NeuroMolecular Medicine
https://doi.org/10.1007/s12017-023-08738-1

REVIEW

The Ubiquitin Proteasome System as a Therapeutic Area in Parkinson's Disease

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Received: 10 November 2022 / Accepted: 28 January 2023

Abstract

Parkinson's disease (PD) is the most common neurodegenerative movement disorder. There are no available therapeutics that slow or halt the progressive loss of dopamine-producing neurons, which underlies the primary clinical symptoms. Currently approved PD drugs can provide symptomatic relief by increasing brain dopamine content or activity; however, the alleviation is temporary, and the effectiveness diminishes with the inevitable progression of neurodegeneration. Discovery and development of disease-modifying neuroprotective therapies has been hampered by insufficient understanding of the root cause of PD-related neurodegeneration. The etiology of PD involves a combination of genetic and environmental factors. Although a single cause has yet to emerge, genetic, cell biological and neuropathological evidence implicates mitochondrial dysfunction and protein aggregation. Postmortem PD brains show pathognomonic Lewy body intraneuronal inclusions composed of aggregated α-synuclein, indicative of failure to degrade misfolded protein. Mutations in the genes that code for α-synuclein, as well as the E3 ubiquitin ligase Parkin, cause rare inherited forms of PD. While many ubiquitin ligases label proteins with ubiquitin chains to mark proteins for degradation by the proteasome, Parkin has been shown to mark dysfunctional mitochondria for degradation by mitophagy. The ubiquitin proteasome system participates in several aspects of the cell's response to mitochondrial damage, affording numerous therapeutic opportunities to augment mitophagy and potentially stop PD progression. This review examines the role and therapeutic potential of such UPS modulators, exemplified by both ubiquitinating and deubiquitinating enzymes.

Keywords Parkin · Parkinson's disease · Mitophagy · Neurodegeneration · Ubiquitin · Mitochondria

Abbreviations

PD	Parkinson's disease
Ub	Ubiquitin
UPS	Ubiquitin–proteasome system
DUB	Deubiquitinase
USP	Ubiquitin-specific protease
COMT	Catechol-o-methyl transferase
TK	Tyrosine Kinase
SIRTs	Sirtuins
GLP-1	Glucagon-like peptide-1
MAO B	Monoamine oxidase B
LRRK2	Leucine rich repeat kinase 2
PROTAC	Proteolysis targeting chimera
AUTAC	Autophagy targeting chimera
OMM	Outer mitochondrial membrane
PINK 1	PTEN-induced kinase 1
pSer^{65}-Ub	Ubiquitin phosphorylated at its Serine-65 position
GDNF	Glial derived neurotrophic factor

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Published online: 05 February 2023

CDNF	Cerebral dopamine neurotrophic factor
mAb	Monoclonal antibody

Background

Parkinson's disease (PD) is a devastating illness with no cure. Its complex etiology is linked by genetic evidence to the mitochondrion, whose response to stress can be overwhelmed, resulting in the lethal accumulation of damaged mitochondria in neuronal cells of the brain (Clark et al., 2021). This response utilizes the two principal mechanisms of protein degradation in cells, proteasomal degradation and lysosomal autophagy, and numerous potential therapeutic targets for PD that are components of these pathways have been identified. The prevalence of PD and related neurodegenerative diseases is increasing as the population ages, commensurate with a diminished capacity to respond to various proteinopathies and the resultant sequelae that lead to neuronal cell death. Currently approved PD drugs, most aimed at increasing dopamine content or activity in the basal ganglia, treat PD symptoms, but not the underlying causes of neurodegeneration, which remain uncertain. These drugs are most effective in the initial stages of the disease, but to date, none can stop disease progression. Clinical trials of new PD experimental therapeutics include drugs that have the potential to halt or reverse the disease rather than to merely ameliorate symptoms. Various novel mechanisms of action are employed by these new drugs. In the preclinical arena, the reversal of PD is being attempted by modulating these mechanisms with experimental agents. The ubiquitin proteasome system (UPS) is involved in several aspects of the cellular response to mitochondrial damage, and various components of the UPS can, in theory, be treated with small molecules to augment mitophagy and stop PD progression. This review presents the landscape of PD therapies, including currently approved therapeutics and examines the role and therapeutic potential of UPS modulators as potential disease-modifying agents.

Combating Parkinson's Disease

Current State of Treatment for PD—Approved Drugs

PD is the second most common progressive neurodegenerative disorder, affecting between 6 and 10 million individuals worldwide including ~ 1 million in the US (Yang et al., 2020). PD is characterized clinically by a wide range of motor symptoms including tremor, rigidity, bradykinesia, and postural instability and a series of non-motor symptoms such as depression, cognitive impairment, constipation, and hyposmia (diminished sense of smell). PD is characterized neuropathologically by the loss of dopaminergic neurons in the *substantia nigra pars compacta*, resulting in decreased dopaminergic neurotransmission via projections to the caudate and putamen, which are necessary for normal movement. The other neuropathological hallmark of PD is the presence of intracellular inclusions (Lewy bodies) in surviving dopaminergic neurons (Wakabayashi et al., 2013). While there remains some debate about whether Lewy bodies are a cause or a consequence of PD, genetic and cell biological evidence suggests that the process of Lewy body formation is coupled to neurodegeneration via disruption of mitochondrial function and other key cellular functions (Mahul-Mellier et al., 2020). PD is considered a multifactorial disease; both genetic and environmental factors contribute to its etiology. Approximately 10% of PD cases are inherited; the remaining cases are idiopathic. There is currently no drug available that can cure PD or stop its progression. Current PD therapies, focused primarily on increasing the level of dopamine within the brain, include drug combinations, *e.g.*, levodopa/carbidopa, designed for this purpose (Table 1).

Although effective in relieving some PD symptoms initially, these drugs become less effective as the disease progresses, and patients develop more severe motor symptoms and often non-motor complications (Goldman & Guerra, 2020; Rascol et al., 2003). Dopamine agonists, monoamine oxidase and COMT inhibitors, deep brain stimulation, and spinal cord stimulation are some of the alternative therapies available.

Recently, a drug with a novel mechanism of action was approved for PD; in August, 2019, the FDA approved Kyowa Hakko Kirin's adenosine A_{2A} receptor antagonist Nourianz®/istradefylline as an add-on treatment to levodopa for idiopathic PD with "OFF" episodes, *i.e.*, the occurrence of symptoms between regular doses (Chen & Cunha, 2020). A_{2A} receptors are located close to dopamine receptors, and adenosine acts as a neuromodulator, coordinating responses to dopamine and other neurotransmitters in regions of the brain governing motor function, mood, and learning and memory (Dunwiddie & Masino, 2001). Selective A_{2A} receptor antagonists enhance the therapeutic effects of L-DOPA and reduce movement abnormalities resulting from long-term L-DOPA treatment. Thus, A_{2A} receptor antagonists have potential advantages over the accepted standard treatments for Parkinson's disease (Shook & Jackson, 2011). It should be noted, however, that, while its mode of action is novel, Nourianz® is an adjuvant treatment that modulates the effects of dopamine therapy to ameliorate symptoms of PD, not to effect a cure. In addition, non-motor symptoms of PD can be treated with NMDA antagonists (Vanle et al., 2018).

Because of the neurodegenerative nature of PD, the purely symptomatic treatment afforded by current therapies is inadequate. By the final stage of PD, patients become

Table 1 Current approved therapies

#	Drug	Mechanism	Brand names	References
1	Levodopa, Carbidopa-levodopa	A dopamine precursor, often prescribed with carbidopa to prevent premature conversion leading to side-effects	Sinemet, Rytary (extended release), Duopa (continuous infusion)	Hauser (2009)
2	Dopamine agonists	Mimics dopamine, stimulating similar effects in the brain	Mirapex, Requip, Neupro (patch), Apokyn (rapid-acting injectables)	Carbone et al. (2019)
3	MAO B inhibitors	Prevents breakdown of brain dopamine via inhibition of monoamine oxidase B, which metabolizes dopamine	Eldepryl, Zelapar, Azilect	Ozdemir et al. (2021)
4	Catechol O-methyltransferase (COMT) inhibitors	Prolongs the effect of Levodopa by blocking COMT, an enzyme that breaks down dopamine	Comtan	Leung et al. (2021)
5	Anticholinergics	Controls tremors via inhibition of acetylcholine activity	Cogentin, Artane	Katzenschlager et al. (2003)
6	Acetylcholinesterase inhibitor	Reduces cholinergic deficits and may improve cognitive function	Rivastigmine (Exelon)	Patel and Gupta (2022)
7	Amantadine	Acts as a receptor antagonist for muscarinic acetylcholine	Symmetrel, Gocovri, Osmolex ER	Rascol et al. (2021)
8	Selective A_{2A} receptor antagonists	Facilitates dopamine receptor signaling, normalizing motor function in animal models of dopamine dysregulation	Nourianz	"Istradefylline (Nourianz) for Parkinson's disease" (2020)
9	Norepinephrine prodrug	To treat light-headedness, dizziness, and prevent falls	Droxidopa	Kulshreshtha et al. (2020)

completely dependent upon caregivers, placing an enormous burden on society. It is estimated that for PD in the US, the total cost includes $25.4 billion in direct medical costs and $26.5 billion in indirect and non-medical costs, *e.g.*, caregiver and disability income costs (Yang et al., 2020). Several new therapeutic agents are in the clinical development for PD, however in this review we will focus on novel therapeutic targets from the UPS with the potential to develop disease-modifying agents.

Mitochondrial Health in PD; New Drugs from the Ubiquitin Proteasome System?

Mitochondrial dynamics, a complex mechanism for maintaining a healthy mitochondrial population in cells, entails fission, fusion, transport, and mitophagy (Cerri & Blandini, 2019). All these mechanisms are affected by cell metabolism (Bozi et al., 2020). Dysregulation of mitochondrial homeostasis has been implicated in neurodegenerative diseases, including PD (Clark et al., 2021). Mitochondrial dysfunction has been regarded as a leading candidate cause of idiopathic PD, particularly since an inhibitor of mitochondrial complex I, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), was found to be the cause of acute permanent parkinsonism in illicit drug users inadvertently self-administering MPTP in a batch of synthetic heroin, nearly 40 years ago (Langston, 2017). Mitochondrial toxins have also been shown to induce PD-like neurodegeneration in animal models (Chanthammachat & Dharmasaroja, 2019) and abnormal mitophagy has been observed in genetic models of PD (Dagda et al., 2009). In addition, genetic evidence from mitochondrial macromolecular complexes and mitochondrial DNA (mtDNA) from dopaminergic neurons of elderly PD patients further implicates mitochondrial dysfunction in neurodegeneration (Bury et al., 2017; Grunewald et al., 2016), although the precise role of mtDNA in PD is still somewhat unsettled (Muller-Nedebock et al., 2019).

Defective mitochondria are primary sources of reactive oxygen species, which produce the types of damage seen in brains of PD patients (Murphy, 2009; Stefanatos & Sanz, 2018). Dopaminergic neurons of the *substantia nigra* require high energy, making them vulnerable to degeneration upon mitochondrial dysfunction (Mamelak, 2018). Finally, mitochondrial dysfunction has been identified as a link between aging and PD pathogenesis (Hou et al., 2019).

Further compelling evidence that mitochondrial dysfunction can cause PD emerged from genetic studies of families with Mendelian patterns of inherited PD. Homozygous loss-of-function mutations in the genes

encoding PTEN-induced kinase 1 (PINK1) and the E3 ubiquitin ligase Parkin cause, with very high penetrance, loss of dopamine neurons in the substantia nigra and motor symptoms clinically indistinguishable from idiopathic PD, except that the onset of symptoms usually occurs at earlier age compared with typical PD (Kitada et al., 1998; Valente et al., 2004). While studies in *Drosophila* suggest PINK1 functions upstream of Parkin, basal PINK1 activity is reduced in the brains of Parkin-deficient mice, suggesting that their activities are mutually coupled (Clark et al., 2006; Park et al., 2006; Yang et al., 2006). As detailed below, the findings that PINK1 and Parkin function in the same pathway, converging on ubiquitin-mediated regulation of mitochondrial health, and that human loss-of-function mutations in either PINK1 or Parkin cause early onset forms of PD, provide strong rationale for developing PD therapeutics targeting this pathway. Thus, augmentation of the clearance of damaged mitochondria through mitophagy is seen as a potential therapeutic avenue for PD and other forms of neurodegeneration (Liu et al., 2019).

A promising means of achieving this outcome is to modulate enzymes of the ubiquitin proteasome system (UPS) that add or remove ubiquitin from mitochondrial substrates, thereby activating or deactivating mitophagy. Before considering the particulars of this strategy, it is useful to review the role of the UPS in mitochondrial function and CNS disease.

The UPS—in Neurodegenerative Disease

The UPS—a rich source of therapeutic targets. The cellular content of a protein is regulated by a combination of its synthesis and degradation rates to ensure proper cell function. Extracellular or membrane-associated proteins are generally transported to lysosomes by the Golgi-endosomal apparatus, where they are degraded. Soluble cytoplasmic proteins are degraded in a regulated fashion by the Ubiquitin (Ub)-proteasomal degradation pathway, also known as the Ub Proteasome System (UPS) (Lee & Goldberg, 1998) (Fig. 1).

Ub, a small (76 amino acids) polypeptide is covalently attached to the lysine side chains of many proteins by the coordinated action of three enzymes—E1 activating enzymes, E2 conjugating enzymes, and E3 ubiquitin ligases (Pickart, 2001; Wilkinson, 2000). A poly-Ub tag is synthesized by the sequential addition of new Ub molecules to the lysine-48 (K48) of the previous Ub. Proteins tagged with K48 poly-Ub are delivered to the proteasome, which degrades them. Ub chains of various lengths and spatial configurations can be formed at other lysines on the Ub molecule (K6, K11, K27, K29, K33, K63) or the N-terminal methionine (M1), giving rise to a multitude of poly-Ubs with characteristic physiological roles governing a protein's cellular content, location, or activity (Trempe, 2011). Ub is cleaved from substrates by deubiquitinating enzymes (DUBs), primarily Ub-specific proteases (Reyes-Turcu et al., 2009). Protein ubiquitination and deubiquitination can serve non-degradative cellular regulatory purposes including

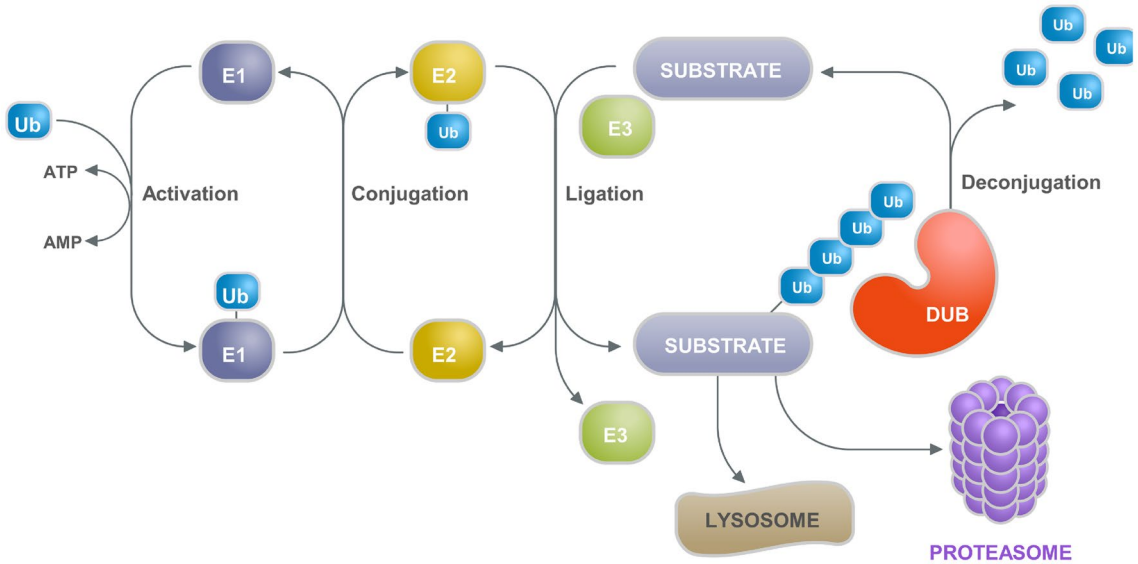

Fig. 1 The Ub Proteasome System (UPS) E1 activates Ub linked to ATP hydrolysis. Activated Ub (Ub-E1) is conjugated to Ub conjugating enzyme E2, and Ub-E2 then interacts with an E3 ligase/substrate complex, facilitating transfer of Ub to the substrate. The substrate conjugated with multiple Ub molecules is subsequently degraded by the proteasome or lysosome. In the opposing reaction, Ub proteases (DUBs) remove Ubs from polyubiquitinated substrates, thereby sparing the substrate protein from degradation or some other Ub-dependent cellular fate

compartmentation (Sommer et al., 2001), endoplasmic-reticulum-associated protein degradation (ERAD)/membrane recycling/endosomal sorting (Preston & Brodsky, 2017), and protein activation/deactivation (Peng et al., 2020). Given the scope of protein regulation in cells by elements of the UPS and the opportunity for selective intervention afforded by the multiplicity of E3 ligases and DUBs, it is not surprising that the entire UPS is being mined for potential therapeutic agents. All Ub pathway mechanisms are temporally and spatially controlled with strict substrate specificity. Of the approximately 600 E3 ligases and 80–100 DUBs identified, many have been linked with disease (Rape, 2018), including neurodegenerative diseases (Fritsch et al., 2019). Thus, enzymes of the UPS have been viewed as promising therapeutic targets for many neurodegenerative diseases (Huang & Dixit, 2016; Schmidt et al., 2021).

Modulators of Therapeutic Targets from the UPS

Therapeutic agents acting on targets from the UPS fall into three major classes: modulators of the proteasome; modulators of Ub conjugating enzymes (E3 ligases); and modulators of Ub deconjugating enzymes (DUBs).

The proteasome. Proteasome activity is a major mechanism employed by cells to regulate their protein content and to clear misfolded, damaged proteins. The proteasome is a large protein complex containing proteases that eliminate Ub-tagged proteins by hydrolyzing their peptide linkages. The proteasome binds the poly-Ub tagged protein to be degraded, removes the poly-Ub chains, unfolds the protein and passes it through the sites of proteolysis, leading to hydrolysis of the protein and release of small peptides (Voges et al., 1999). The proteasomal proteases are prime therapeutic targets (Park et al., 2020) and the proteasome inhibitor bortezomib/Velcade was approved for cancer treatment (multiple myeloma and similar cancers) in 2004. Several other proteasome inhibitors, including carfilzomib/Kyprolis and Ixazomib/Ninlaro, have subsequently been approved (Fricker, 2020; Robak & Robak, 2019).

E3 Ligases. E3 ligases are a family of enzymes that facilitates the transfer of Ub from an E2 enzyme to a specific protein substrate (Fang & Weissman, 2004) (Fig. 1). Approximately 600 E3 ligases have been identified, and many of them are associated with disease pathologies (Rape, 2018); selective modulators of these E3 ligases have been pursued for development as therapeutic agents for treating cancer and other diseases, including neurodegenerative diseases (Masumoto & Kitagawa, 2016; Tomoshige et al., 2017). Despite considerable effort, very few E3 modulators have entered clinical trials—Nutlin-3A, an antagonist of MDM2-p53 binding (Zhang et al., 2015), serdemetan, an inhibitor of E3 ligase and activator of p53 (Tabernero et al., 2011), GDC-0152, an inhibitor of an IAP E3 ligase (Flygare et al., 2012), and NX-1607, a Cbl-b inhibitor (A Study of NX-, 1607 in Adults With Advanced Malignancies, 2021)—and none of these has yet been approved by the FDA. After their approval for cancer treatment in combination with other agents, the IMiD (immunomodulatory) drugs thalidomide and related molecules lenalidomide and pomalidomide were shown to bind to cereblon, the substrate-recognizing subunit of cullin-4 type E3 ligase (Ito et al., 2010). Revlimid and other IMiDs work as molecular glues by promoting the interaction of the E3 ligase to its target proteins, the transcription factors Ikaros (IKZF1) and Aiolos (IKZF3), which control the cancer by immune stimulation (Ito & Handa, 2020). IMiDs selectively ubiquitinate these targets, leading to their degradation by the proteasome.

DUBs. DUBs are a family of approximately 80 cysteine proteases that reverse the action of E3 ligases by catalyzing the hydrolytic removal of conjugated Ubs (Fig. 1). Like E3 ligases, they are major regulators of cellular proteins and are associated with multiple pathological states by genetic and biochemical studies (Antao et al., 2020). Although potent and highly selective modulators of several physiologically important DUBs have been reported, no DUB inhibitor has been approved by the FDA, and only one, VLX1570, an inhibitor of proteasome-associated DUBs, has advanced to clinical trial (Rowinsky et al., 2020).

The UPS in CNS Disease—Roles for the Proteasome, E3 Ligases, and DUBs

It has been known since the late 1980s that Ub is intimately associated with the brain and the central nervous system. The detection of Ub in the brain and cerebrospinal fluid by immunohistochemical visualization or biochemical methods is a hallmark of neurodegenerative disease pathology (Alves-Rodrigues et al., 1998; Petrucelli & Dawson, 2004). For example, PD is characterized by Lewy bodies, conspicuous inclusions containing both α-synuclein and Ub (Kordower et al., 2008). The DUB UCHL-1 is reduced in patients with Lewy body dementia (Barrachina et al., 2006). Neurofibrillary tangles of Tau protein in Alzheimer's disease contain Ub (Perry et al., 1987), and Ub intraneuronal inclusions in the *substantia nigra* are a feature of motor neuron disease with dementia (Al-Sarraj et al., 2002). Abnormal levels of Ub are found in cerebrospinal fluid in Creutzfeldt-Jakob disease (Manaka et al., 1992), and Ub-containing inclusions are often found in ALS (Ince et al., 1998). Thus, it seems reasonable to assume that the UPS contributes to the pathology of neuronal diseases. More recently, ubiquitination was recognized as an important component of postsynaptic function and plasticity (Mabb & Ehlers, 2010) as well as the cellular response to pathological protein aggregates (Harris & Rubinsztein, 2012).

At present, both proteasomal and non-proteasomal components of the UPS are recognized as potential therapeutic targets for various neuronal pathologies, including PD (Table 2). The core 20S proteasome has been shown to act in older neurons on neurodegeneration-relevant substrates such as α-synuclein and tau, and pharmacological activation of the 20S proteasome has been shown to accelerate removal of aggregation-prone proteins in some models (Opoku-Nsiah & Gestwicki, 2018). E3 Ligases are key regulators of critical regulatory proteins in the CNS, and some of these ligases have been associated with mitophagy and neurodegeneration (Harper et al., 2018; Koyano et al., 2019). Ub de-conjugation catalyzed by DUBs is also important in controlling pathways critical to neurodegeneration, including mitophagy (Chakraborty & Ziviani, 2020).

Current Therapeutic Targets from the UPS in PD and Other Forms of Neurodegeneration: The E3 Ligase Parkin and The DUB USP30

Genetic and cellular evidence accumulating over the past several years links both the etiology of PD and the mitophagic response to this disease with mitochondrial damage and the subsequent activation of the Ub E3 ligase Parkin and its translocation from the cytosol to the disease-stressed mitochondrion. Interactions among phosphorylation, ubiquitination, and deubiquitination of Parkin and its various substrate proteins are complex, involving several types of Ub chains, and the complete mechanism is not yet fully understood (Dikic & Bremm, 2014; Durcan & Fon, 2015a). Nevertheless, considerable effort has been expended to find UPS-based experimental therapies that could augment mitophagy (Clark et al., 2021; Truban et al., 2017). Among the more extensively characterized of these potential treatments are activators of Parkin (Ge et al., 2020) and inhibitors of the DUBs that oppose Parkin-mediated mitophagy such as USP30 (Bingol et al., 2014) (Table 2). The DUBs USP33 (Niu et al., 2020), USP8 (Durcan et al., 2014), and USP15 (Cornelissen et al., 2014) have also been associated with mitophagy by removing Ub from either mitochondrial proteins ubiquitinated by Parkin or Parkin itself (Dikic & Bremm, 2014).

E3 Ligase Parkin

Parkin is a RING-in-between-RING (RBR) E3 Ub ligase that consists of an N-terminal Ub-like (Ubl) domain and four zinc finger domains (RING0, RING1, IBR, and RING2). In addition, the linker between the IBR and RING2 domains (REP, repressor element of Parkin) acts as a regulatory element. Parkin's RING1 domain binds Ub-charged E2 enzymes and transfers Ub from the E2 to the active site cysteine (Cys431 in RING2) and subsequently to lysine on Parkin itself (autoubiquitination) or on a substrate protein (substrate ubiquitination) (Gladkova et al., 2018; Wauer et al., 2015). Parkin exists in an auto-inhibited 'off' state

Table 2 Examples of UPS components implicated in neurodegenerative disease

#	Target	Link to neurodegenerative disease	References
1	Proteasome		
	20S Proteasome	The catalytically active core subunit responsible for degradation of most cellular proteins and important for clearance of pathogenic proteins such as α-synuclein	Opoku-Nsiah and Gestwicki (2018),
2	E3 Ligases		
	Hrd-1/Synoviolin	ERAD ligase which promotes APP ubiquitination and degradation; downregulated in brain cells of Alzheimer's disease patients	Kaneko et al. (2010)
	MARCH5	Ubiquitinates mitochondrial outer membrane proteins with subsequent PINK1-mediated phosphorylation of ubiquitin as the initial step in Parkin recruitment and activation of mitophagy	Koyano et al. (2019)
	PARKIN	Recruited to dysfunctional mitochondria and activated by PINK1-mediated phosphorylation to promote mitophagy. Loss-of-function mutations in Parkin and PINK1 cause recessively inherited forms of PD	Ge et al. (2020)
3	DUBs		
	USP30	Negatively regulates mitophagy by reversing ubiquitination of mitochondrial outer membrane proteins	Bingol et al. (2014)
	USP33	Deubiquitinates Parkin, antagonizing its pro-mitophagy effects	Niu et al. (2020)
	USP8	Acts on multiple substrates and cell functions. Removes noncanonical K6-linked Ub chains from autoubiquitinated Parkin, but general effect of pharmacological intervention is unclear	Durcan and Fon (2015b)
	USP15	Negatively regulates mitophagy by reversing ubiquitination of mitochondrial outer membrane proteins	Cornelissen et al. (2014)

that is mediated by multiple intramolecular interactions; as a result, the active site Cys^{431} domain is occluded by the RING0-RING2 interactions, and the E2 binding site is blocked by Ubl-RING1 and REP-RING1 interactions (Wauer & Komander, 2013). Engineered point mutations that disrupt these inhibitory interactions robustly activate Parkin and facilitate its translocation to dysfunctional mitochondria (Trempe et al., 2013; Youle & Narendra, 2011). Depolarization of mitochondria with ionophores, such as CCCP or valinomycin, causes both accumulation of PINK1 on the outer mitochondrial membrane and PINK1-dependent phosphorylation of ubiquitin at serine 65 (pS65-Ub), which leads to the re-localization of cytosolic Parkin to the outer mitochondrial membrane and the dis-inhibition of the ubiquitin E3 ligase activity of Parkin, which promotes mitophagy to clear cells of dysfunctional mitochondria (Koyano et al., 2014; Narendra et al., 2008; Okatsu et al., 2012). PINK1 is apparently the only kinase that phosphorylates ubiquitin at serine 65, as evidenced by the absence of pS65-Ub in cells derived from PINK1-deficient animal models of PD (Barodia et al., 2019). Notably, the N-terminus of Parkin is homologous to ubiquitin and PINK1 also phosphorylates serine 65 in the ubiquitin-like domain of Parkin, increasing the E3 ubiquitin ligase activity of Parkin (Kondapalli et al., 2012). In functional mitochondria with normal membrane potential, the PINK1 content is negligible owing to proteasomal degradation upon mitochondrial import and processing of PINK1 (Yamano & Youle, 2013). Upon mitochondrial depolarization, PINK1 turnover ceases and the enzyme accumulates at the outer mitochondrial membrane (OMM) and phosphorylates serine-65 moieties of Ub conjugated to various OMM proteins as well as serine-65 present in the ubiquitin-like domain of Parkin. This "seed" ubiquitination of PINK1 substrates is likely catalyzed by various Ub ligases, one of which, March5 (known also as MITOL), has been described in detail (Koyano et al., 2019) (Fig. 2). Parkin is activated on damaged mitochondria by binding to pUb through its RING1 domain, resulting in a conformational change in RING1 and exposure of the Ubl domain for phosphorylation (pSer65) by PINK1 (Koyano et al., 2014). Phosphorylation of Parkin on its Ser65 moiety by PINK1 and binding of Parkin to phospho-Ser65-Ub (pSer65Ub) result in full activation of Parkin (Kondapalli et al., 2012).

Activated pSer65Ub-Parkin then ubiquitinates a variety of OMM proteins (Sarraf et al., 2013), including TOM20 and other TOM complex members, CISD1, VDAC1, and Mitofusin2. Parkin-mediated ubiquitination plays two roles in mitophagy. First, the complex poly-Ub tag containing phosphorylated Ub is a signal for mitophagy, wherein autophagosomes engulf the damaged mitochondria selectively (Lazarou et al., 2015). Second, some of the OMM proteins ubiquitinated by Parkin, including fusins, are degraded in the proteasome, with effects possibly including the removal of damaged mitochondrial fragments (Glauser et al., 2011).

Evidence suggests additional mitochondrion-related roles for Parkin and PINK1 in neuroprotection. Parkin overexpression was shown to be protective in dopaminergic neurons and PD models (Jiang et al., 2004; Lo Bianco et al., 2004). In addition to promoting mitochondrial biogenesis, Parkin exerts a pro-survival effect on neuronal cells by downregulating the cell death inducer AIMP2 (Lee et al., 2013).

Parkin and Neuroinflammation

Evidence suggests that neuroinflammation, the activation of the brain's innate immune system, plays a role in PD (Tansey & Goldberg, 2010). PD patients have abnormally high levels of pro-inflammatory cytokines in their cerebrospinal fluid. Microglia, the principal immune cells of the brain, are activated in PD, and the loss of microglial Parkin contributes to neuroinflammation (Dionisio et al., 2019). Elevated α-synuclein may activate microglia in PD models. Parkin/PINK1-mediated mitophagy has been shown to restrain innate immunity in vivo, and dysfunction in mitophagy leads to an inflammatory phenotype that ultimately may result in dopaminergic neuronal loss (Sliter et al., 2018). In addition, innate immunity is activated in patients with Parkin mutations. Parkin and PINK1 have also been shown to mediate mitochondrial antigen presentation and trigger adaptive immune responses through mitochondrial-derived vesicles (Matheoud et al., 2016). Parkin knockout increases vulnerability to inflammation-related nigral degeneration by LPS injections (Frank-Cannon et al., 2008). All these results suggest a cycle in which inflammation increases mitochondrial damage which, in turn, exacerbates the inflammatory process. Thus, augmenting Parkin activity may confer additional benefit in PD by preventing or reducing neuroinflammation. Other aspects of UPS involvement in neuroinflammation signaling are discussed in detail in a recent review (Schmidt et al., 2021).

Search for Parkin Activators

Genetic data support the development of Parkin activators for augmenting mitophagy. Parkin mutants with impaired auto-inhibition are recruited to mitochondria much faster than wild-type Parkin after treatment with the mitochondrial uncoupler CCCP (Trempe et al., 2013), suggesting that Parkin activation is a limiting step in the mitophagy pathway and that small molecule activators will not only turn on the enzymatic activity of Parkin, but also promote its recruitment to dysfunctional mitochondria. Recently, various human Parkin mutants, including pathogenic mutants such as G284R and R275W, have been characterized functionally using cell-based mitophagy assays

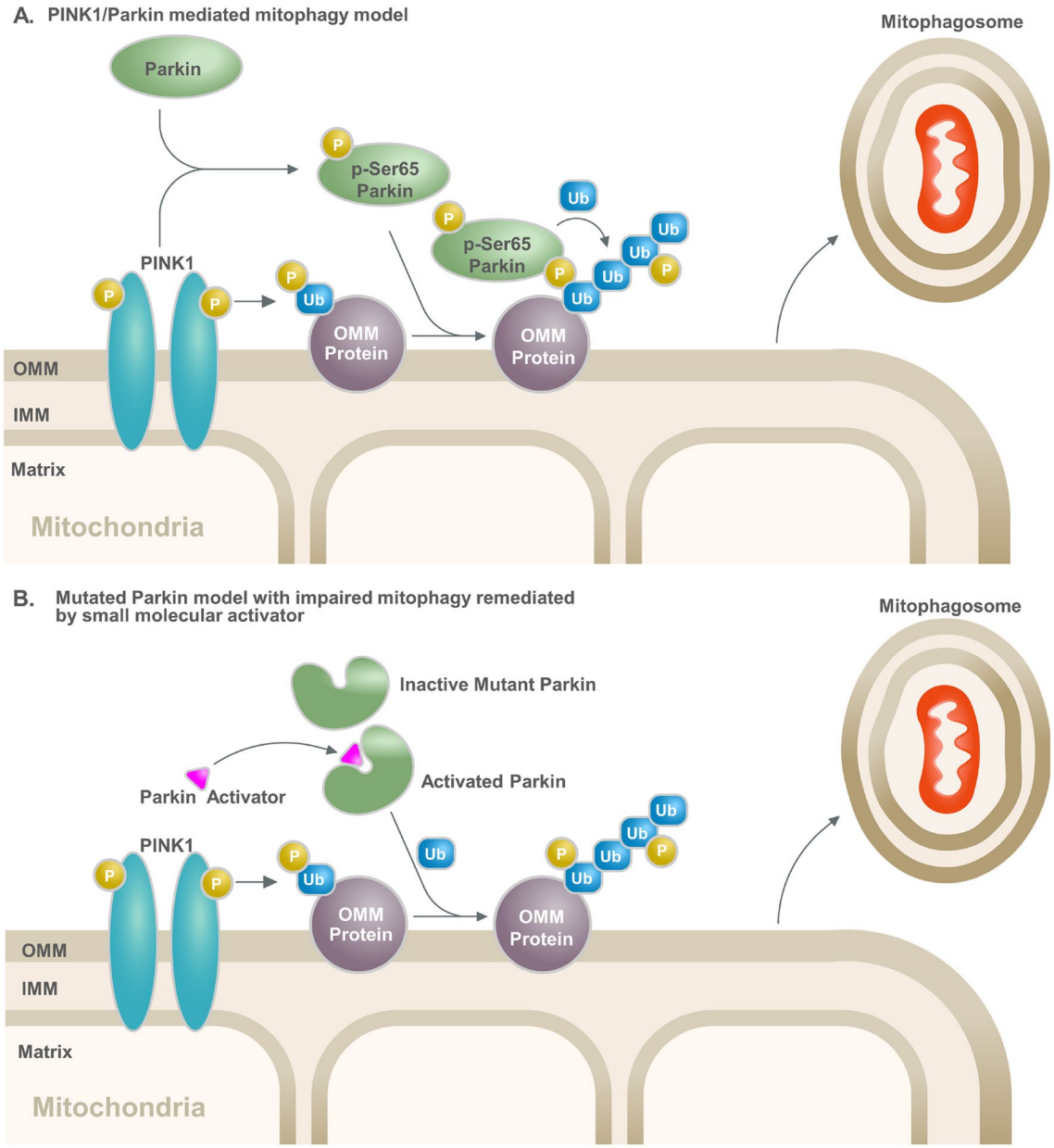

Fig. 2 Parkin activator therapeutic hypothesis Autoinhibited parkin is activated by PINK1 in dysfunctional mitochondria (**A**); this activation can also be achieved by small molecules (**B**). Activated Parkin promotes ubiquitination and PINK1-dependent phosphorylation of ubiquitination of OMM substrates. Recruitment of autophagy adapters (not depicted) leads to enhanced mitophagy. Ultimately, clearance of damaged mitochondria leads to dampened inflammation and prevention of neuronal death (see text for details)

(Yi et al., 2019). R275W disrupts pUb binding, keeping Parkin inactive (Ordureau et al., 2014). Co-expression of naturally occurring activating variants (V224A) as well as engineered activating mutants (W403A, F146A) in cells, however, can completely rescue the loss of Parkin activity resulting from human Parkin mutations causally linked to PD (Yi et al., 2019). Most importantly, naturally occurring Parkin variants that are 2–3 times more active than the wild-type protein have been observed in PD-free human subjects (Yi et al., 2019). This evidence strongly indicates that selective small molecule Parkin activators are likely to be safe.

A Parkin activator drug could be disease-modifying by intervening in the multipronged pathogenic mechanism leading to PD. While there has been considerable activity on the part of pharmaceutical companies and academic researchers to identify small molecule Parkin activators, this effort has so far not led to clinical candidates. In 2013, Regnstrom et al., described a surface plasmon resonance (SPR) based fragment screening method for identifying small molecules

that bind Parkin (Regnstrom et al., 2013). More recently, patents covering a series of Parkin activators have been published (Johnston and Garofalo, 2017; Springer et al., 2019). Most recently, Shlevkov et al., reported compounds that activated Parkin in in vitro biochemical assays (EC_{50} = 170 nM). However these compounds failed to activate Parkin in cell-based assays (Shlevkov et al., 2022). Parkin activator compounds capable of promoting mitophagy in cells and in vivo are necessary to validate the Parkin therapeutic hypothesis in preclinical PD models.

Protein Degrader Molecules—PROTACs

Ub E3 ligase activity is central to the recently developed PROTAC (PROteolysis TArgeting Chimera) therapeutic strategy. PROTACs are chimeric molecules consisting of binders of various E3 ligases tethered to binders of an intended cellular target protein to degrade the protein selectively in situ rather than inhibit it pharmacologically, thereby increasing efficiency and minimizing the development of resistance (Nalawansha & Crews, 2020). PROTACs developed to degrade anticancer targets have recently entered Phase I/II clinical trials (Mukhamejanova et al., 2020). PROTACs are also being developed for degrading pathogenic forms of the tau protein, a target for treating neurodegenerative diseases (Lu et al., 2018). Tau degraders that recruit Cereblon E3 ligase (FMF-06-038 and FMF-06-049) were shown to reduce pathogenic Tau more than 50% at 100 nM within 24 h in A152T and P301L tauopathy neuronal models (Wang et al., 2021).

A PROTAC strategy has also been applied to combat neuroinflammatory components of neurodegeneration. The Kelch-like ECH-associated protein 1 (Keap1)-Nuclear factor erythroid 2-related factor 2 (Nrf2) signaling pathway is important in the cellular defense system against oxidative stress by inducing antioxidant and anti-inflammatory effects (Choi et al., 2021). While activation of the Keap1 (E3 ligase) component of this pathway is beneficial in cell and in vivo models of PD (Kim et al., 2020), a PROTAC approach is also possible. Lu et al. utilized Keap1, a substrate adaptor protein for the E3 ligase involved in oxidative stress regulation, as a novel candidate for PROTACs that can degrade the tau protein. A peptide ligand resulting from their work showed strong in vitro binding to Keap1 and tau substrate, and it was subsequently validated in cell studies as a proteasome-directed degrader of tau (Lu et al., 2018).

Takahashi et al. described a PROTAC-like strategy for degrading fragments of damaged mitochondria (Takahashi et al., 2019). Autophagy targeting chimera 4 (AUTAC4), is a PROTAC-like chimeric molecule combining a ligand of an OMM protein linked to a guanine tag to induce Lys63-linked poly-ubiquitination, which will result in autophagic degradation.

DUBs Involved in Mitophagy

DUBs associated with mitochondria have been shown to negatively regulate mitophagy by reversing the ubiquitination of michondrial proteins and affecting Parkin's translocation to damaged mitochondria (Dikic & Bremm, 2014). Additional roles for this enzyme class in mitophagy have been described (Clark et al., 2021). Four DUBs (USP30, USP33, USP8, and USP15) have recently been the subject of translational studies in mitophagy and neurodegeneration. One of them, USP30, is an active developmental target, while therapeutic hypotheses have been proposed for the other three.

USP30. USP30 is an OMM-localized DUB that was recognized more than a decade ago to play a role in regulating mitochondrial morphology (Nakamura & Hirose, 2008). This protease was subsequently found to be a negative regulator of the Parkin/PINK1 mitophagy pathway (Bingol et al., 2014). Overexpression of USP30 reverses Parkin-mediated ubiquitination of proteins in damaged mitochondria (Liang et al., 2015) and blocks Parkin's ability to drive mitophagy, leading to accumulation of dysfunctional mitochondria. In contrast, knockdown of USP30 rescues the defective mitophagy caused by Parkin- or PINK1-deficiency (Bingol et al., 2014). Numerous substrates for deubiquitination by USP30 have been identified, and some of these act upstream of Parkin (Marcassa et al., 2018), suggesting that USP30 plays a multifaceted role in regulating mitophagy. It is clear from cellular studies combining chemical biology with genetic ablation that USP30 is an attractive therapeutic target that can be exploited to augment mitophagy in diseased neuronal cells (Marcassa et al., 2018). For example, Liang et al. (2015) reported that USP30 interacts with TOM20, a component of the TOM (**t**ranslocase of **o**uter **m**embrane) receptor complex, which imports mitochondrial precursor proteins synthesized in the cytosol. Some recent models postulate that in damaged mitochondria, activated PINK1 is associated with ubiquitinated TOM complex components. The ubiquitin tag on TOM20 is phosphorylated by PINK 1, leading to Parkin recruitment, and promoting mitophagy (TOM20 being an example of OMM proteins 1, Fig. 3). USP30 can block this process by removing ubiquitin from TOM20 (Rusilowicz-Jones et al., 2020).

A covalent cyano pyrrolidine inhibitor of USP30 (FT3967385/FT385, in vitro IC_{50} ~ 1 nM) was shown to antagonize deubiquitination of TOM20 and increase the accumulation of phosphorylated ubiquitin and the level of basal mitophagy, recapitulating results from cells lacking USP30 (Rusilowicz-Jones et al., 2020). However, FT385 inhibited USP6 at higher concentrations and a related compound was shown to have off-target effects in cells thereby limiting its utility as USP30 inhibitor (Phu et al., 2020). A more selective benzosulphonamide inhibitor Compound 39

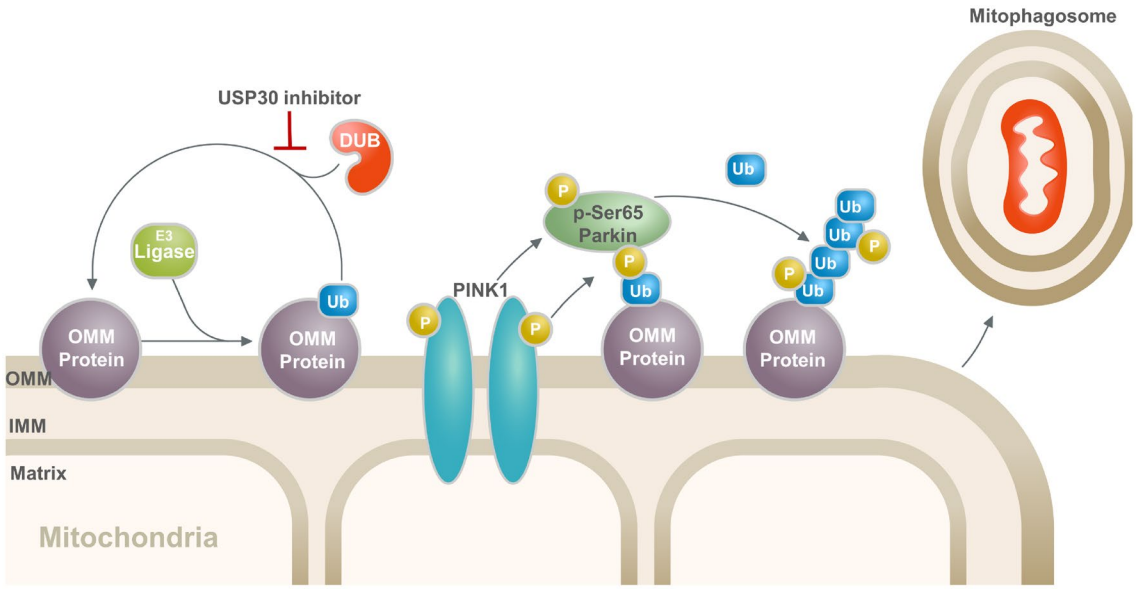

Fig. 3 Model for enhancement of PINK1/parkin-mediated mitophagy by a USP30 inhibitor At the OMM, PINK1, which accumulates on depolarized, dysfunctional mitochondria, phosphorylates Ub conjugated to one of multiple OMM proteins 1 by an E3 ligase (e.g., March5). The ubiquitination step is reversed by USP30, preventing PINK1 mediated phosphorylation and subsequent Parkin recruitment. Phosphorylated Ub (pSer65) recruits Parkin to the OMM and activates parkin. PINK1 phosphorylates Parkin at Ser65, and Parkin is further activated. In parallel, various OMM proteins 2 are ubiquitinated by activated Parkin, amplifying the cycle of ubiquitination by Parkin, phosphorylation by PINK1 and recruitment of more Parkin to the OMM. These proteins participate in the recruitment of autophagic vesicles and other steps leading to mitophagy [summarized in (Clark et al., 2021)]. In the absence of a USP30 inhibitor, the scheme is countered by USP30-mediated deubiquitination, and damaged mitochondria accumulate, leading to cell death

(CMPD-39) with an in vitro IC_{50} of ~ 20 nM was shown to promote mitophagy in SHSY5Y cells at 1 µM. COMPD-39 also rescued defective mitophagy in dopaminergic iNeurons (induced neurons) generated from PD patient derived fibroblasts (Rusilowicz-Jones et al., 2022). A racemic phenylalanine derivative ST-539 was also reported as a USP30 inhibitor that promoted mitophagy in HeLa cells stably expressing Parkin. Importantly, ST-539 showed an in vivo half-life of 3.85 h (C_{max} 1427 ng/mL) in mice and promoted mitophagy in cardiac tissue of mito-Keima expressing mice after dosing daily with 25 mg/kg via intraperitoneal injections (Luo et al., 2021). Notably, the compound did not promote mitophagy in the brain likely due to inadequate compound exposure (Luo et al., 2021). Thus, a potent, selective and brain penetrable inhibitor of USP30 would be expected to augment mitophagy in diseased neurons; in fact, various classes of small molecule USP30 inhibitors are currently in preclinical development for PD (Kluge et al., 2018). Several patent applications (for example, from Mission Therapeutics, Genentech, and Mitobridge Inc.) have been filed covering these potential PD therapeutics (https://www.uspto.gov/patents/search), although to our knowledge no clinical trials have been initiated.

USP33. USP33/VDU1 is localized to the OMM, where it interacts with and deubiquitinates auto-ubiquitinated Parkin (Niu et al., 2020). Cellular and in vitro assays have shown that USP33 removes K6, K11, K48, and K63-linked ubiquitin conjugates from Parkin, primarily at Lys435. USP33 knockdown increased both Parkin protein stabilization and its translocation to depolarized mitochondria, leading to the enhancement of mitophagy (Niu et al., 2020). Thus, inhibitors of USP33 could have therapeutic effect by rescuing Parkin ubiquitination.

USP8. USP8 was associated originally with endosomal trafficking (Row et al., 2006). Subsequent studies demonstrated that USP8 preferentially removes noncanonical K6-linked ubiquitin chains from Parkin, a process required for the efficient recruitment of Parkin to depolarized mitochondria (Sun et al., 2020). Durcan et al. (Durcan & Fon, 2015b; Durcan et al., 2014) reported that USP8 is required for the efficient recruitment of Parkin to depolarized mitochondria and subsequent mitophagy, preferentially removing K6-linked Ub conjugates from Parkin but having little effect on the ubiquitination or stability of known mitochondrial substrates of Parkin. In addition, recent work has shown that USP8-catalyzed deubiquitination of α-synuclein may reduce its lysosomal degradation, increasing its accumulation in Lewy bodies (Alexopoulou et al., 2016). Genetic screening and in vivo studies, moreover, have shown that USP8 inhibition can correct mitochondrial elements of the PINK1 knockout phenotype (von Stockum et al., 2019). Because of USP8's pleiotropic nature (Dufner & Knobeloch, 2019) and

seemingly contradictory effects observed in various studies, it is premature to propose a therapeutic hypothesis based on this DUB as a therapeutic target for PD.

USP15. A DUB having both potential clinical utility and pleiotropic cellular effects (Georges et al., 2021), USP15 was found to attenuate Parkin-linked mitophagy under conditions in which other DUBs or catalytically dead USP15 did not. In these studies, knockdown of USP15 restored mitophagy in fibroblasts of PD patients with Parkin mutations and diminished Parkin content (Cornelissen et al., 2014). USP15 does not deubiquitinate Parkin but, rather, reverses Parkin-mediated ubiquitination of mitochondrial protein substrates, and knockdown of the closest *Drosophila* homolog of USP15 rescues the mitochondrial and behavioral defects in Parkin RNAi flies (Cornelissen et al., 2014). Thus, while USP15, like USP8, can potentially affect many cellular substrates, no data reported to date would contra-indicate USP15 inhibition as a therapeutic strategy for PD.

Conclusions/Perspectives

The need for curative therapy for PD remains, and it is hoped that work with various molecular targets will yield new drugs that will fulfill this need. Among these targets are two enzymes from the UPS, Parkin and USP30, both of which are validated for their potential to augment mitophagy in neuronal cells in which mitophagy is overwhelmed by the scale of mitochondrial damage. Medicinal chemistry efforts are underway to optimize effectors of both targets. There is a similar compendium of evidence from genetics and cellular studies indicating that mitophagy is overwhelmed in dying neuronal cells in Alzheimer's disease (AD) patients (Pradeepkiran & Reddy, 2020), suggesting that USP30 and Parkin may be useful therapeutic targets for this neurodegenerative disease as well.

A potential issue faced in the development of any small molecule therapeutic is the evolution of resistance. An obvious cause of resistance to drugs that treat neurodegenerative diseases such as PD and AD is related to their dependence on the blood brain barrier to achieve the intended pharmacological effect. It has been reported that multidrug resistance transporters such as P-glycoprotein (Toornvliet et al., 2006) and breast cancer resistant protein (BCRP) (Xiong et al., 2009) are active in the blood brain barrier and are affected in AD, leading to altered CNS distribution of drugs that bind these reporters. Thus, multidrug resistance mechanisms are a potential obstacle to treatment of neurodegenerative disease using small molecules; adjuvant treatment with pump inhibitors may be beneficial in these cases (Pan et al., 2019). In addition, drugs that act on molecular (particularly enzymic) targets face resistance generated by functional degeneracy, or compensation of the targeted enzyme by a second enzyme that is not inhibited by the therapeutic agent but can perform the enzyme's function if necessary. This issue could be addressed proactively using combinations of drugs with separate, independent mechanisms, for example, combining an inhibitor of USP30 with a drug that acts on a molecular target unrelated to deubiquitination. The availability of experimental drugs from several molecular target classes currently in development affords the opportunity to pursue this avenue of combating drug resistance in treating neurodegenerative diseases. Successful development of a PROTAC drug could also diminish the likelihood of resistance, although this potential awaits clinical confirmation.

Additional effort is needed to improve in vivo models and biomarkers for PD. In the case of PD, the numerous biochemical and genetic in vivo models available are somewhat difficult and controversial, although they can address subsets of PD symptoms (Koszla et al., 2021). For example, the loss of PINK1 or Parkin in mice results in no overt PD phenotype, which may reflect compensatory mechanisms in the organism (Goldberg et al., 2003; Perez & Palmiter, 2005). In addition, Parkin KO rats do not undergo progressive nigral neurodegeneration, while PINK1 KO rats exhibit gait abnormalities, α-synuclein aggregates, and dopaminergic neuron loss at 8 months of age (Creed & Goldberg, 2018a, 2018b). Moreover, Parkin-independent mitophagy pathways mediated by receptors and ubiquitin E3 ligases mitochondrial E3 ubiquitin ligase 1 (MUL1) and ariadne RBR E3 ubiquitin ligase 1 (ARIH1) have been described (Yao et al., 2020) and the impact of alternative mitophagy pathways on therapeutic strategies in PD remains to be evaluated. Attempts to study mitophagy in vivo, using *Drosophila* and rodent models that express fluorescent mitophagy reporters such as mito-Keima and mito-QC have begun to yield results (Kim et al., 2019). Phospho-ubiquitin (pS65-Ub) has been detected in postmortem brains from elderly individuals and patients with Lewy body disease, implicating the Parkin-PINK1 pathway in aging and neurodegeneration (Hou et al., 2018). Phospho-Ub is considered a potential biomarker in Parkinson's disease (Fiesel et al., 2015).

Translational research supports the development of small molecule effectors targeting UPS mediators of mitochondrial health such as Parkin, PINK1, and USP30. To advance such molecules to the clinic, however, it will be necessary to establish pharmacokinetics, pharmacodynamics, and in vivo efficacy, in addition to safety and tolerability.

Acknowledgements This work was supported by Progenra R & D funds and The Michael J. Fox Foundation for Parkinson's Research, New York, USA.

Author Contributions TB conceived the idea and revised the article. KS, MM, MG wrote the article.

Data Availability Data sharing not applicable to this article as no datasets were generated or analysed during the current study.

Declarations

Conflict of interest KS, MM, TB are employees of Progenra Inc., Progenra aims to develop small molecule Parkin activators. MG has no competing interests.

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