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Mechanisms of *VPS35*-Mediated Neurodegeneration in Parkinson's Disease

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Abstract

Parkinson's disease is a sporadic and common neurodegenerative movement disorder resulting from the complex interplay between genetic risk, aging and environmental exposure. Familial forms of PD account for ~10% of cases and are known to result from the inheritance of mutations in at least 15 genes. Mutations in the *vacuolar protein sorting 35 ortholog* (*VPS35*) gene cause late-onset, autosomal dominant familial PD. VPS35 is a key suunit of the pentameric retromer complex that plays a role in the retrograde sorting and recycling of transmembrane cargo proteins from endosomes to the plasma membrane and trans-Golgi network. A single heterozygous Asp620Asn (D620N) mutation in *VPS35* has been identified in multiple families that segregates with PD, and a number of experimental cellular and animal models have been developed to understand its pathogenic effects. At the molecular level, the D620N mutation has been shown to impair the interaction of VPS35 with the WASH complex, that plays an accessory function in retromer-dependent sorting. In addition, the D620N mutation has been linked to the abnormal sorting of retromer cargo, including CI-M6PR, AMPA receptor subunits, MUL1, LAMP2a and ATG9A, as well as to LRRK2 hyperactivation. At the cellular level, data support an impact of D620N VPS35 on mitochondrial function, the autophagy-lysosomal pathway, Wnt signaling and neurotransmission via altered endosomal sorting. The relevance of abnormal retromer sorting and cellular pathways to PD-related neurodegenerative phenotypes induced by D620N VPS35 in rodent models is not yet clear. There is also uncertainty regarding the mechanism-of-action of the D620N mutation and whether it manifests pathogenic effects in animal models and PD through a gain-of-function and/or a partial dominant-negative mechanism. Here, we discuss the emerging molecular and cellular mechanisms underlying PD induced by familial *VPS35* mutations, going from structure to cellular function to neuropathology. We further discuss studies linking reduced retromer function to other neurodegenerative diseases and potential therapeutic strategies to normalize retromer function to mitigate disease.

Keywords

Parkinson's disease (PD); retromer; endosome; VPS35; LRRK2; Golgi; Lysosome; Mitochondria; vesicular sorting

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Introduction

Parkinson's disease (PD) is a common neurodegenerative movement disorder characterized by motor symptoms such as resting tremor, bradykinesia, rigidity and postural instability, in addition to myriad often prodromal non-motor symptoms (Poewe et al., 2017). The motor symptoms are caused by the progressive loss of dopaminergic neurons in the substantia nigra pars compacta leading to a loss of dopamine in the caudate putamen (Poewe et al., 2017; Przedborski, 2017). At present, disease-modifying therapies are not available to slow or stop disease progression. Identifying the underlying mechanisms of neuronal degeneration is critical for the discovery and validation of new therapeutic drug targets for treating PD. PD typically occurs as a sporadic disease most likely due to complex interactions between genetic risk, environmental exposure and aging, yet ~10% of cases are familial and are known to be caused by mutations in at least 15 different genes (Blauwendraat, Nalls, & Singleton, 2020; Przedborski, 2017). Understanding how familial mutations cause monogenic PD has provided important insight into disease pathophysiology and has nominated many novel drug targets.

Among monogenic forms of PD, a single mutation (D620N) in the *Vacuolar Protein Sorting 35 ortholog* (*VPS35*) gene causes late-onset, autosomal dominant PD in multiple families worldwide with a clinical and pathological spectrum similar to sporadic PD (Williams, Chen, & Moore, 2017). VPS35 is a key component of the retromer complex involved in the sorting and recycling of transmembrane cargo proteins from endosomes to the *trans*-Golgi network (TGN) or plasma membrane. Since the first discovery of *VPS35* mutations in 2011, a number of distinct yet overlapping mechanisms have been proposed suggesting that PD-linked mutations cause defects in the recycling or transport of specific cargo or receptors implicated in the function of lysosomes or mitochondria, or in neurotransmission and cell death pathways, that will be reviewed in this chapter. A potential mechanism involving the activation of leucine-rich repeat kinase 2 (LRRK2), the most common PD-linked gene, has recently been reported and may provide new insight into how D620N VPS35 causes neurodegeneration (Mir et al., 2018). Although a link between LRRK2 activation and neurodegeneration remains to be demonstrated in D620N VPS35 animal models, this discovery links these two late-onset PD genes that operate within the endolysosomal pathway and could provide new insight into converging mechanisms underlying disease pathophysiology. Furthermore, LRRK2-directed therapies currently in clinical trials for PD might also be of interest for patients harboring *VPS35* familial mutations. Notably, recent studies suggest that non-mutated VPS35 could be implicated in Alzheimer's disease (AD), Pick's disease or amyotrophic lateral sclerosis (ALS) (Muzio et al., 2020; Small et al., 2005; Vagnozzi et al., 2019), suggesting that understanding retromer biology could provide insight more broadly into neurodegenerative disease. In this chapter, we review the discovery, structure and functions of VPS35 and the possible mechanisms of neurodegeneration due to PD-linked mutations. We discuss emerging therapeutic strategies that are being developed to restore retromer function, and how new treatments developed for PD patients harboring *LRRK2* mutations may potentially benefit *VPS35*-linked PD.

I) Discovery of PD-linked *VPS35* mutations

Mutations in the *VPS35* gene as a cause of autosomal dominant familial PD were first discovered by exome sequencing in 2011. A heterozygous Asp620Asn (D620N) mutation was identified to segregate with disease in a multi-generational Swiss family with late onset PD (Vilarino-Guell et al., 2011). The same mutation has been reported in PD families from the US, Tunisia and in Yemenite Jews from Israel (Vilarino-Guell et al., 2011). A second study identified the D620N mutation in several Austrian families with PD with high but incomplete penetrance (Zimprich et al., 2011). The D620N mutation has now been confirmed in PD families from around the world including parts of Asia, although this mutation is particularly rare in China (Williams et al., 2017; Y. Zhang et al., 2012). While additional mutations in VPS35 have been reported in PD subjects, such as G51S, P316S, R524W or L774M, they are of unclear pathogenicity as their segregation with disease in families has not been unambiguously demonstrated (Williams et al., 2017). The D620N mutation is estimated to account for 0.1 to 1% of familial PD cases (Deng, Gao, & Jankovic, 2013), suggesting it is relatively rare compared to more common *LRRK2* or *Parkin* familial mutations.

The clinical specturm of *VPS35*-linked PD is broadly similar to sporadic PD with an average age-of-onset of 52–53 years, and manifesting as L-Dopa-responsive parkinsonism associated with resting tremor with slow progression often with mild cognitive impairment (Ishiguro et al., 2021; Kumar et al., 2012; Struhal et al., 2014; Wider et al., 2008). Fluorodopa PET imaging revealed an asymmetric uptake deficiency in the caudate putamen, similar to sporadic PD (Wider et al., 2008), suggesting the loss of dopaminergic innervation. The neuropathological spectrum however remains to be properly evaluated since only one autopsy of a *VPS35* mutation carrier has been reported, and included the cortex and basal ganglia but without the substantia nigra, locus coruleus or brainstem (Wider et al., 2008). No Lewy bodies, α-synuclein pathology or any intraneuronal inclusions were detected in these two brain regions suggesting that any pathology, if it is indeed present, is likely confined to the substantia nigra and/or brainstem. Recently, a PD subject harboring mutations in two distinct genes, *VPS35* (c.102+33G>A) and *FBXO7* (c.540A>G), was reported with depigmentation of substantia nigra, Lewy bodies in nigral dopaminergic neurons, as well as α-synuclein pathology in the pons and amygdala, tau pathology in the hippocampus (AT8-positive) and sparse β-amyloid plaques in the occipitotemporal gyrus (Menšíková et al., 2019). This *VPS35* mutation was also found in a cousin who developed PD (Bartonikova et al., 2016). However, the pathogenicity of these two variants remains to be confirmed and whether this complex pathology results from digenic inheritance cannot be ruled out. At this juncture, it remains unclear whether *VPS35*-linked PD recapitulates the neuropathological hallmarks of sproadic PD.

VPS35 mRNA expression is decreased in the substantia nigra of sporadic PD compared to unaffected control brains using a meta-analysis of microarray gene expression data sets, as well as in laser-microdissected nigral dopaminergic neurons from PD brains (MacLeod et al., 2013). However, VPS35 protein levels are unaltered in caudate putamen or frontal cortex from sporadic PD/DLB brains (Tsika et al., 2014), yet reported to be reduced in PD brains in a second study (Zhao et al., 2018). Interestingly, retromer protein subunits

are reported to be decreased in affected brain regions of other neurodegenerative diseases, including the entorhinal cortex in AD (Small et al., 2005), frontal cortex and hippocampus in Progressive Supranuclear Palsy (PSP) and Pick's disease (Vagnozzi et al., 2019), and spinal cord motor neurons in ALS (Muzio et al., 2020). These observations support a broad role for the retromer in neurodegenerative disease, and suggest that therapeutic strategies targeting retromer dysfunction could have potential applications beyond PD.

II) From VPS35 structure to function

VPS35 is a key component of the retromer, a pentameric protein complex that mediates the retieval and sorting of transmembrane cargo proteins from endosomes to the plasma membrane or TGN for recycling. Retromer is composed of two sub-complexes: the cargo-selective complex (CSC) containing VPS35, VPS26 and VPS29, and a sorting nexin (SNX) dimer (Williams et al., 2017). There are two VPS26 subunits, VPS26A and VPS26B, that compete to form distinct retromer complexes (Bugarcic et al., 2011). For example, VPS26A-retromer can interact with the cation-independent mannose 6-phosphate receptor (CI-M6PR) whereas no interaction between the VPS26B-retromer and CI-M6PR has been detected (Bugarcic et al., 2011). VPS35 is the largest subunit of the CSC consisting of 796 amino acids that forms an α-solenoid structure. Retromer interactions with endosomal membranes are facilitated by SNX proteins that contain *phox* homology (PX) and bin/amphiphysin/rvs (BAR) domains (SNX1 or SNX2 and SNX5 or SNX6, corresponding to VPS5 and VPS17 in yeast), or with SNX proteins lacking BAR domains such as SNX3 or SNX27 (Feng et al., 2017; Gallon et al., 2014). SNX proteins are implicated in cargo recognition and in mediating membrane curvature leading to the formation of endosomal tubules that faciltate cargo partitioning and sorting (Carlton et al., 2004; Kovtun et al., 2018; Yong et al., 2020).

Cryo-electron tomography studies in yeast revealed that retromer recruitment to tubular-shaped membranes depends on VPS5 which forms a homodimer that interacts with the membrane via the positively-charged ends of the BAR and PX domains (Kovtun et al., 2018). VPS26 forms a homodimer on the VPS5 array that serves as an anchor for the α-solenoid structure of VPS35 that interacts with the N-terminus of VPS26 via its own N-terminal region. The C-terminal region of VPS35 interacts with VPS29. This study also shows VPS35 dimerization occuring at the apex of an arch formed by two retromer complexes, on the opposite face to the C-terminal VPS29 binding site. Notably, in this model, the yeast D694 residue corresponding to D620 in humans, is localized at the apex where VPS35 homodimerizes (Kovtun et al., 2018). More recently, the dimeric structure of mammalian retromer was shown to be similar to yeast retromer but with a flatter conformation (Kendall et al., 2020). Mammalian retromer is able to assemble into trimers, tetramers or even chains *in vitro* based on cryo-EM studies and these assemblies can be modulated by salt concentration (Kendall et al., 2020). This study revealed the importance of charged amino acids in VPS35 at the interface between two retromer monomers by identifying key residues within an acidic patch (E615A, D616A and E617A) and three lysines (K659E, K662E, and K663E) that are required for the capacity to form dimers and multimers (Kendall et al., 2020). D620 is located adjacent to this acidic patch and both studies suggest that the D620N mutation (a substitution of a negatively-charged aspartate

for a polar uncharged asparagine) could disrupt the electrostatic interaction between VPS35 monomers within a retromer dimer.

Additional proteins are recruited to stabilize the retromer on the endosomal membrane such as Rab7a (Seaman, 2012). At endosomal membranes, retromer can recognize distinct cargo proteins such as CI-M6PR, sortilin-1, sorLA, insulin-like growth factor receptor 1 (IGF1R), glucose transporter 1 (GLUT1) or β2-adrenergic receptor (β2AR) and sort them for retrieval to the TGN (CI-M6PR, sortilin-1, sorLA) or plasma membrane (β2AR, GLUT1, IGF1R) (Cui, Yang, & Teasdale, 2018; Seaman, Gautreau, & Billadeau, 2013; Steinberg et al., 2013). Retromer sorting of certain cargos (i.e. β2AR, GLUT1) is facilitated by F-actin patches on endosomes formed by the pentameric Wiskott-Aldrich syndrome and SCAR homolog (WASH) complex that interacts with VPS35 in the retromer via its FAM21 subunit (Cui et al., 2018; Seaman et al., 2013). The different cargo sorted by the retromer suggest that retromer dysfunction can have a wide impact on multiple cellular pathways.

In mammals, VPS35 is ubiquitously expressed throughout the body with highest mRNA levels in the brain, heart, spleen, skeletal muscle, small intestine, ovary, testis and placenta (Haft et al., 2000; P. Zhang et al., 2000) (https://www.proteinatlas.org/ENSG00000069329-VPS35/tissue). In keeping with its wide expression, the retromer is essential for life. *VPS35* knockout mice die before birth at embryonic day 10 (Wen et al., 2011). In mouse brain, VPS35 is expressed widely across brain regions, in multiple cell types with highest levels in neurons, oligodendrocytes or oligodendrocyte progenitor cells, and within different neuronal populations with enrichment in serotonergic, cholinergic, noradrenergic and dorsal root ganglia populations according to RNA-Seq data (Sargent et al., 2021; Tsika et al., 2014; Wen et al., 2011). In neurons, the retromer is localized within the cell soma, axons and dendrites, mainly upon endosomes, the TGN and small sorting vesicles (Choy et al., 2014; Munsie et al., 2015; Tsika et al., 2014).

III) Mechanisms of neurotoxicity associated with *VPS35* mutations

i) VPS35 and cell death in animal models: Although formal proof of neurodegeneration in *VPS35*-linked PD brains at autopsy is lacking, but expected based upon neuroimaging and clinical studies (Wider et al., 2008), several rodent models have now confirmed that the D620N mutation is sufficient to induce progressive neuronal loss (X. Chen et al., 2019; Chiu et al., 2020; Niu et al., 2021; Tang, Erion, et al., 2015; Tang, Liu, et al., 2015; Tsika et al., 2014). The overexpression of human D620N VPS35 by intranigral delivery of AAV2/6 vectors induces axonal damage and nigral dopaminergic neuronal loss in adult rats to a greater extent that wild-type (WT) VPS35 (Tsika et al., 2014). Three independent studies confirm the degeneration of dopaminergic neurons in heterozygous and homozygous *D620N VPS35* knockin (KI) mice between 13–16 months of age, associated with motoric deficits and the widespread accumulation of abnormal hyperphosphorylated tau pathology in the brain (X. Chen et al., 2019; Chiu et al., 2020; Niu et al., 2021). The somatic accumulation of total α-synuclein in the substantia nigra of KI mice has also been reported, yet it is not known whether this represents abnormal or pathological α-synuclein (Chiu et al., 2020; Niu et al., 2021).

In *Drosophila* models, the overexpression of fly D620N VPS35 can successfully rescue the lethality of *VPS35* null flies, supporting the concept that D620N VPS35 is mostly a functional protein (Inoshita et al., 2017; Malik, Godena, & Whitworth, 2015). Similar studies in compound hetereozygous mice containing one D620N KI and one KO allele of *VPS35*, reveal that a single copy of D620N VPS35 is sufficient to overcome embryonic lethality and early dopaminergic neuronal loss that has been observed in germline or conditional *VPS35* KO mice (X. Chen et al., 2019). While this organismal genetic data is compelling, studies in primary ventral mesencephalic neuronal models suggest that D620N VPS35 expression is less protective than WT VPS35 following MPP^+-induced neurotoxicity (Bi, Li, Huang, & Zhou, 2013). Yet, in primary cortical neurons, human D620N VPS35 expression was not different to WT VPS35 in oppositely rendering neurons more susceptible to toxicity induced by MPP^+, rotenone, hydrogen peroxide or MG132 exposure (Tsika et al., 2014). Furthermore, yeast WT or D686N VPS35 expression was sufficient to rescue *VPS35* null yeast cells from cadmium-induced toxicity or confer increased sensitivity to nickel (Tsika et al., 2014). These studies, especially complementation studies, suggest that the retromer is broadly functional when VPS35 harbors a D620N or equivalent mutation.

Several studies suggest that modulating the endogenous expression of VPS35 is also sufficient to cause similar PD-like phenotypes. Heterozygous *VPS35* null mice are viable and reproduce some aspects of PD pathology, such as late-onset dopaminergic neuronal loss at 12 and 18 months of age, decreased dopaminergic innervation in the striatum, motor deficits, and the accumulation of insoluble α-synuclein in the substantia nigra (Tang, Erion, et al., 2015). Since *VPS35* KO mice are embryonic lethal (X. Chen et al., 2019; Wen et al., 2011), the homozygous conditional deletion of *VPS35* in specific neuronal populations is able to reproduce pathological features observed in neurodegenerative diseases. *VPS35* deletion in dopaminergic neurons using DAT-Cre mice induced dopaminergic neurodegeneration by 2–3 months of age, motor impairment and the accumulation of Ser129 phosphorylated α-synuclein (Tang, Liu, et al., 2015). These studies suggest that a *VPS35* loss-of-function can cause PD-like neurodegeneration, yet there are important differences between *VPS35* KO and KI mice such as embryonic lethality vesus normal survival, and the timing of neuronal loss. Another important consideration is whether *VPS35* deficiency selectively affects nigral dopaminergic neurons in germline (heterozygous) or conditional (DAT-Cre) mice and therefore produce relevant models of PD (Tang, Erion, et al., 2015; Tang, Liu, et al., 2015). For example, conditional deletion of *VPS35* in embryonic cortical pyramidal neurons in mice produces a frontotemporal dementia (FTD)-like model with atrophy of the neocortex, a terminal differentiation deficit, neuronal cell death, protein accumulation (TDP-43 and ubiquitin-protein conjugates) and gliosis within weeks after birth (Tang et al., 2020). *VPS35* deletion is poorly tolerated by different neuronal populations and it remains unknown whether certain neurons are more vulnerable than others to toxicity induced by *VPS35* deletion. Given that VPS35 levels are reduced in affected brain regions from AD, tauopathy and ALS subjects (Muzio et al., 2020; Small et al., 2005; Vagnozzi et al., 2019), and VPS35 is expressed by a range of different brain cells (Tsika et al., 2014; Wen et al., 2011), is it unclear which cell types are affected by *VPS35* deficiency and might drive susceptibility in these diseases. *VPS35* deletion in microglia using inducible CX3CR1-Cre mice increased hippocampal microglial density and

activity, altered dendritic morphology and density of newborn neurons in the dentate gyrus, and produced depressive-like behavior and memory impairment in young mice (Appel et al., 2018). *VPS35* deletion in microglia also increased the phagocytic activity of these cells including toward postsynaptic markers, such as PSD95, supporting a critical role for VPS35 in regulating dendrite outgrowth via microglial activity (Appel et al., 2018). The impact of *VPS35* deletion in other glial cell types in the brain such as oligodendrocytes or astrocytes remains to be studied, and other distinct neuronal populations remain to be evaluated.

A number of distinct mechanisms have been proposed to explain neuronal degeneration induced by *VPS35* mutations or *VPS35* deficiency in neurons and other cell types. The major consequence of retromer dysfunction is impaired cargo sorting and recycling, but which specific cargo are responsible for the pathogenic effects of the D620N mutation and in which cell types remains obscure. While *VPS35* mutations or deletion in mice can phenocopy the loss of dopaminergic neurons, it is likely that *VPS35* deficiency adversely affects many neuronal and glial populations whereas the D620N mutation acts in a more subtle and specific manner (Appel et al., 2018; X. Chen et al., 2019; Tang, Erion, et al., 2015; Tang, Liu, et al., 2015; Tang et al., 2020; Vagnozzi et al., 2019).

ii) Autophagy and lysosomal function: Autophagy is a fundamental mechanism that involves the capacity to recycle intracellular components via bulk degradation. Autophagy impairment has long been implicated in PD and several PD-related gene products have been shown to be involved in regulating or altering autophagic function, including α-synuclein (*SNCA*), β-glucocerbrosidase (*GBA1*), ATP13A2, FBXO7, LRRK2 and VPS35 (Lu, Wu, & Yue, 2020). There are three types of autophagy: microautophagy, macroautophagy, and chaperone-mediated autophagy (CMA). In microautophagy, cargo enters directly into the lysosome by invagination of the membrane, whereas macroautophagy involves the generation of a double membrane around targeted cargo and its transport to the lysosome. In CMA, a HSC70 chaperone selectively binds to substrate proteins and transports them directly across the lysosomal membrane via a CMA complex containing LAMP2a. These autophagy pathways culminate in a common last step involving delivery to lysosomes, an acidic organelle containing multiple hydrolases and proteases capable of degrading organelles, membranes and proteins.

D620N VPS35 has been reported to induce macroautophagy and CMA impairment in different cellular models. D620N VPS35 protein exhibits a reduced interaction with the FAM21 subunit of the WASH complex, reducing its recruitment to endosomal membranes, and resulting in the abnormal sorting of the autophagy protein ATG9A in HeLa cells (Zavodszky et al., 2014). Abnormal ATG9A sorting may account in part for the impaired autophagosome formation induced by VPS35 D620N overexpression in these cells (Zavodszky et al., 2014).The reduced binding of VPS35 to FAM21 caused by the D620N mutation has now been confirmed by other groups and remains one of the only consistent molecular defects reported to date (X. Chen et al., 2019; McGough et al., 2014). It remains to be determined whether ATG9A sorting defects and impaired macroautophagy are sufficient to induce neuronal cell death in D620N VPS35 models.

The D620N mutation is also implicated in impairing the degradation of aggregation-prone proteins such as α-synuclein or tau albeit via an indirect mechanism. CI-M6PR is a well known retromer cargo that is responsible for the delivery of its ligand cathepsin D, a lysosomal protease linked to α-synuclein degradation, to the late endosome where it undergoes processing into a mature form. D620N VPS35 interacts normally with CI-M6PR but alters the sorting of cathepsin D leading to α-synuclein accumulation in cell lines and fibroblasts derived from PD subjects harboring the D620N mutation (J. Follett et al., 2014). Other studies report that reducing VPS35 expression by gene silencing lowers the levels of CI-M6PR and cathepsin D proteins in brains of P301S tau transgenic mice, leading to the accumulation of phosphorylated tau (Vagnozzi et al., 2019). In *Drosophila*, deletion of retromer subunits impairs lysosomal degradation and induces the accumulation of autolysosomes, preventing the function of lysosomal cathepsin L (Maruzs et al., 2015). *VPS35* deletion in cortical pyramidal neurons of mice resulted in neurodegeneration with an increase in p62 and LC3B-II levels and protein aggregation (TDP-43) (Tang et al., 2020). In this study, the retromer cargo, sortilin-1, was shown to accumulate in neurons and was identified as potentially mediating lysosomal dysfunction. For example, selectively targeting sortilin-1 expression to lysosomes was sufficient alone to induce a similar lysosomal phenotype, whereas the silencing of sortilin-1 expression partly reversed the phenotype of conditional *VPS35* KO mice (Tang et al., 2020). In addition to α-synuclein and tau, reduced VPS35 expression is also linked to the accumulation Aβ in cells (Ansell-Schultz, Reyes, Samuelsson, & Hallbeck, 2018). D620N VPS35 has also been implicated in CMA dysfunction where *VPS35* deficiency or D620N VPS35 expression in primary neuronal cultures similarly impaired the endosome to TGN recycling of LAMP2a, a mechanism potentially linked to α-synuclein accumulation in dopaminergic neurons of *VPS35*-deficient mice (Tang, Erion, et al., 2015).

iii) Mitochondrial function: The role of mitochondrial dysfunction in PD has been studied for many decades (Haelterman et al., 2014). Dopaminergic neurons of the substantia nigra exhibit a unique morphology consisting of long and highly branched axons with a high bioenergetic demand, making mitochondrial function critical for their maintenance and survival (Pissadaki & Bolam, 2013). In PD subjects, mitochondrial respiratory complexes (particularly Complex I and NADH cytochrome c reductase) are reduced in the substantia nigra compared to healthy controls (Schapira et al., 1990). Likewise, nigral dopaminergic neurons in PD brains exhibit increased mitochondrial DNA deletions and loss of mitochondrial proteins (Bender et al., 2006; Kraytsberg et al., 2006). A number of proteins linked to familial PD, such as parkin, PINK1, DJ-1, α-synuclein or LRRK2, are either directly implicated in mitochondrial function or quality control, or can indirectly impact mitochondrial function (Biskup et al., 2006; Canet-Aviles et al., 2004; Hsieh et al., 2016; Narendra, Tanaka, Suen, & Youle, 2008; Sanders et al., 2014; Stevens et al., 2015; Thomas et al., 2011; X. Wang et al., 2019).

The dynamic regulation of mitochondrial morphology is essential for optimal mitochondrial function, full respiratory capacity and for cell survival (Uo et al., 2009). Retromer plays an unexpected role in the turnover of proteins implicated in mitochondrial fission or fusion. Retromer is able to sort cargos (such as MUL1 or DLP1) from mitochondria

via mitochondrial derived vesicles (MDV) to peroxisomes or lysosomes for degradation (Braschi et al., 2010; Sugiura, McLelland, Fon, & McBride, 2014; W. Wang et al., 2016). Indeed, *VPS35*-deleted midbrain dopaminergic neurons exhibit mitochondrial fragmentation and dysfunction in conditional *VPS35* $KO^{DAT\text{-}Cre}$ mice (Tang, Liu, et al., 2015). These *VPS35*-deficient neurons display increased levels of the E3 ubiquitin ligase MUL1 (MAPL) which promoted the degradation of mitofusin 2 (Mfn2), a protein important for mediating mitochondrial fusion (H. Chen et al., 2003; Tang, Liu, et al., 2015). Importantly, overexpression of WT VPS35, but not D620N VPS35, rescued mitochondrial fragmentation suggesting that the D620N mutation may act in a loss-of-function manner in relation to mitochondrial morphology (Tang, Liu, et al., 2015).

Retromer is also reported to mediate the clearance of mitochondrial DLP1 complexes, a key complex required for mitochondrial fission, by relocalizing them from mitochondria to MDVs for sorting to lysosomes for degradation (Pitts, Yoon, Krueger, & McNiven, 1999; W. Wang et al., 2016). D620N VPS35 has increased interactions with DLP1 (compared to WT VPS35) that increases mitochondrial DLP1 complex turnover and mitochondrial fragmentation in human cell lines and rat cortical neurons (W. Wang et al., 2016). Interestingly, the VPS35-DLP1 interaction is reported to be increased in the brains of idiopathic PD subjects and following oxidative stress induced by paraquat or hydrogen peroxide in neural cells (W. Wang et al., 2016). Increased mitochondrial fragmentation could also be observed in dopaminergic neurons after delivery of lentiviral vectors expressing D620N VPS35 in the ventral tegmental area of mice, or in nigral dopaminergic neurons of aged *D620N VPS35* KI mice (Chiu et al., 2020; W. Wang et al., 2016). Intriguingly, mitochondrial fragmentation and cell death induced by lentiviral D620N VPS35 delivery in mice could be rescued by treatment with the mitochondrial fission inhibitor, mdivi-1, that selectively targets dynamin-1 and DLP1 (W. Wang et al., 2016). D620N VPS35 is therefore suggested to induce mitochondrial fragmentation and cell death via three distinct mechanisms involving inhibition of mitochondrial fusion (Mfn2), increased mitochondrial fission (DLP1), and/or via enhanced MDV-dependent sorting to lysosomes.

iv) Wnt/β-catenin signaling pathway: A potential mechanism linking *VPS35* mutations to cell death involves the Wnt/β-catenin pathway (Chiu et al., 2020). The Wnt/β-catenin pathway plays an important role in dopaminergic neuronal differentiation and survival in the substantia nigra (Arenas, 2014). Wnt1 activates a neuroprotective cascade by promoting the nuclear translocation of β-catenin leading to the upregulation of the anti-apoptotic protein survivin (Jiang et al., 2014; Wheatley & Altieri, 2019). Genes implicated in the Wnt signaling pathway are down-regulated in midbrain dopaminergic neurons from PD patients, as well as in cells treated with MPP^+, suggesting that this pathway is implicated in cell death mechanisms related to PD (L. Zhang et al., 2016). When associated with SNX3, the retromer regulates the trafficking of Wntless, a receptor that binds and transports Wnt from the TGN to the plasma membrane where it is released (Harterink et al., 2011). The retromer mediates the sorting and recycling of Wntless from endosomes to the TGN (Harterink et al., 2011). A recent study found that Wnt1, β-catenin and survivin levels were down-regulated and caspases-8 and −9 were up-regulated in the substantia nigra of 16 month-old *D620N VPS35* KI mice, an age when these KI mice exhibited pronounced

dopaminergic neurodegeneration (Chiu et al., 2020). While a direct effect of D620N VPS35 on the retromer-mediated sorting of Wntless has not yet been shown, data from KI mice suggests that the Wnt/β-catenin pathway could be relevant for dopaminergic cell death.

v) Neurotransmission: Different aspects of neurotransmission are modulated by VPS35 and the retromer. VPS35 colocalizes with synaptic markers (Choy et al., 2014) and is detected in synaptosomes following subcellular fractionation of brain tissue (Tsika et al., 2014). Several studies report that *D620N VPS35* KI and transgenic mice exhibit impaired striatal dopamine release (Cataldi et al., 2018; Ishizu et al., 2016; Vanan et al., 2020). In one study, a reduction of dopamine transporter (DAT) levels in the striatum was found in 3 month-old *D620N VPS35* KI mice (Cataldi et al., 2018). The recycling of DAT was shown to be retromer-dependent in a human neural cell line since the endocytic recycling of DAT is impaired by VPS35 knockdown (Wu et al., 2017). In *Drosophila*, expression of the D647N VPS35 ortholog (equivalent to the D620N mutation) failed to rescue the impairment of synaptic vesicle recycling and dopamine release induced by *VPS35* deficiency, whereas WT VPS35 could rescue these synaptic phenotypes (Inoshita et al., 2017). In addition to the nigrostriatal dopaminergic pathway, the retromer has also been implicated in other neurotransmitter systems. In cultured rat neurons, the retromer can localize to dendrites and plays a role in the recycling of the AMPA receptor subunit GluR1 and β-adrenergic receptors (β2AR) (Choy et al., 2014). D620N VPS35 disturbs GluR1 recycling and causes an impairment of glutamatergic neurotransmission in iPSC-derived dopaminergic neurons from PD subjects harboring a D620N mutation and in mouse cortical neuronal cultures (Munsie et al., 2015). AMPA receptors, but also β2AR and NMDA receptors, are reduced by *VPS35* gene silencing in cultured neurons or hippocampal slices (Choy et al., 2014). Similar observations reveal a decrease in GluR1 and GluR2 receptors in primary neuronal cultures from *VPS35*-deficient mice (Tian et al., 2015). *VPS35* deficiency in mice also impairs the maturation of dendritic spines that can be partially rescued by overexpression of the GluR2 receptor (Tian et al., 2015). Together, these studies suggest that D620N VPS35 causes the mislocalization of cell surface receptors and transporters involved in neurotransmission, including dopaminergic neurotransmission that is impaired in PD.

IV) VPS35 and LRRK2 may operate in a common pathway

A number of common pathologic pathways are emerging that functionally connect gene products linked to familial PD. *LRRK2* or *VPS35* mutations have similar effects on the autophagy-lysosomal pathway in different models suggesting a common molecular defect. The overexpression of G2019S LRRK2 or D620N VPS35 similarly lead to reduced sorting of CI-M6PR to lysosomes and the TGN, and both mutant proteins impair neurite outgrowth in rat primary neuronal cultures (MacLeod et al., 2013). G2019S LRRK2 overexpression is also reported to induce a retromer deficiency in mammalian cells and mouse brain where it leads to reduced levels of VPS35, VPS26 and VPS29 proteins. WT VPS35 expression can rescue G2019S LRRK2-induced neurite defects in neuronal cultures, whereas D620N VPS35 cannot (MacLeod et al., 2013). In *Drosophila*, WT VPS35 overexpression rescues the motor deficits and reduced lifespan induced by the overexpression of PD-linked LRRK2 mutations (Y1699C, I2020T) (Linhart et al., 2014). These two studies suggest that the D620N mutant impairs the neuroprotective function of VPS35, and places VPS35 and

retromer function downstream of mutant LRRK2-mediated neurotoxicity. Alternatively, a recent study has demonstrated that D620N VPS35 expression in KI mice induces the hyperactivation of LRRK2 in cells and tissues, as revealed by increased LRRK2-dependent Rab10 phosphorylation at Thr73 in lung, spleen, kidney and brain (Mir et al., 2018; Nguyen et al., 2020). Similarly, primary neutrophils and monocytes derived from PD subjects with a *D620N* mutation reveal elevated LRRK2 kinase activity compared to control or idiopathic PD subjects (Mir et al., 2018). In mouse embryonic fibroblasts (MEF) derived from *D620N VPS35* KI mice, Rab10 phosphorylation was increased to a greater extent (~6-fold) than in MEFs from *R1441C* or *G2019S LRRK2* KI mice (2–4-fold) (Mir et al., 2018). The contribution of LRRK2 to the elevated Rab10 phosphorylation in *VPS35* KI tissues or cells was confirmed by LRRK2 gene silencing or kinase inhibition using MLi-2 (Mir et al., 2018). This study provides compelling evidence that VPS35 lies upstream of LRRK2 in a common pathway, and suggests that VPS35 may modulate LRRK2 activity through a direct interaction with LRRK2 or indirectly via an unknown mechanism. At present, there is evidence that mutant LRRK2 may operate by inducing a retromer deficiency, and that mutant VPS35 induces LRRK2 hyperactivation, and it remains unclear whether these mechanisms are compatible or mutally exclusive events. Nevetheless, the activation of LRRK2 in *VPS35*-linked PD has therapeutic implications.

V) Therapeutic strategies targeting VPS35 and the retromer

The role of VPS35 and the retromer in neurodegenerative disease has only emerged in recent years, and as such therapeutic molecules and strategies are now beginning to be identified and validated using cellular and preclinical animal models. Unlike some PD-linked gene products such as LRRK2, parkin or PINK1, retromer subunits lack enzymatic activity but instead serve as protein scaffolds with the capacity to recognize and bind distinct cargo and vesicular membranes. In addition to these molecular properties, the D620N mutation in VPS35 tends to act in a subtle manner with evidence for both a gain-of-function and a partial dominant-negative mechanism of action. However, pathological effects induced by D620N VPS35 in cell and animal models are often similar but clearly distinct from those produced in *VPS35* KO or knockdown models (X. Chen et al., 2019; Tang, Erion, et al., 2015; Tang, Liu, et al., 2015). Studies in cells and rodent brain indicate that D620N VPS35 does not alter the assembly or stability of the retromer complex, as the levels of the VPS26 and VPS29 subunits and their interactions with VPS35 remain normal (X. Chen et al., 2019; J. Follett et al., 2014; Munsie et al., 2015; Tsika et al., 2014). Also, D620N VPS35 does not robustly alter overall retromer function, as the sorting of cargos such as CI-M6PR, sortilin-1 and SorLA are unaltered in PD patient-derived D620N primary fibroblasts or rodent primary cortical neurons (Tsika et al., 2014). However, D620N VPS35 expression can subtly alter cargo sorting, including CI-M6PR, AMPA receptor subunits, LAMP2A, ATG9A and DLP-1, depending on the cellular model being used (Jordan Follett et al., 2014; Munsie et al., 2015; Tang, Erion, et al., 2015; W. Wang et al., 2016; Zavodszky et al., 2014). Whether this abnormal cargo sorting is necessary and sufficient to drive neuronal damage and degeneration remains to be demonstrated in PD-relevant neuronal populations and animal models. Likewise, the D620N mutation impairs the interaction of VPS35 with the WASH complex in human cell lines but whether this defect is maintained or relevant in brain cells is not known (X. Chen et al., 2019; McGough et al., 2014; Zavodszky et al., 2014). What

is also unclear at this juncture is whether distinct neuronal or glial cell populations contain specialized or novel retromer cargo, or have an altered dependency on the WASH complex or even specific retromer subunits (i.e. VPS26A versus VPS26B retromer complexes), compared to what is currently known from studies in yeast or mammalian cell lines. Recent Cryo-EM data has revealed that the retromer can assemble into dimers and multimers, and the D620 residue is localized at the dimer interface between retromer monomers (Kendall et al., 2020; Kovtun et al., 2018). Understanding whether the D620N mutation can disrupt retromer function by altering its capacity to multimerize around endosomal tubules would be an important next step. If this effect is confirmed, an approach aimed at stabilizing retromer dimers using small molecules could prove beneficial for PD subjects harboring the D620N mutation.

A potential role for non-mutated VPS35 in sporadic PD is poorly defined. While it is unclear at present whether VPS35 protein levels are altered in brain tissue from sporadic PD subjects due to conflicting reports (Tsika et al., 2014; Zhao et al., 2018), VPS35 mRNA expression was reduced in laser-captured dopaminergic neurons from the substantia nigra of PD subjects whereas *VPS35* deletion in mice can also recapitulate PD-relevant pathology (MacLeod et al., 2013; Tang, Erion, et al., 2015). In support of a potential neuroprotective role, the overexpression of VPS35 extended the lifespan of flies exposed to MPP^+-induced neurotoxicity or transgenic flies overexpressing mutant LRRK2 (Linhart et al., 2014). Lentiviral vectors expressing VPS35 delivered to the hippocampus of human WT α-synuclein transgenic mice provided neuroprotection in this model, and also reduced α-synuclein accumulation in primary cortical neurons induced by α-synuclein pre-formed fibrils (Dhungel et al., 2015). In contrast, the overexpression of WT VPS35 using AAV2/6 vectors delivered directly to the substantia nigra induced an intermediate level of dopaminergic neuronal degeneration in adult rats, compared to D620N VPS35, suggesting that VPS35 gain-of-function is neurotoxic (Tsika et al., 2014). These studies might suggest a therapeutic window exists for the benefical effects of VPS35 overexpression in animal models. Additional preclinical studies are required in animal models to evaluate the impact of different species, neuronal populations, viral vectors, viral titer, and promoter strength and specificity, in eliciting the neuroprotective versus detrimental effects resulting from VPS35 overexpression. Since retromer subunits are reduced in affected brain regions of distinct neurodegenerative diseases (Muzio et al., 2020; Small et al., 2005; Vagnozzi et al., 2019), strategies to restore functional retromer would be of interest for attenuating neuropathology, as was recently demonstrated in tauopathy models (Vagnozzi et al., 2019).

In addition to restoring individual retromer subunits, an alternative strategy based on stabilizing the retromer complex has been developed. Pharmacological chaperones have been identified that stabilize the retromer and increase its overall levels. The chemical chaperones, R55 and R33, are reported to elicit neuroprotection in mouse models of AD or ALS and in human iPSC-derived neurons (Muzio et al., 2020; Young et al., 2018). R55 and R33 were identified to bind to the VPS35-VPS29 interface and increase retromer stability, which reduced the accumulation of Aβ in mouse primary neuronal cultures as well as reducing the accumulation of phosphorylated tau in human iPSC-derived neurons (Mecozzi et al., 2014; Young et al., 2018). A recent study developed compound 2a, a retromer chaperone based upon the chemical structure of R55/R33, that also binds at

the VPS29-VPS35 interface and exhibits improved blood brain barrier permeability, that revealed promising neuroprotective effects in a G93A SOD1 transgenic mouse model of ALS (Muzio et al., 2020). While chemical chaperones targeting the retromer appear promising for attenuating neurodegeneration, additional studies are required to confirm the specificity of these compounds as well as their effects on peripheral organs given that VPS35 is broadly expressed.

Retromer chaperones await evaluation in animal models of PD, such as LRRK2 or α-synuclein models where retromer deficiency might play an important role (Dhungel et al., 2015; MacLeod et al., 2013). It is unclear whether retromer chaperones will prove effective in *D620N VPS35* animal models given that this mutation does not result in an obvious retromer deficiency (X. Chen et al., 2019). Would stabilizing a mutant retromer complex further magnify its pathogenic effects including further enhancing LRRK2 activation in these models? While a better understanding of how or whether LRRK2 activation contributes to neuropathology in *D620N VPS35* KI mice is now required, therapeutic strategies aimed at inhibition of LRRK2 kinase activity or the CNS-restricted reduction of LRRK2 expression could be of interest for treating *VPS35*-linked PD subjects. For example, clinical trials are underway with the intrathecal delivery of the LRRK2 antisense oligonucleotide BIIB094 developed by Biogen/Ionis, or the LRRK2 kinase inhibitors DNL151 and DNL201 developed by Denali Therapeutics in sporadic and *G2019S LRRK2* PD subjects. Future preclinical studies will aim to establish whether LRRK2 plays a pivotal role in mediating the neurodegenerative phenotypes that develop in *D620N VPS35* animal models.

VI) Conclusion

Since the first identification of the D620N mutation in *VPS35* as a cause of late-onset, autosomal dominant familial PD almost 10 years ago (Vilarino-Guell et al., 2011; Zimprich et al., 2011), a number of mechanisms of neuronal toxicity have been nominated that largely involve the abnormal sorting of different retromer cargo implicated in a range of cellular pathways. However, a number of important questions remain to be addressed, such as whether the impaired sorting of one or more specific cargo is sufficient to induce neuronal death or is critical for the survival of dopaminergic neurons. Depending on the specific nature of this cargo sorting defect in particular neuronal populations, it remains unclear whether *VPS35* mutations primarily influence the autophagy-lysosomal pathway, mitochondrial function or the sorting of receptors to dendrites, and whether these cellular events are interrelated. In addition, the mechanisms and contribution of LRRK2 hyperactivation to the pathogenic effects of *D620N VPS35* remain to be further elucidated. *D620N VPS35* could conceivably induce a specific activation of LRRK2 that is distinct from the mechanisms of familial *LRRK2* mutations, such as through altering LRRK2 protein interactions and complexes, membrane occupancy or location, and/or access to its substrates. Confirmation of LRRK2-dependent neuronal toxicity as the major pathogenic mechanism of *VPS35* mutations would provide important rationale for the use of LRRK2-directed therapeutics in *D620N VPS35* PD subjects. In addition to pathology induced by *D620N VPS35* in familial PD, the observation that *VPS35* and retromer deficiency are linked to AD, different tauopathy and ALS brains supports restoring VPS35 expression by gene therapy or

retromer stabilization using chemical chaperones as promising strategies for treating these neurodegenerative diseases. Whether such strategies would be beneficial for treating *D620N VPS35* or sporadic PD subjects requires further investigation, especially given that D620N VPS35 is a mostly functional protein. The identification of *VPS35* mutations as a cause of familial PD have provided important new insight by highlighting a role for the retromer complex and endosomal sorting pathways in disease pathophysiology. Additional studies are required to further clarify the mechanism-of-action of familial mutations and the potential role of LRRK2 in *VPS35*-linked PD.

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