/critique of the manuscript.

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SS:

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KB:

2012-2016 : NHMRC Australia, GNT 1022812, Chemerin, a New Therapeutic to Treat Obesity

2012-2016 : NHMRC Australia, GNT1020285, Exome Sequencing by NGS to Identify Rare Variants Affecting Type 2 Diabetes

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Figure Legends

Figure 1: RAB GTPase activity and regulation.

RAB-GTP participates in intracellular trafficking by interacting with effector proteins, membranes and vesicles (1). GAP catalyzes GTP hydrolysis and inactivates RAB (2). GDI stabilizes RAB-GDP (3). GEF removes GDP via guanine exchange, allowing RAB binding to GTP (4).

Figure 2: The role of RAB GTPases in PD associated pathways.

The function of RAB GTPases are closely associated with those of established PD genes (in red). These relationships and pathways are depicted in a schematic model of intracellular trafficking in midbrain dopaminergic neurons.

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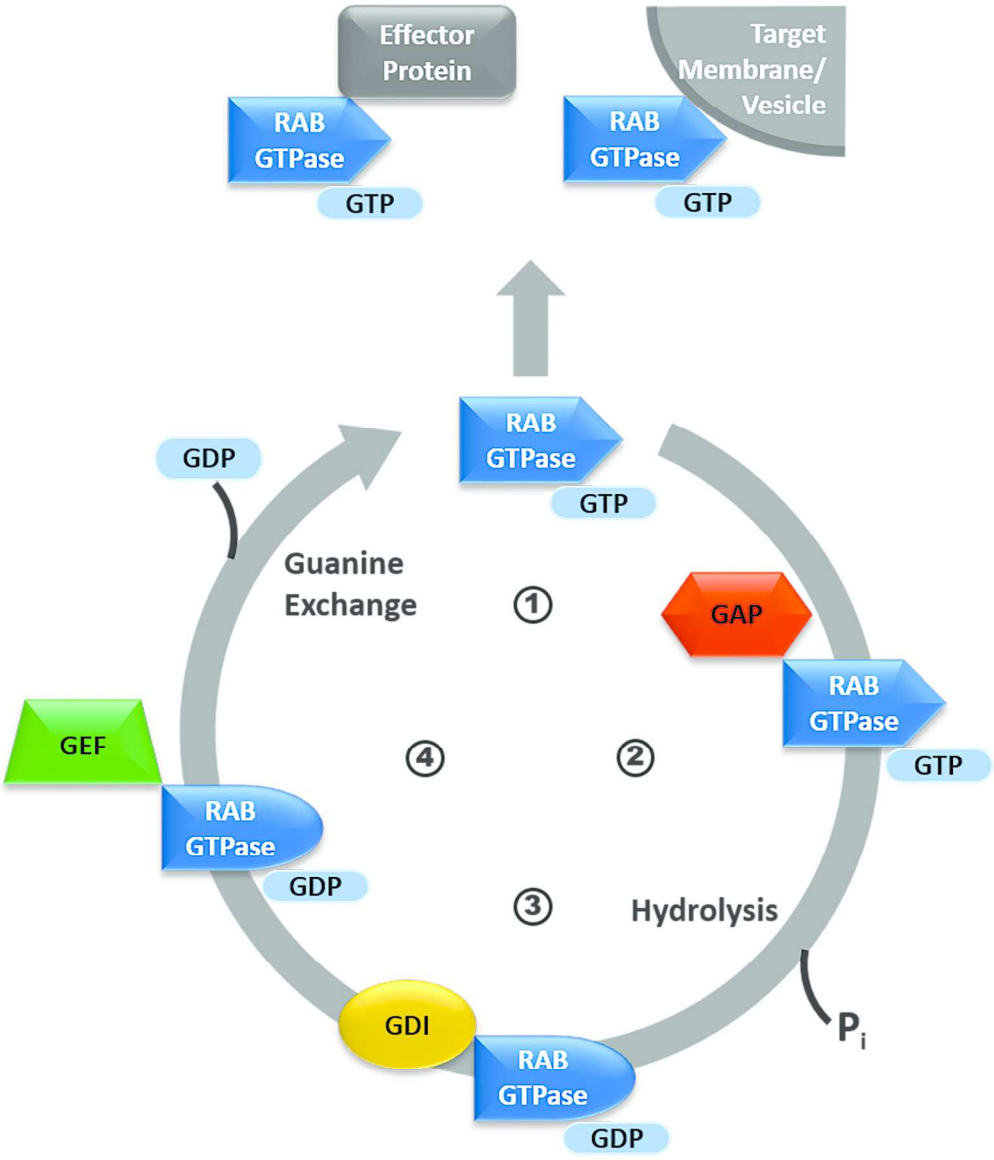

Figure 1: RAB GTPase activity and regulation.

167x196mm (300 x 300 DPI)

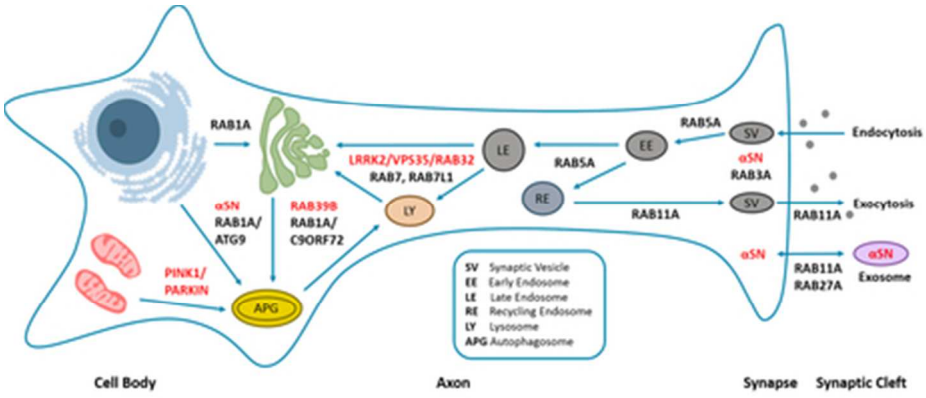

Figure 2: The role of RAB GTPases in PD associated pathways.

39x16mm (300 x 300 DPI)

Table 1: Summary of PD associated RAB GTPases and their therapeutic potential.

RAB GTPase	Known and proposed functions [15]	Interaction with PD associated proteins/ proposed role in PD	Evidence for therapeutic potential	References
RAB1A	ER-Golgi trafficking Autophagy	Inhibited by αSN OE Colocalizes with αSN aggregates Substrate of LRRK2 phosphorylation	OE rescues αSN induced defects in macroautophagy and trafficking OE rescues αSN induced dopaminergic neuron loss and motor deficits in animal model	[40, 48, 68-73]
RAB3A	Synaptic vesicle transport Exocytosis	Colocalizes with αSN Regulate αSN distribution at synapse		[62-65]
RAB5A	Early endosome trafficking Endocytosis	Substrate of LRRK2 phosphorylation		[48]
RAB7	Late endosome-Lysosome trafficking	Colocalizes with αSN aggregates Interacts with Retromer complex (VPS35) Negatively regulated by LRRK2 Substrate of LRRK2 phosphorylation	OE rescues αSN aggregation	[48, 73-74, 87-88, 107]
RAB8A	TGN trafficking Exocytosis Autophagy	Colocalizes with αSN aggregates Modulates αSN oligomerization and aggregation Substrate of LRRK2 phosphorylation Phosphorylated by PINK1 mediated mechanism	OE rescues αSN aggregation	[40, 48, 66, 73, 101]
RAB10	TGN trafficking Exocytosis	Colocalizes with αSN aggregates Substrate of LRRK2 phosphorylation		[48, 73]
RAB11A	Recycling endosome trafficking Exocytosis	Colocalizes with αSN aggregates Exocytosis and exosomal release of αSN Modulates αSN oligomerization and aggregation	OE rescues αSN aggregation OE rescues dopaminergic neuron loss in animal model	[38, 73, 75-78]
RAB12	Centrosome trafficking Exocytosis	Substrate of LRRK2 phosphorylation		[48]
RAB13	TGN trafficking Tight junction formation	Modulates αSN oligomerization and aggregation Phosphorylated by PINK1 mediated mechanism	OE rescues αSN aggregation	[38, 101]
RAB27A	Exocytosis, Melanosome trafficking	Exocytosis and exosomal release of αSN		[78]

RAB7L1/RAB29	TGN trafficking	Regulates lysosomal trafficking with LRRK2 Regulates Retromer complex (VPS35) Substrate of LRRK2 phosphorylation	OE rescues LRRK2 induced lysosomal defects and neurite shortening OE rescues dopaminergic neurons loss in animal model	[48, 89, 91, 93]
RAB32	TGN trafficking Mitochondrial dynamics	Mutations cause PD Regulates LRRK2 endosomal transport		[47, 49]
RAB39B	TGN trafficking Autophagy	Mutations cause PD Regulates αSN homeostasis Modulates αSN oligomerization and aggregation Substrate of LRRK2 phosphorylation		[27-33, 38, 40, 48]
Abbreviations: αSN alpha synuclein, ER endoplasmic reticulum, TGN trans-golgi network, OE overexpression.				

The emerging role of Rab GTPases in the pathogenesis of Parkinson's disease.

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Abstract

The identification of pathogenic mutations in *RAB39B* and *RAB32* that cause Parkinson's disease (PD) has highlighted the emerging role of protein trafficking in disease pathogenesis. Rab GTPases function as master regulators of membrane trafficking, including vesicle formation, movement along cytoskeletal networks and membrane fusion. Recent studies have linked Rab GTPases with alpha-synuclein, Leucine-rich repeat kinase 2 and Vacuolar protein sorting 35, three key proteins in PD pathogenesis. In this review, we will discuss the various RAB GTPases associated with PD, current progress in the research and potential future directions. Investigations into the function of RAB GTPases will likely provide significant insight into the etiology of PD and identify novel therapeutic targets for a currently incurable disease.

Parkinson's disease (PD) is a common neurodegenerative disorder affecting up to 5% of elderly individuals.[1, 2] Further, it is the most common cause of Parkinsonism, a spectrum of clinical disorders that present with a similar range of motor deficits, including bradykinesia, resting tremor, cogwheel rigidity and postural instability.[3] In PD, these symptoms are mediated by the pathological hallmark features, including a loss of dopaminergic neurons in the substantia nigra *pars compacta* (SNpc-a region in the midbrain responsible for the initiation and fine-tuning of voluntary movement) and the presence of intracellular protein inclusions (Lewy bodies) composed primarily of alpha-synuclein (αSN) in surviving dopaminergic neurons.[4] Current therapeutics for PD provide symptomatic relief but do not halt disease initiation or progression and lose efficacy over time, with undesirable side effects such as dyskinesia.[5] Therefore, a greater understanding of the etiology and pathological mechanisms underlying PD is required for the development of more targeted and effective therapies.

A large proportion of affected individuals have idiopathic PD, however a causal mutation can be identified in ~5-10% of patients. Due to the clinical similarities between genetic and idiopathic PD, it is hypothesized that they share common pathological pathways. Therefore, understanding the role of PD-associated genes in disease initiation and progression may elucidate common underlying pathological mechanisms. Currently, mutations in over 20 genes and loci have been shown to be associated with or causative of PD.[6, 7] Functional studies of the encoded proteins has revealed common themes in the mechanisms of PD pathology, including dysfunctions in protein degradation, mitochondria, and apoptotic pathways. More recently, a role has emerged for protein trafficking in the pathogenesis of PD. In particular, the dysregulation of members of a family of proteins essential for all aspects of cellular trafficking,

called Ras Analog in Brain (RAB) GTPases, have been shown to be directly causative or associated with PD. The underlying mechanisms involve RAB regulation of αSN and interactions with a range of other PD associated genes. In this review, we discuss the evidence linking the RAB protein family to PD and consider how functional studies of RAB GTPases can provide insights into disease pathogenesis and potential therapeutics.

RAB GTPase Proteins

RAB proteins are small GTPases that belong to a superfamily of over 60 distinct proteins in humans. They act as molecular switches during vesicular transport and membrane trafficking, by cycling between a GDP-bound inactive and GTP-bound active form. This switching activity is tightly controlled by protein regulators and involve a conformational change in two regions of the protein that interact with effectors, switch I and II.[8] Specifically, the GTP-bound active GTPase can interact with protein effectors or target membranes to alter downstream signaling cascades. The main regulators of GTPases include the GTPase activating protein (GAP), which inactivates GTPases by catalyzing GTP to GDP hydrolysis. The inactive GTPase, predominantly localized to the cytoplasm, is stabilized by a GDP dissociation inhibitor (GDI). GDI binds inactive GTPases with high affinity and prevents its reactivation by Guanine exchange factor (GEF). GEF activates the GTPase by promoting GDP to GTP exchange (Figure 1).[9] A single RAB GTPase can display a multitude of functions through the use of different effector proteins and vice versa. For example, more than 20 proteins have been identified to directly or indirectly interact with GTP-RAB5, a core component of the endosomal docking apparatus.[10] GTPases can also bind to protein cargos enclosed within transport vesicles. For example, RAB27A is a key component of

the vesicular trafficking machinery in melanocytes and is known to transport melanosomes, vesicles in which melanin is synthesized and stored.[11]

Commonly, RAB GTPases are ubiquitously expressed in all cell types and are responsible for the regulation of fundamental aspects of cellular trafficking that are highly conserved in all eukaryotes.[12] They include participation in various steps in trans/cis-Golgi transport, endosomal transport, lysosomal transport, vesicle transport, endocytosis and autophagy. These activities are vital for the regulation of the spatiotemporal localization and thus function of intracellular proteins, the degradation or recycling of various protein components and the establishment of membrane identity. As these functions have previously been comprehensively reviewed they will not be described further.[13-16]

~~Conversely, RAB GTPases that are enriched in certain tissues, or are regulated by tissue-specific effectors, can confer functional specificity.~~Conversely, RAB GTPases that are enriched in certain tissues, or which regulate tissue-specific effectors, can confer functional specificity. For example, RAB GTPases that are expressed predominantly in the brain are involved in the regulation of neural development, neurite growth, axonal transport and synaptic vesicles.[17] Dysregulation of these specialized RAB proteins can contribute to the pathogenesis of disease.[18] For example, mutations in *RAB27A* cause Griscelli syndrome and mutations in *RAB7* cause Charcot-Marie-Tooth type 2B.[19, 20] Additionally, mutations in RAB regulatory (GAP/GEF) or effector proteins, which dysregulate RAB GTPase localization and/or function, can also result in disease. For example, mutations in *GDI1*, encoding a GDI common to all RAB GTPases (αGDI), have been shown to cause X-linked non-specific mental retardation.[21] Both loss of and reduced function of αGDI leads to altered synaptic vesicle biogenesis and recycling in the

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hippocampus.[22] Thus, both causative mutations and more subtle 'secondary' mechanisms are evident in the contributions of RAB proteins to disease pathogenesis.

Mutations in *RAB39B* and *RAB32* cause PD

RAB39B was first identified in 2002 [23] and loss of function mutations were initially linked to X-linked mental retardation (XLID).[24] Subsequently, multiplication of *RAB39B* was associated with XLID, indicating altered *RAB39B* dosage contributes to neurological disease.[25, 26] More recently, *RAB39B* was the first RAB GTPase to be causally linked to the development of PD, with loss of function mutations shown to cause an X-linked recessive form of early onset PD.[27] Two families with loss of function mutations in *RAB39B* were identified. The affected individuals presented with typical PD associated clinical features, including bradykinesia, resting tremors and rigidity. Additional features, including XLID and macrocephaly, matched those previously described in the XLID study.[24] In the post mortem brain, there was neuron loss and the presence of αSN positive Lewy bodies and Lewy neurites.[27] Subsequently, additional studies have confirmed the role of *RAB39B* in PD [28-33] although negative results in several large mutation screens suggest germline mutations in *RAB39B* are a rare in PD.[31, 34-37]

The function of *RAB39B* is currently being investigated. *RAB39B* has been shown to be highly expressed in neurons compared to other cell types [24] but its intracellular localization remains uncertain. Different studies have shown RAB39B to localize to the Golgi, early endosome, recycling endosome, and plasma membrane in various *in vitro* models.[24, 27, 28] These results suggest a function in the trans-Golgi network (TGN), which directs the distribution of newly synthesized proteins from the Golgi to various subcellular compartments, such as endosomes,

or the plasma membrane. Functional studies of RAB39B have indicated a role in the regulation of αSN homeostasis. Downregulation of *Rab39b* in *in vitro* models (P19 cells and primary murine hippocampal neurons) resulted in reduced steady state levels of αSN and reduced density of αSN immunoreactive puncta in dendritic processes.[27] In a second study, knockdown of *RAB39B* in human neuroglial (H4) cells did not affect steady state levels, but did result in increased oligomerization of an αSN reporter.[38] How these *in vitro* observations relate to the observed αSN pathology in RAB39B-mediated PD is an active area of current research, but has been suggested to reflect differences between simple cell models and the complex and prolonged cellular alterations associated with end-stage disease.[27] Indeed, it is currently unknown if there is a direct or indirect interaction between RAB39B and αSN and this warrants further investigation. Further, RAB39B may modulate synaptic plasticity and the regulation of neuronal outgrowth, as both downregulation and overexpression of *Rab39b* in primary neurons results in reduced neuronal branching and synapse formation.[24, 25, 39] Most recently, RAB39B has been linked to autophagy. C9ORF72 was shown to complex with the WD Repeat Domain 41 (WDR41) and Smith-Magenis Syndrome Chromosome Region Candidate 8 (SMCR8) proteins to act as a GEF protein for RAB39B and RAB8A.[40] Repeat expansion affecting *C9ORF72* results in the linked neurodegenerative disease familial amyotrophic lateral sclerosis and fronto-temporal dementia.[41] C9ORF72 is also a known RAB1A effector, and regulates RAB1A dependent trafficking of the ULK1 complex, which is responsible for the initiation of autophagy.[42] It was proposed that C9ORF72 participates in a novel RAB signaling cascade, in which RAB1A recruits the C9ORF72/SMCR8/WDR41 complex to activate RAB39B and RAB8A. Subsequently, these

activated RAB GTPases regulate the maturation of autophagosomes by directly or indirectly interacting with the downstream autophagy pathway.

Mutations in a second RAB gene (*RAB32*) have now been shown to cause late onset familial PD. RAB32 was previously linked to cellular processes including autophagy, mitochondrial dynamics, phagocytosis and inflammation in the brain.[43-46] Our very recent study showed a p.Ser71Arg mutation in *RAB32* segregated with late onset autosomal dominant PD in three unrelated families.[47] This serine/threonine residue is found in the switch II domain of most rabs, and is differentially phosphorylated by LRRK2.[48] Of note a direct interaction between RAB32 and LRRK2 was previously demonstrated and shown to be important for late endosomal transport and sorting.[49] These studies implicate *RAB32*, in association with *LRRK2*, in the pathogenesis of PD and are likely to be an area of intense study in the near term.

Functional linkages between RAB GTPases and αSN

The gene encoding αSN (*SNCA*) was the first gene causally linked to PD in 1997.[50] Subsequently, additional point mutations as well as gene dosage increases were identified, demonstrating that both qualitative and quantitative changes in *SCNA* expression can cause PD.[51-55] Recent genome-wide association studies (GWAS) have indicated variants in the *SNCA* promotor and 3' UTR regions, associated with elevated expression, also correlate with a strong risk of developing sporadic PD.[56-58] Despite its fundamental role in the pathogenesis of PD, the function of αSN in the brain or the periphery remains largely unknown. The protein is abundant in the mammalian brain, comprising up to 1% of total cytoplasmic protein lysate [59] and is enriched in presynaptic

nerve terminals, where it is thought to play a role in the regulation of synaptic vesicle transport.[60, 61]

One of the first studies linking αSN and RAB GTPases utilized a transgenic mouse overexpressing PD associated mutant *p.Ala30Pro αSN*, (NM_000345.3: c.88G>C, p.Ala30Pro), and showed abnormal interaction of p.Ala30Pro αSN with RAB3A, RAB5A and RAB8A by co-immunoprecipitation analysis of brain homogenates.[62] Further, abnormal binding of wildtype (WT) αSN to RAB3A was found in brain homogenates from individuals with idiopathic PD, diffuse Lewy body disease or multiple system atrophy.[63, 64] These interactions suggest that unconventional binding of WT or mutant αSN to RAB proteins could disrupt the intricate control of RAB function and alter the homeostasis of αSN. Interestingly, RAB3A is involved in synaptic vesicle transport and thus the two proteins may possess complementary functions.[17] Indeed, a study demonstrated that active GTP-bound RAB3A directly interacted with and stabilized WT αSN to the membranes of presynaptic vesicles. Concurrently, when RAB3A is removed from these membranes following GTP hydrolysis, it also acts to free αSN during synaptic activity.[65] WT αSN has also been shown to interact with RAB8A and this is enhanced by phosphorylation of αSN at the serine 129 (S129), promoting binding to the switch I and II regions of RAB8A.[66] Given that up to 90% of αSN present in Lewy bodies is phosphorylated at S129,[67] this interaction potentially implicates RAB8A in the formation of αSN aggregates and the clearance of toxic oligomeric αSN species in the cell.

Yeast models have proven to be a powerful platform for elucidating the functional relationships between αSN and RAB proteins. Overexpression of *WT* or *p.Ala53Thr αSN* (NM_000345.3: c.157G>A, p.Ala53Thr) caused an inhibition of ER to Golgi trafficking due to impaired

docking/fusion of vesicles to Golgi membranes. This phenotype could be rescued by overexpression of RAB GTPase *Ypt1p* (Yeast homolog of *Rab1*) which promotes ER to Golgi transport.[68] Further, in both *C. elegans* and rat primary midbrain cultures, overexpression of *Rab1a* was able to rescue dopaminergic neuron loss induced by overexpression of *WT* or *p.Ala53Thr αSN*.[69] Consistent with a potential neuroprotective role for RAB1A, overexpression has also been shown to rescue other aspects of αSN toxicity. For example, some cell and animal models with overexpression of *WT αSN* show impaired macroautophagy, a process that degrades and recycles cellular contents by autophagosome formation and lysosome fusion. This deficit can be rescued by *RAB1A* overexpression.[70] However, autophagy impairment was not observed when *p.Ala53Thr* and *p.Ala30Pro* αSN mutants were overexpressed, suggesting this mechanism is associated with toxicity induced by increased dosage of αSN. Furthermore, the authors demonstrated that increased dosage of αSN impairs autophagy by inhibiting RAB1A activity, culminating in the mislocalization of ATG9, a protein that regulates the formation of autophagosome precursors.[70] In another study, overexpression of human *WT αSN* in a rat model showed dopaminergic neuron loss, Golgi fragmentation in surviving dopaminergic neurons and motor impairments.[71] *RAB1A* overexpression in this model improved motor behavior and rescued Golgi fragmentation, but did not ameliorate neuronal loss. In dopaminergic neurons generated from iPSC with triplication of *αSN*, overexpression of *RAB1A* rescued phenotypes induced by increased *αSN*, including Golgi fragmentation and disruptions in lysosomal hydrolase trafficking, as well as reduced αSN levels.[72] The authors proposed that accumulation of αSN in the cell body resulted in the mislocalization of RAB1A from its normal ER-Golgi localization, thus disrupting its function. In support, a study in yeast has shown that

WT αSN overexpression induces mislocalization and accumulation of many RAB GTPase proteins to αSN positive aggregations or vesicles. These include RAB proteins of various functions, Ypt1p, Ypt6p, Ypt7p Sec4p, Ypt10p and Ypt32p, homologs to RAB1A, RAB6 and RAB7, RAB8A, RAB10 and RAB11 in mammals respectively.[73] The authors therefore proposed that αSN was dysregulating general trafficking events, rather than interacting specifically with each RAB protein, a highly probable hypothesis. For example, RAB7 and RAB11A are crucial effectors of the late and recycling endosomes respectively. In drosophila models overexpressing *p.Ala53Thr αSN*, *RAB7* overexpression has been shown to clear αSN aggregates via autophagy, much like RAB1A, and increase the size and acidity of RAB7 positive vesicles.[74] In mammalian cell models with overexpression of *WT αSN*, *RAB11A* overexpression has been shown to reduce αSN aggregation and toxicity by facilitating the secretion of αSN via exocytosis.[75, 76] *RAB11* overexpression was also shown to rescue dopaminergic neuron loss and abnormal synaptic signaling in a drosophila model overexpressing *WT αSN*, presumably by decreasing insoluble αSN in the cell.[77] Thus, there is considerable evidence that a range of endosomal trafficking events, including autophagy and exocytosis, are implicated in αSN toxicity.

In addition to exocytosis, αSN is also known to be secreted via exosomes in a process regulated by *RAB11* and *RAB27.*[78] Exosomes, or extracellular vesicles, are released into the extracellular environment by many cell types and function in intercellular communication. In a human cell line overexpressing *SNCA*, αSN was shown to be secreted via exosomes in a calcium dependent manner, and this secreted or extracellular αSN propagated its cytotoxicity when taken up by neighboring cells.[79] *RAB11* has been shown to participate in this process by recycling and secreting extracellular αSN via exocytosis.[75] Taken together, this suggests that αSN secretion via

exocytosis and exosomes may facilitate the spreading of αSN from cell to cell in PD, much like in Prion related pathologies. Beyond PD, *RAB11* has been linked to other neurodegenerative diseases characterized by misaggregation of protein, suggesting dysfunctions in protein trafficking and secretion may be a common pathogenic pathway. For example, overexpression of *RAB11* is known to correct defects in synaptic signaling and vesicle formation in a Huntington's disease (HD) drosophila model.[80] RAB11 been shown to interact with Presenilin 1 and 2 in Alzheimer's Disease (AD), suggesting a role in the processing of amyloid precursor protein (APP).[81]

More recently, Gonçalves *et al.* performed an RNAi screen of over 1300 genes involved in trafficking and phosphotransferase activity to identify proteins that modulate αSN oligomerization and aggregation.[38] The screen was performed in human neuroglial cells overexpressing *WT SNCA*, using a biomolecular fluorescence complementation assay. Four of nine genes identified in the screen encoded RAB GTPases (*RAB8B*, *RAB11A, RAB13* and *RAB39B*). In *RAB8B* and *RAB13* KD cells, αSN oligomerization was correlated with a dysregulation in the level of αSN and an increase in cell cytotoxicity. However, αSN oligomerization occurred independent of these phenotypes in *RAB11A* and *RAB39B* KD cells. Moreover, overexpression of *RAB8B*, *RAB11A* and *RAB13* was able to rescue αSN aggregation, possibly by increasing the endocytic recycling of αSN. It is currently unknown whether these RAB GTPases function independently, however they should be considered as potential targets for PD therapeutics.

Functional linkages between RAB GTPases and LRRK2

Mutations in the Leucine-rich repeat kinase 2 gene (*LRRK2*) are the most common genetic cause of late onset autosomal dominant PD. The most frequent mutation (NM_198578.3:c.6055G>A, p.Gly2019Ser) is present in 1% of patients with sporadic PD and 4% of patients with PD showing autosomal dominant inheritance.[4, 56] LRRK2 is a large multidomain protein and there is considerable evidence suggesting the activity of the LRRK2 Ras-like GTPase and kinase domains mediates toxicity.[82] Several *LRRK2* mutations, including p.Gly2019Ser, have been shown to increase LRRK2 kinase activity and are toxic when overexpressed in cellular models.[83, 84] Previously, LRRK2 was shown to be important in the regulation of neurite outgrowth and autophagy.[85, 86] LRRK2 is predominantly localized to the cytoplasm, but is present at the ER, Golgi apparatus, early endosomes, lysosomes and synaptic vesicles, suggesting dysfunctional LRRK2 could disrupt normal axonal protein trafficking.[83] A study in drosophila demonstrated that the LRRK2 homolog Lrrk functionally interacts with RAB7 in the transport of late endosomes.[87] Moreover, in transiently transfected cells, Lrrk was shown to physically interact with Rab7, and WT Lrrk showed a markedly stronger interaction with the inactive form of Rab7 compared to the both wildtype and constitutively active forms. This difference was not observed with Lrrk p.Gly2019Ser. Thus, the authors suggested that Lrrk may play a role in stabilizing the inactive form of Rab7, resulting in negative regulation of Rab7-dependent lysosomal positioning and transport.[87] PD-causing *LRRK2* mutations dysregulate this interaction, thus abrogating the ability of Lrrk to negatively regulate Rab7-induced lysosomal trafficking and clustering. This is consistent with evidence from fibroblasts derived from PD patients (n=5) expressing *LRRK2 p.Gly2019Ser*. A reduction in RAB7 activity was associated with a delay in late endosome to lysosome trafficking of the epidermal growth factor receptor.[88]

A second RAB GTPase shown to be associated with *LRRK2* is *RAB7L1/RAB29.*[89] *RAB7L1* maps to PARK16, a locus of five genes that is a genetic risk factor for idiopathic PD.[90] MacLeod *et al.* showed that overexpression of either WT or constitutively active *RAB7L1* orthologue in a drosophila model rescued dopamine neuron loss and premature mortality induced by LRRK2 p.Gly2019Ser. In addition, overexpression of WT or constitutively active *RAB7L1* in rat primary neuronal cells overexpressing LRRK2 p.Gly2019Ser rescued phenotypes such as lysosomal swelling and reduced neurite process length during outgrowth.[89] In contrast, overexpression of WT Rab7L1 in WT primary mouse cortical cultures caused neurite shortening, perhaps due to dysregulation of the stoichiometric ratio of components of the LRRK2 complex that are required for normal LRRK2 function.[91] In humans, interaction of variants at additional genetic loci that can influence PD susceptibility may explain why *Rab7L1* has been associated with both elevated and reduced risk of PD.[92] In a *C. elegans* model, the protein complex 3 (AP3), a known regulator of endosome to lysosome trafficking, was shown to be a downstream effector of LRRK2 and RAB7L1. Thus, RAB7L1 together with LRRK2 can act to regulate AP3 and its various functions, including lysosomal trafficking and the coordination of axonal morphology.[93] Overall, defects in the LRRK2-RAB7L1 pathway are proposed to alter endo-lysosomal trafficking, with detrimental impacts on protein degradation and abnormal neuron growth.

The close functional relationship between LRRK2 and RAB proteins was further demonstrated by Steger and colleagues, who identified a subset of RAB GTPases as key LRRK2 substrates. LRRK2 was shown to phosphorylate target RAB GTPases at an evolutionarily conserved residue in the switch II domain.[48] It was shown *in vitro* that RAB proteins such as RAB1B, RAB8A and RAB10, which encode a threonine at this particular residue, were more efficiently

phosphorylated than those possessing a serine residue, such as RAB5B, RAB7A, RAB7L1, RAB12 and RAB39B. Further, LRRK2 mutants that display an increase in kinase activity, such as p.Gly2019Ser, were able to increase RAB phosphorylation by up to threefold compared to WT LRRK2. Importantly, the authors found that LRRK2 phosphorylation could potentially compromise RAB protein function. Using RAB8A as an example, LRRK2 phosphorylated RAB8A showed a decreased interaction with GEF protein Rabin8, inhibiting GDP exchange fourfold and resulting in decreased RAB8A activation. Further, phosphomimetics of RAB10 and RAB12 showed an inability to bind GDI proteins, which could potentially disrupt the subcellular distribution and/or activity of the RAB protein. These results are consistent with a model for *LRRK2*-mediated PD, where LRRK2 mutants with increased kinase activity dysregulate RAB protein cycling by altering affinity for cognate regulatory proteins. The predicted outcome would be dysregulated cellular trafficking, such as RAB5 and RAB7 mediated endosomal pathways and RAB7L1-mediated lysosomal trafficking. It will be important to further investigate the mechanisms underlying these LRRK2-RAB interactions, especially for *RAB7L1, RAB32* and *RAB39B*, which have shown independent associations with PD.

Interactions between RAB GTPase and Parkin/PINK1

Biallelic loss of function mutations in *PARK2*, encoding the Parkin protein, cause early onset PD, typically prior to age 40. Loss of function mutations account for up to 15% of early onset patients showing autosomal recessive inheritance, a proportion that decreases significantly with increasing age of onset.[94] Parkin is an E3 ubiquitin ligase that functions in the ubiquitin proteasomal system (UPS) and ubiquitinates proteins to mark them for degradation.[1, 95] Loss of

function mutations in *PARK2* impair the protein's ability to interact with the E2 ligase and its protein substrates, effectively abolishing its ability to target the correct substrates for degradation. After *PARK2*, mutations in the gene encoding PTEN-induced putative kinase 1 (*PINK1*) are the second most common genetic cause of early onset PD. *PINK1* mutations account for up to 8% of early onset PD showing an autosomal recessive inheritance.[96] PINK1 and Parkin play a prominent role in the regulation of mitophagy, a specialized form of autophagy for mitochondria. Early studies suggested that *PARK2* and *PINK1* knockout animals exhibited similar mitochondrial dysfunctions, including increased oxidative stress and reduced mitochondrial respiratory capacity.[97] Later, it was shown in drosophila models that *PINK1* is functionally upstream of Parkin and recruits Parkin to damaged mitochondria, where it promotes mitophagy.[98] *PINK1* encodes a mitochondrial targeting sequence to the outer mitochondrial membrane. On healthy mitochondria, PINK1 levels are very low due to cleavage by voltage-dependent proteolysis. However, when mitochondria are damaged and the membrane potential is reduced, PINK1 begins to accumulate and subsequently recruits and activates Parkin by phosphorylating ubiquitin and the ubiquitin-like domain of Parkin.[99, 100]

Recently, another function for PINK1 was uncovered. Analogous with LRRK2, PINK1-mediated kinase activity regulates RAB GTPases by phosphorylation. A report showed that activation of PINK1 kinase activity was required for downstream phosphorylation of RAB8A, RAB8B and RAB13 at a highly conserved serine residue, Ser111.[101] Unlike LRRK2 phosphorylation of RAB proteins, this was not shown to be a direct kinase-substrate interaction, suggesting intermediate kinases or phosphatases that are regulated by PINK1 are directly targeting these RAB GTPases. Further, this appears to occur independent of Parkin phosphorylation at Ser65,

and thus is proposed to be independent of the PINK1 mitophagy pathways. The functional outcome of Ser111 phosphorylation of RAB8A was impaired interaction with GEF protein Rabin8 in cell models.[101] Given that Ser111 lies within a region of the protein involved with effector binding, interactions with additional proteins are also predicted to be disrupted. Therefore, the loss of PINK1 activity observed in autosomal recessive PD not only prevents the turnover of damaged mitochondria, but may also have a detrimental impact on the activity of RAB GTPases by disrupting the function of intermediary proteins, kinases or phosphatases that directly interact with downstream RAB proteins. The preliminary data suggest additional pathways exist for *PINK1*-mediated PD, and understanding the specific mechanism of these pathways could potentially improve current disease models.

Interactions between RAB GTPase and VPS35.

Vacuolar sorting protein 35 (*VPS35*) is a subunit of the retromer complex, which is made up of the VPS26-VPS29-VPS35 trimer and a sorting nexin (SNX) dimer. The retromer complex plays a vital role in cargo selection in the retrograde transport pathway from endosomes to the Golgi,[102] and the identification of PD-causing mutations in VPS35 strongly implicated these pathways in disease pathogenesis. Currently, the only *VPS35* variant with proven pathogenicity is the p.Asp620Asn mutation (NM_018206.5:c.1858G>A, p.Asp620Asn).[103, 104] The most well studied retromer cargo is the cation-independent mannose-6-phosphate receptor (CI-M6PR), which participates in the sorting of acidic lysosomal hydrolase precursors from the TGN to endosomes and lysosomes. The retromer functions to recycle the receptor back to the TGN and thus ensures proper regulation of lysosomal enzymes.[105] This process is disrupted by the

p.Asp620Asn VPS35 mutation, which is in the region of VPS35 that directly interacts with CI-M6PR.[106] This process can also be impaired by dysregulation of RAB7, a known interactor of the VPS26-29-35 trimer which mediates the recruitment of retromers to late endosomes.[107] Dysfunctions in retromer trafficking could lead to functional consequences in downstream pathways carried out by its cargoes. For example, the CI-M6PR ligand cathepsinD, is a protease that has been shown to degrade αSN.[108, 109] Expression of CathepsinD degrades αSN and is neuroprotective in cells and animals overexpressing *αSN*. This suggests upregulating retromer activity is a potential avenue for PD therapeutics, and indirectly reduces the level of toxic αSN in cells via activity of its protein cargoes. The function of the retromer complex in the endo-lysosomal pathway overlaps with that of LRRK2 and RAB7L1. In cell models with reduced RAB7L1 activity, or cell and animal models overexpressing PD associated mutant forms of LRRK2, there is a significant decrease in the steady state levels of VPS26, VPS29 and VPS35.[89] This implies a deficiency in retromer activity may contribute to the neuropathology underlying LRRK2-mediated PD, and requires further investigation.

In addition to endo-lysosomal trafficking, VPS35 functions in synaptic transmission and recycling, and regulation of the trafficking of neurotransmitter receptors such as the GluA1 subunit of alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptors (AMPARs) at neuron synapses.[110] This function is perturbed in neuron models differentiated from pluripotent stem cells encoding *p.Asp620Asn VPS35*. Rab39b has been reported to regulate trafficking of the AMPAR subunit GluA2 to the cell surface,[39] raising the possibility that VPS35 and RAB39B converge on the same cellular pathways. Beyond PD, the retromer complex has been implicated in neurogenetic disorders such as Alzheimer disease and Down syndrome,

which are characterized by protein aggregation. Indeed upregulation of retromer in cell and animal models has been shown to improve recycling and rescue pathological features associated with these disorders.[111]

Conclusions and Future Directions

RAB GTPase proteins are implicated in multiple pathological mechanisms underlying PD (Table 1), and it is an exciting time in the field with an acceleration of novel discoveries being reported. Many PD associated proteins have a role in trafficking pathways or interact directly with RAB GTPases (Figure 2). Dysregulation of these proteins has been shown to disrupt essential steps in intracellular trafficking, in particular affecting the late endosome, lysosome and autophagy pathways. Other genes associated with Parkinsonism including *ATP13A2*, *VPS13C*, *GBA1*, *MAPT* and *DCTN1*, not discussed in this review due to space constraints, have also been shown to be involved in similar trafficking events,[112-117] highlighting the role of protein trafficking in the development of disease. More broadly, these data demonstrate that it is highly plausible that impaired intracellular trafficking is a key contributor to idiopathic PD. [118] Although it is unclear why certain neuronal populations, such as DA neurons in the ventral tier of the SNpc, are selectively lost by the early symptomatic stage of PD, there are several compelling possibilities. It has been proposed that dopamine might induce oxidative and nitrosative damage of axon terminals through the production of reactive species.[119] In addition, the vulnerability of DA neurons may be impacted by the energy costs required to maintain transport/synaptic activity in the massive, unmyelinated arbors typical of these cells.[120, 121] However, these features cannot entirely account for the selectivity, given other neuronal

populations with similar architecture, such as DA of the ventral tegmental area, are much less affected during disease. Most recently, it has been suggested that susceptible neurons play a key role in neuromodulatory networks, and have distinctive features including reduced calcium buffering capacity and slow, tonic activity which may result in increased reactive oxygen species and dysregulated calcium homeostasis.[122] Notably, all of these cellular processes are intricately connected, and vulnerable to, disruption in protein trafficking pathways. It is possible that relatively minor perturbations or reductions in the efficiency of trafficking pathways may have cumulative effects over the lifetime of a neuron, ultimately reaching a threshold that causes neurodegeneration and cell death.

The recent discoveries that mutations in *RAB39B* or *RAB32* can cause PD provides the most direct functional linkage to RAB proteins. Moreover, given that a substantial genetic contribution to Parkinsonism remains to be identified,[123] the application of modern genomic technologies is likely to identify additional causal genetic variants in RAB GTPases and associated proteins. Studying the function of these RAB proteins will expand our growing knowledge on the effect of trafficking defects in PD, ultimately leading to a better understanding of disease mechanisms and better therapeutics. Indeed, as discussed in this review, manipulating the expression of several RAB GTPases has already been shown to ameliorate disease-associated features in various cell and animal models of PD. There is also the possibility that RAB GTPases could confer neuroprotective effects. In transgenic mouse models overexpressing *αSN*, reduced dopamine release and reuptake could be observed prior to any signs of axonal damage or degeneration of dopaminergic neurons.[124, 125] This was associated with alterations in the distribution and recycling of synaptic vesicles.[125-127] The

potential for RAB proteins to rescue these early trafficking defects and protect neurons from irreversible damage warrants further investigation. Therefore, studies into the RAB GTPase family of proteins, in association with cellular trafficking pathways, hold great potential for the discovery of novel PD therapeutics, to slow or halt the disease process.

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Author Roles

YG: Research project execution and writing the first draft of the manuscript.

GW: Research project execution and review/critique of the manuscript.

SS: Research project execution and review/critique of the manuscript.

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Figure Legends

Figure 1: RAB GTPase activity and regulation.

RAB-GTP participates in intracellular trafficking by interacting with effector proteins, membranes and vesicles (1). GAP catalyzes GTP hydrolysis and inactivates RAB (2). GDI stabilizes RAB-GDP (3). GEF removes GDP via guanine exchange, allowing RAB binding to GTP (4).

Figure 2: The role of RAB GTPases in PD associated pathways.

The function of RAB GTPases are closely associated with those of established PD genes (in red). These relationships and pathways are depicted in a schematic model of intracellular trafficking in midbrain dopaminergic neurons.

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Table 1: Summary of PD associated RAB GTPases and their therapeutic potential.

RAB GTPase	Known and proposed functions [15]	Interaction with PD associated proteins/ proposed role in PD	Evidence for therapeutic potential	References
RAB1A	ER-Golgi trafficking Autophagy	Inhibited by αSN OE Colocalizes with αSN aggregates Substrate of LRRK2 phosphorylation	OE rescues αSN induced defects in macroautophagy and trafficking OE rescues αSN induced dopaminergic neuron loss and motor deficits in animal model	[40, 48, 68-73]
RAB3A	Synaptic vesicle transport Exocytosis	Colocalizes with αSN Regulate αSN distribution at synapse		[62-65]
RAB5A	Early endosome trafficking Endocytosis	Substrate of LRRK2 phosphorylation		[48]
RAB7	Late endosome-Lysosome trafficking	Colocalizes with αSN aggregates Interacts with Retromer complex (VPS35) Negatively regulated by LRRK2 Substrate of LRRK2 phosphorylation	OE rescues αSN aggregation	[48, 73-74, 87-88, 107]
RAB8A	TGN trafficking Exocytosis Autophagy	Colocalizes with αSN aggregates Modulates αSN oligomerization and aggregation Substrate of LRRK2 phosphorylation Phosphorylated by PINK1 mediated mechanism	OE rescues αSN aggregation	[40, 48, 66, 73, 101]
RAB10	TGN trafficking Exocytosis	Colocalizes with αSN aggregates Substrate of LRRK2 phosphorylation		[48, 73]
RAB11A	Recycling endosome trafficking Exocytosis	Colocalizes with αSN aggregates Exocytosis and exosomal release of αSN Modulates αSN oligomerization and aggregation	OE rescues αSN aggregation OE rescues dopaminergic neuron loss in animal model	[38, 73, 75-787]
RAB12	Centrosome trafficking Exocytosis	Substrate of LRRK2 phosphorylation		[48]
RAB13	TGN trafficking Tight junction formation	Modulates αSN oligomerization and aggregation Phosphorylated by PINK1 mediated mechanism	OE rescues αSN aggregation	[38, 101]
RAB27A	Exocytosis, Melanosome trafficking	Exocytosis and exosomal release of αSN		[78]

RAB7L1/RAB29	TGN trafficking	Regulates lysosomal trafficking with LRRK2 Regulates Retromer complex (VPS35) Substrate of LRRK2 phosphorylation	OE rescues LRRK2 induced lysosomal defects and neurite shortening OE rescues dopaminergic neurons loss in animal model	[48, 89, 91, 93]
RAB32	TGN trafficking Mitochondrial dynamics	Mutations cause PD Regulates LRRK2 endosomal transport		[47, 49]
RAB39B	TGN trafficking Autophagy	Mutations cause PD Regulates αSN homeostasis Modulates αSN oligomerization and aggregation Substrate of LRRK2 phosphorylation		[27-33, 38, 40, 48]
Abbreviations: αSN alpha synuclein, ER endoplasmic reticulum, TGN trans-golgi network, OE overexpression.				