The Landscape of *Parkin* Variants Reveals Pathogenic Mechanisms and Therapeutic Targets in Parkinson's Disease

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19 **Abstract**

20 Mutations in *Parkin* (*PARK2*), which encodes an E3 ubiquitin ligase implicated in
 21 mitophagy, are the most common cause of early onset Parkinson’s Disease (PD).
 22 Hundreds of naturally occurring *Parkin* variants have been reported, both in PD
 23 patient and population databases. However, the effects of the majority of these
 24 variants on the function of Parkin and in PD pathogenesis remains unknown. Here we
 25 develop a framework for classification of the pathogenicity of *Parkin* variants based
 26 on the integration of clinical and functional evidence – including measures of
 27 mitophagy and protein stability, and predictive structural modeling – and assess 51
 28 naturally occurring *Parkin* variants accordingly. Surprisingly, only a minority of
 29 *Parkin* variants, even among those previously associated with PD, disrupted Parkin
 30 function. Moreover, a few of these naturally occurring *Parkin* variants actually
 31 enhanced mitophagy. Interestingly, impaired mitophagy in several of the most
 32 common *pathogenic* Parkin variants could be rescued both by naturally-occurring
 33 (p.V224A) and structure-guided designer (p.W403A; p.F146A) hyperactive Parkin
 34 variants. Together, the findings provide a coherent framework to classify *Parkin*
 35 variants based on pathogenicity and suggest that several *pathogenic Parkin* variants
 36 represent promising targets to stratify patients for genotype-specific drug design.

37

38 Introduction

39 Parkinson's disease (PD) is the second most common neurodegenerative disease.
 40 Although most PD cases are sporadic, a fraction are familial and caused by mutations
 41 in different genes (1). Mutations in the *Parkin* (*PARK2*) gene are the most common
 42 cause of autosomal recessive early-onset parkinsonism (EOPD) and are believed to
 43 result in a loss of Parkin protein function (2). *Parkin* variants include rearrangements
 44 and copy number variations, such as deletions and duplications of exons, as well as
 45 single nucleotide variants (SNVs) that cause missense, nonsense, or splice site
 46 mutations (3-5). Of these, missense variants are the most frequently reported in PD
 47 patients and, because they likely impede Parkin protein function rather than disrupting
 48 protein expression, may represent viable targets for therapies that enhance Parkin
 49 activity.

50 To envisage such genotype-specific therapies, the pathogenicity of the many
 51 *Parkin* variants in the population first needs to be determined. The American College
 52 of Medical Genetics and Genomics for Molecular Pathology (ACMG-AMP) has
 53 outlined five standard terminologies to describe variants identified in genes that cause
 54 Mendelian disorders (6). "*Pathogenic*" and "*likely pathogenic*" indicate a clear or
 55 very likely disease-causing effect of a variant, respectively. Conversely, "*likely benign*,"
 56 and "*benign*" indicate variants that are not disease-causing. Variants that cannot be
 57 assigned to one of these four groups are designated as "*uncertain significance*". Over
 58 200 *Parkin* missense variants have been deposited in public repositories (4, 5, 7, 8).

59 However, to date, only a minority have been clearly annotated based on formal
60 criteria.

61 A clear assignment of a *Parkin* variant requires integrating different lines of
62 evidence that fall into two broad categories (9). Clinical evidence consists of the
63 association or segregation of the variant with disease (or the absence of) in human
64 cohorts or within families with multiple affected individuals. Functional evidence
65 refers to the consequence(s) of the variant, using experimental assays that measure
66 biochemical and cellular properties, as well as computational algorithms that model
67 the effect(s) of the variant based on protein structure and function. Clinical evidence
68 has a hierarchical relationship relative to functional evidence and prevails when a
69 discrepancy or conflict arises between clinical and functional observations (9, 10).

70 Parkin is a basally autoinhibited E3 ubiquitin (Ub) ligase, which contains an
71 N-terminal Ub-like (Ubl) domain, connected through a linker to four
72 zinc-coordinating domains, RING0, RING1, In-Between-RING (IBR), and RING2,
73 which form a core designated as the R0RBR (11). Parkin is activated by PINK1, a
74 mitochondrial kinase that is also implicated in EOPD (1). PINK1 accumulates on
75 damaged mitochondria upon depolarization, where it phosphorylates nearby Ub
76 (12-15). Parkin binds to phospho-Ub (pUb), which recruits Parkin to mitochondria
77 and facilitates PINK1 phosphorylation of the Parkin Ubl, which in turn fully activates
78 Parkin (16-18). Parkin then ubiquitinates multiple outer mitochondrial membrane
79 targets, triggering a feed-forward amplification loop, that leads to the clearance of
80 damaged mitochondria via autophagy (mitophagy) (reviewed in (19)). Some *Parkin*

missense variants found in patients have been shown to affect protein folding, Parkin autoubiquitination, protein-protein interactions or recruitment to mitochondria (20-25). However, as the pathogenic nature of most of these variants was not clear, the disease relevance of the functional alteration remained to be determined.

Here, we characterized all *Parkin* missense variants found in public databases, according to ACMG-AMP criteria. We then used a cell-based assay to quantify Parkin-mediated mitophagy and Parkin protein levels. We also applied structural simulations to explore the mechanisms underlying the observed functional alterations. Integrating these data, we find that only a minority of *Parkin* variants can be considered *pathogenic*. Interestingly, we identified several naturally-occurring *Parkin* variants in the population that increase mitophagy in cells. Remarkably, such hyperactive *Parkin* variants were able to rescue the impaired function of several common *pathogenic Parkin* variants. Our study suggests that several *pathogenic* PD-linked *Parkin* mutations represent promising targets amenable to genotype-specific drug design.

Results

Most *Parkin* missense variants lack sufficient clinical evidence to establish pathogenicity

To classify *Parkin* variants, we utilized Sherlock (semiquantitative, hierarchical evidence-based rules for locus interpretation), a classification framework that translates the ACMG-AMP standards to a set of discrete, but related, rules with refined weights (9). Clinical evidence was examined first, as it most directly relates to

disease (9). Briefly, data from population databases, including minor allele frequency (MAF) and homozygote counts, and clinical records in PD-specific databases that report PD patients or unaffected family members carrying missense variants in *Parkin*, were examined to assign weighted points as pathogenic or benign to each variant (Supplementary Fig. 1). The pathogenic points and benign points were summed-up separately and compared to preset thresholds to assign the variants to one of the five ACMG-AMP categories (Supplementary Fig. 2).

From the PD-specific databases, PDmutDB (3, 4) and MDSgene (5, 26), we identified a total of 75 *Parkin* missense variants in PD patients (Fig. 1A). Most were absent or very rare in the control cohorts from the original reports, possibly due to the small sizes of the control cohorts. Therefore, we searched for the 75 variants in public population databases and found that 51 of them were reported in dbSNPs (7) and in the Exome Aggregation Consortium (ExAC), including high-quality variant calls across 60,706 human exomes (8) (Fig. 1A). In ExAC, we also found an additional 164 *Parkin* missense variants (Fig. 1A). The classification of the missense variants using clinical evidence allowed us to clearly designate thirteen variants in PD as *pathogenic* or *likely pathogenic* and ten variants as *likely benign* or *benign* (Fig. 1A and Table 1). The details of the points assigned to each variant are summarized in Supplementary Table 1. Remarkably, large numbers of remaining variants lacked sufficient data to be assigned to either the *benign* or *pathogenic* categories and were therefore designated as of *uncertain significance* (Fig. 1A, Table 1 and Supplementary Table 1).

124 ***Parkin* variants can be classified functionally in cells**

125 Next, to determine the effects of the variants on Parkin function, we employed a
126 cell-based assay to monitor and quantify mitophagy using fluorescence-activated cell
127 sorting (FACS) in U2OS cells stably expressing inducible mt-Keima, a pH-sensitive
128 fluorescent protein that is targeted to mitochondria and exhibits a large shift in its
129 emission wavelength upon engulfment in the acidic compartment of lysosomes (27).
130 U2OS cells express very low level of endogenous Parkin, which is insufficient to
131 mediate mitophagy in response to the mitochondrial potential uncoupler CCCP (28).
132 In cells transiently expressing either wildtype (WT) Parkin or one of the Parkin
133 variants fused to GFP, the shift in mt-Keima emission was measured upon four hours
134 of mitochondrial depolarization with CCCP (Supplementary Fig. 3A). The GFP
135 intensity in untreated cells was also quantified as a measure of steady-state Parkin
136 protein levels (Supplementary Fig. 3B). The level of CCCP-induced Parkin-mediated
137 mitophagy and GFP intensity of the Parkin variants were normalized to that of WT
138 Parkin.

139 We analyzed all the variants that were designated clinically as *pathogenic* or *likely*
140 *pathogenic*, *benign* or *likely benign* (Fig. 1B). We also analyzed an additional
141 twenty-eight variants of *uncertain significance*, including variants that were reported
142 as homozygous or compound heterozygous in either the disease-specific databases or
143 in population databases (Fig. 1C and Supplementary Table 1). These variants
144 represented the most common missense variants reported in the human population
145 from public databases. Moreover, they were reported in 283 out of 309 families or

146 isolated patients carrying Parkin missense variants in PD-specific databases.

147 The thirteen *Parkin* variants classified as *pathogenic* or *likely pathogenic* based on
148 clinical evidence all showed significantly decreased mitophagy (Fig. 1B). Seven
149 variants (p.R42P, p.V56E, p.C212Y, p.C253Y, p.C238W, p.R275W, and p.C441R)
150 also showed decreased GFP intensity (Fig. 1B), suggesting reduced protein stability.
151 Of these, all the variants in the R0RBR of Parkin formed inclusions to different
152 degrees, detected by fluorescence microscopy (Supplementary Fig. 4). In contrast, the
153 p.R42P and p.V56E variants in the Ubl domain showed lower overall GFP intensity
154 without visible inclusions. Thus, for a subset of *pathogenic* and *likely pathogenic*
155 *Parkin* variants, the observed defects in mitophagy are likely to stem from abnormal
156 protein folding and reduced protein stability. In contrast, all ten *Parkin* variants
157 classified as *benign* and *likely benign* based on clinical evidence exhibited similar
158 GFP intensity as WT and most, with three exceptions, also exhibited WT levels of
159 Parkin-mediated mitophagy (Fig. 1B). p.Q34R and p.A46T displayed decreased
160 mitophagy, whereas p.R334C showed an almost three-fold increase (Fig. 1B). Of the
161 twenty-eight variants of *uncertain significance* based on clinical evidence, fourteen
162 displayed similar GFP intensity and mitophagy as WT Parkin (Fig. 1C). Nine
163 displayed impaired mitophagy, three of which also showed decreased GFP intensity.
164 Surprisingly, five variants showed increased mitophagy (Fig. 1C).

165 The wide range of changes in Parkin levels and mitophagy prompted us to ask
166 whether we could classify the different variants into discrete groups. Variants that
167 significantly decreased Parkin protein levels and mitophagy were assigned to Group 1

(Fig. 2A and Table 1). Variants that severely (0-30% of WT) or moderately (30-60% of WT) reduced Parkin-mediated mitophagy but displayed normal protein levels were assigned to Groups 2 and 3, respectively. Group 4 consisted of variants that were similar to WT, whereas Group 5 consisted of variants with increased (>140% of WT) Parkin-mediated mitophagy. Interestingly, all the *pathogenic* or *likely pathogenic* variants, classified based on clinical evidence, were assigned to group 1 or 2, whereas all the *benign* or *likely benign* variants fell into group 3, 4, or 5 (Fig. 2A-B). This suggested that the two functional measurements in our cell model could faithfully discriminate the pathogenic variants from the “non-pathogenic” variants.

Integration of clinical and functional evidence refines the classification of *Parkin* variants

We next examined which clinical features were most strongly correlated with functional alterations. Among the variants that segregated with PD in families (Supplementary Table 1), all but one (p.R33Q) severely altered Parkin function and were assigned to groups 1 or 2 (Fig. 2C). However, it is important to note that segregation analysis was only possible in ~30 families, as most reports only involved single case reports without related family information (Supplementary Table 1). Conversely, variants that were reported as homozygotes in ExAC were frequent in Groups 3, 4 and 5 and not assigned to Groups 1 or 2 (Fig. 2D). Notably, several variants that were reported in PD patients as homozygous or compound heterozygous, nonetheless displayed WT Parkin levels and function (Fig. 2E). Most had relatively high MAFs in ExAC (Supplementary Table 1), suggesting their presence in patients

190 was due to their high prevalence rather than pathogenicity. Taken together, our data
191 shows that variants segregating with disease in families impair Parkin function
192 (Groups 1 and 2), whereas variants that occur as homozygotes in ExAC did not
193 severely reduce Parkin function (Groups 3, 4 and 5).

194 Next, we devised a scoring scheme, based on Sherlock, to assign benign or
195 pathogenic points (Supplementary Fig. 5), according to the functional group to which
196 the *Parkin* variants were assigned in the cellular assays (Fig. 2A). The pathogenic
197 points and benign points were then added to the corresponding points from the
198 clinical evidence in order to obtain combined *pathogenic* and *benign* scores from all
199 evidence for the final annotation of each variant (Table 1 and Supplementary Table 1).
200 Using these combined clinical and functional scores, all the *likely pathogenic* variants
201 from clinical evidence were reclassified as *pathogenic* (Table 1). Additionally, three
202 *likely benign* variants were reclassified as *benign* (Table 1). Most of the variants of
203 *uncertain significance* were reclassified as *likely benign*, while six were reclassified as
204 *pathogenic* and nine remained of *uncertain significance* (Table 1). In summary, all
205 variants that caused functional alterations assigned to groups 1 and 2, as measured in
206 our experimental assay, were reclassified as *pathogenic*, whereas most variants
207 assigned to functional groups 3, 4 and 5 were reclassified as either *benign* or *likely*
208 *benign* (Fig. 2F).

209 **Structural analysis of *Parkin* variants reveals pathogenic mechanisms**

210 As the structure of Parkin is known, we modeled the effects of variants on the
211 reported crystal structures of Parkin to gain insight into the mechanisms underlying

the functional changes. The structures of autoinhibited Parkin, pUb-bound Parkin, p-Ub-bound phospho-Parkin, pUb-E2 enzyme-bound phospho-Parkin were used as they depict Parkin in its different states of activation (17, 18, 29, 30).

Most variants in functional groups 1 and 2 were predicted to introduce steric clashes with nearby residues in at least one of the Parkin structures. In contrast, most variants in Group 4, which displayed similar function as WT Parkin, introduced no major clashes and did not affect interactions (Fig. 3A-B). However, mild clashes were observed in a few cases, suggesting that some degree of steric clashing could be tolerated and possibly be compensated for by local conformation changes to maintain overall protein function. For example, three variants at Arg42 were analyzed. Mutation of Arg42 to proline introduced major clashes, whereas substitution to histidine introduced mild clashes and there was no clash introduced by substitution to cysteine (Fig. 3C-E). Congruently, only p.R42P was classified as *pathogenic* based on clinical criteria and functional impairment in cell-based assays (Fig. 1B-C), consistent with the simulations that showed this mutation unfolded the Ubl domain (22, 31). These simulated steric clashes nicely illustrate how different amino acid substitutions at a given residue could lead to distinct functional impairment.

The effects of variants in Groups 1 and 2 likely disrupt several different aspects of Parkin function (Fig. 4A). Seven variants involve cysteine residues that coordinate zinc, and their mutation would result in overall misfolding of the Parkin protein (29). Five variants alter key motifs mediating ubiquitination of substrates, including steric clashes with the E2 binding site on RING1 (p.T240R and p.T240M), and residues

234 implicated in thioester transfer of Ub in the catalytic RING2 domain (p.T415N,
235 p.G430D, p.C431F) (32). These types of alterations are likely to cause a complete loss
236 of Parkin function in either PINK1-Parkin mediated mitophagy or other potential
237 Parkin pathways. Four variants specifically localize to motifs implicated in the
238 conformational change that occurs during activation by PINK1. p.K161N and
239 p.K211N introduce no or mild steric clashes, but substitution of the basic lysine
240 residue to the neutral asparagine eliminates the interaction with the acidic phosphate
241 in pUbl (Fig. 4B and (17, 18)). p.G284R introduces major clashes with pUb, thus
242 impairing binding and recruitment to mitochondria (Fig. 4C). p.R275W disrupts
243 interaction with the helix that mediates pUb binding (Fig. 4D). This is predicted to
244 destabilize Parkin, consistent with the observed decreased steady-state protein level
245 and the presence of cellular inclusions (Fig. 1B and Supplementary Fig. 4). This helix
246 becomes more exposed following pUb binding and may also be involved in the
247 allosteric release of the Ubl during activation (Fig. 4D and (17, 18)). The clashes
248 caused by p.R42P and p.V56E are predicted to misfold the Ubl domain and thus
249 destabilize the protein, as demonstrated earlier for p.R42P (22). Concurrently, p.R42P
250 and p.V56E may also hinder conformational change during activation by preventing
251 Ubl phosphorylation (33) and the binding of pUbl to RING0 (17). One variant,
252 p.P437L, introduced very mild steric clashes in RING2 in the Parkin structure, and the
253 exact molecular mechanism of p.P437L in causing decreased mitophagy remains
254 unclear.

255 Several variants in functional Group 3 that moderately decreased

256 Parkin-mediated mitophagy might also act by hindering Parkin activation by PINK1.
 257 p.A46T could disrupt the interaction of pUbl with RING0 (Supplementary Fig. 6A).
 258 p.R104W induced clashes to the newly identified activation element (ACT), which
 259 binds RING0 and helps stabilize the interaction with pUbl and RING0
 260 (Supplementary Fig. 6B and (18)). p.G359D disrupted the glycine-rich loop in the
 261 IBR domain that interacts with pUb (Supplementary Fig. 6C). These clashes may be
 262 compensated by minor local conformation changes, and thus lead to milder
 263 disruptions in mitophagy and protein stability.

264 **Structure-guided *designer* hyperactive Parkin mutants can rescue** 265 **mitophagy in pathogenic variants**

266 We next hypothesized that some of the *pathogenic* variants may represent targets
 267 for genotype-specific therapy. As a proof of concept, we introduced two artificially
 268 designed mutations, W403A and F146A, which destabilized the REP (repressor
 269 element of Parkin):RING1 and the RING0:RING2 interfaces, respectively. Both
 270 mutations have been shown to accelerate mitophagy by promoting the conformational
 271 changes that occur at these interfaces during Parkin activation by PINK1 (28, 29). We
 272 tested whether these *hyperactive* mutations could rescue the mitophagy defects seen
 273 in the *pathogenic* Parkin variants. As reported previously, mutating F146A or W403A
 274 alone enhanced mitophagy compared to WT Parkin (Fig. 5A) (28). Remarkably,
 275 introducing F146A or W403A *in cis* with p.R42P, p.V56E, p.K161N, p.K211N,
 276 p.R275W p.P437L or p.T240M rescued mitophagy (Fig. 5A). Variants p.R42P,
 277 p.V56E and p.R275W each lower Parkin levels, likely by disrupting protein stability

(Fig. 1B). However, introduction of F146A or W403A did not restore Parkin levels to WT (Supplementary Fig. 7), suggesting the rescue of mitophagy was mediated by Parkin activation *per se* rather than by enhancing Parkin protein stability. The *pathogenic* variants p.K161N and p.K211N are involved in binding the pUbl during activation (17, 18). The fact that both these variants can be rescued by F146A or W403A suggests that destabilization of the REP:RING1 or the RING0:RING2 interface can bypass the tethering of the Ubl to RING0, which occurs during Parkin activation.

Introducing F146A or W403A *in cis* with the variants could not rescue any of the seven *pathogenic* cysteines variants involved in zinc coordination (Fig. 5A). Thus, the severe disruption of Parkin folding and stability induced by mutating these cysteines are likely to preclude them from being good candidates for therapeutic rescue by hyperactivation (Fig. 1B-C). Similarly, most variants that disrupted key catalytic sites could not be rescued (Fig. 5A). Also, p.G284R could not be rescued as it disrupts binding to pUb, which is an essential receptor for recruiting Parkin to damaged mitochondria (34). Interestingly, whereas both p.T240M and p.T240R are predicted to interfere with E2 Ub-conjugating enzyme binding to RING1 and showed similar severe defects in mitophagy, only the former could be rescued by F146A or W403A (Fig. 5A). Both variants created clashes in the E2 binding site (Supplementary Fig. 8A-B). However, Arg240 created a positive charge at the interface, increasing the electrostatic repulsion of E2. Methionine is less bulky and neutral, and its flexibility

could allow some weak interactions with E2 to remain, perhaps explaining its rescue by F146A or W403A (Supplementary Fig. 8C-E).

Overall, the defects in mitophagy of seven of the 19 *pathogenic* variants could be rescued by the designed activating mutations. These seven variants are responsible for over 75% of reported PD patients carrying *pathogenic* missense variants (Fig. 5B) and were the most frequent *pathogenic* missense variants in the general population (Fig. 5C). Mimicking the effects of F146A or W403A could therefore be a useful starting point for designing treatments for patients with PD caused by these *Parkin* variants.

Characterization of naturally-occurring hyperactive Parkin variants

We identified six naturally-occurring variants that, considering all the evidence, were classified as *likely benign* or *benign* and showed enhanced Parkin-mediated mitophagy (Fig. 1B, 1C and 2G). The Parkin structure shows that Arg234 and Arg256 are located at the interface between the REP and RING0. The p.R234Q and p.R256C variants are predicted to destabilize the interface, thus mimicking the W403A *designer* mutant used above (Fig. 6A). Additionally, p.M458L may destabilize the RING0:RING2 interface, mimicking the effects of our other *designer* mutation, F146A (Fig. 6B). Thus, based on structural predictions, three of the six naturally-occurring variants are likely to activate Parkin via mechanisms akin to those involved in the hyperactive mutants designed previously (28). Because these variants occur naturally in the population, our findings demonstrating rescue of mitophagy suggest that targeting these sites and mechanisms are likely to be tolerated and potentially therapeutic in PD. p.P37L also moderately increased Parkin-mediated

321 mitophagy, but the structural basis of the increased activity was unclear, as this
322 variant does not create any steric clash and thus should not affect interactions of the
323 Ubl with RING1 or interactions of the pUbl with RING0 (Supplementary Fig. 9A-B).
324 Mutation of Arg334 to a cysteine could affect the coordination of a nearby zinc in the
325 IBR, which may stabilize the interaction with pUb and thereby enhance Parkin
326 activity (Supplementary Fig. 9C).

327 Unlike the five other naturally-occurring hyperactive variants, p.V224A has not
328 been reported in PD patients (Supplementary Table 1) and showed the highest (almost
329 3-fold above WT) Parkin-mediated mitophagy activity (Fig. 1C). The Val224 residue
330 is localized in the pUb binding pocket, with its side-chain facing towards the
331 phosphorylated Ser65 residue of pUb, and the mutation to alanine could modulate the
332 affinity of Parkin for phospho-ubiquitin (Fig. 6C). We therefore examined the ability
333 of hyperactive p.V224A to rescue the function of the *pathogenic* variants. Introducing
334 the p.V224A variant *in cis* did not affect the protein level of most *pathogenic* variants,
335 except for p.G284R (Fig. 7A). This may stem from an additive destabilizing effect of
336 the double mutant on Parkin folding as both p.V224A and p.G284R are located within
337 the same pUb binding motif. Introducing the p.V224A variant partially rescued the
338 mitophagy defects of p.R42P, p.V56E and p.K161N (Fig. 7B). How the predicted
339 effects of V224A on pUb-binding could partially compensate for defects in Ubl- and
340 pUbl-mediated activation by p.R42P, p.V56E and p.K161N remains to be elucidated.
341 p.V224A could not rescue the mitophagy deficit in p.R275W and p.G284R variants,
342 nor could it rescue any of the remaining *pathogenic* variants that directly damaged

343 catalytic activity and zinc coordination (Fig. 7B). Compared with F146A or W403A,
344 p.V224A was less effective at rescuing the *pathogenic* variants, suggesting that the
345 pUb-binding site may be a less promising target for activating mitophagy than
346 releasing the autoinhibited conformation of Parkin.

347 Discussion

348 *Parkin* mutations are the most common cause of recessive early-onset PD
 349 (EOPD) (2). Although *Parkin* loss-of-function is well established in EOPD (5, 35),
 350 causality for any given missense variant has been more difficult to ascertain. In this
 351 study, we have integrated clinical, experimental and structural modeling approaches
 352 to map out the landscape of *Parkin* variants in the general population and in PD
 353 patients. Our hope is that this work will help provide a more cohesive framework to
 354 guide basic science studies exploring the molecular and cellular functions of the
 355 *PINK1/Parkin* pathway and to guide clinicians caring for patients carrying specific
 356 *Parkin* variants. We also hope that the work will inform structure-based drug design
 357 to develop *Parkin* activators and help guide *Parkin* allele- and genotype-specific
 358 clinical studies.

359 For the over 200 *Parkin* variants reported in public databases (3, 5, 8), we found
 360 that only a minority of variants could be clearly designated as *likely pathogenic*,
 361 *pathogenic*, *likely benign* or *benign* based on clinical evidence alone. While this may
 362 not seem surprising for variants found only in population databases such as ExAC,
 363 where accompanying clinical information is scant, we found a similar situation for
 364 variants reported in patients. Indeed, 52 out 75 variants reported in PDmutDB and
 365 MDSgene remained of *uncertain significance* after having been subjected to the
 366 Sherlock algorithm, the variant classification framework derived from ACMG
 367 standards that we used in this study. The overarching message from these
 368 observations is that the mere presence of *Parkin* variants in PD patients, PD kindreds

369 or PD-specific databases should be interpreted with caution and not automatically
370 taken to imply pathogenicity. Rather, we propose that clinical evidence available for
371 new variants should be subjected to the same rigorous classification scheme presented
372 here to determine pathogenicity.

373 In addition to analyzing clinical evidence, we extensively characterized the
374 cellular effects of the 51 *Parkin* variants most commonly found in patient and
375 population sequencing databases. To our knowledge, a systematic analysis integrating
376 clinical evidence with cellular function, on this scale, has not been reported previously
377 for *Parkin* (20-25, 36, 37). Notably, all the variants designated as *pathogenic* or *likely*
378 *pathogenic* based on clinical evidence also displayed severe mitophagy defects in
379 cells (functional groups 1 and 2). Conversely, all variants designated clinically as
380 *benign* or *likely benign* displayed mitophagy function in the WT range or showed only
381 a slight reduction (functional groups 3, 4 and 5). While this may seem *a priori* as
382 self-evident, several alternative functions of Parkin in cells have been proposed and
383 the role of mitophagy in PD has yet to be definitively established (38-41). Thus, while
384 this work does not refute the biological importance of such alternative functions, the
385 tight correlation between the clinical impact of the variants and their effects on
386 mitophagy provides further evidence that mitophagy can be used as a robust and
387 disease-relevant readout of Parkin function that likely reflects a key pathogenic
388 process in PD.

389 Assignment of the *Parkin* variants to functional groups in cells allowed us to
390 determine which clinical features best correlate with and could be used to predict

391 pathogenicity. Segregation of variants with PD in families and observation of
 392 homozygotes in ExAC turned out to be very strong predictors for pathogenicity or the
 393 absence of pathogenicity, respectively. In contrast, the mere report of PD patients with
 394 one or two *Parkin* variants or the absence of these variants in control cohorts or
 395 population databases should not be automatically taken to imply pathogenicity.
 396 Perhaps more importantly, integrating clinical with functional evidence from cells
 397 allowed us to re-assign 19 of the 28 variants, designated as of *uncertain significance*
 398 based on clinical evidence alone, to one of the benign and pathogenic categories. It
 399 also allowed us to reclassify 6 of the “*likely*” variants to their respective more
 400 definitive *benign* and *pathogenic* categories. Together, these findings attest to the
 401 power of using this sort of iterative combined clinical and experimental approach to
 402 stratify variants.

403 Given what is already known about the structure and function of the Parkin
 404 protein, the work enables in-depth mechanistic exploration of how *pathogenic*
 405 variants can lead to dysfunction. This is something that has been sorely lacking and
 406 may have important implications for how best to target Parkin and design activators
 407 for future therapy. For most of the *pathogenic* variants, the mechanisms by which they
 408 interfere with function can be rationalized based on the Parkin structure. As an
 409 important proof of concept, we showed that the function of several pathogenic *Parkin*
 410 variants, defective in mitophagy in cells, could be rescued when expressed *in cis* with
 411 mutations that have been previously designed to enhance Parkin activity (28, 29). This
 412 provides further stratification according to therapeutic potential. For instance,

413 alterations in residues involved in zinc coordination, in catalytic activation or in
414 pUb-binding could not be rescued. In contrast, alterations in residues involved in Ubl
415 folding or in the pUbl-RING0 interface in the active Parkin structure could be fully
416 rescued, suggesting that therapeutics that disrupt the REP-RING1 or the
417 RING0-RING2 interfaces could potentially bypass these defects (17, 18). Importantly,
418 many of the most commonly occurring variants were the ones that could be rescued,
419 something that bodes well for patients carrying these variants, should a therapeutic
420 mimicking W403A or F146A become available.

421 One of the most surprising findings of our study was that several
422 naturally-occurring variants exhibited a 1.5- to almost 3-fold enhancement in
423 Parkin-mediated mitophagy in our assay. This could not simply be explained by
424 increased Parkin protein levels or by the fact that our assay involved overexpression.
425 Indeed, except for certain pathogenic variants that destabilized Parkin, most variants,
426 including the hyperactive ones, displayed steady-state levels that were very close to
427 WT levels. These hyperactive variants provide an important proof of principle that
428 there are, presumably healthy, individuals in the population living with enhanced
429 Parkin activity. The strongest activating variant was V224A, which increases
430 mitophagy by nearly 3-fold and occurs very near the site for pUb-binding. This was
431 surprising as pUb-binding serves as a critical receptor to recruit Parkin to
432 mitochondria and, to date, every reported mutation in this motif, abolished or
433 dramatically reduced mitophagy. Moreover, when expressed *in cis*, V224A partially
434 rescued certain, but not all, of the mutants that were rescued by W403A and F146A.

435 In the future, it will be important to test whether this involves an enhancement in
436 pUb-binding or some other downstream allosteric effect. Similarly, it will be
437 important to determine the mechanisms by which the two remaining hyperactive
438 variants, P37L and R334C, enhance Parkin function. Moreover, as we only sampled
439 51 of the over 200 *Parkin* variants in the population in this study, it is conceivable that
440 other yet-to-be-discovered hyperactive variants will provide further mechanistic
441 insights into Parkin activation and help identify additional therapeutic sites within the
442 protein.

443 **Materials and methods**

444 **Classification of *Parkin* missense variants**

445 We utilized Sherloc (semiquantitative, hierarchical evidence-based rules for locus
446 interpretation), a variant classification framework derived from ACMG standards to
447 assign *Parkin* missense variants into five categories: *pathogenic*, *likely pathogenic*,
448 *benign*, *likely benign* and of *uncertain significance* (9). We considered two broad
449 categories of evidence for the classification, clinical and functional. The procedures
450 for evaluating and scoring these lines of evidence are summarized as root-decision
451 trees in Supplementary Fig. 1, 2, and 5.

452 For clinical evidence, information regarding missense variants in *Parkin* reported
453 in the population database ExAC
454 (<http://exac.broadinstitute.org/gene/ENSG00000185345>) (8) and the disease-specific
455 databases, PDmutDB (<http://www.molgen.vib-ua.be/PDmutDB>) (3), and MDSgene

(<http://www.mdsgene.org/>) (5) were searched. We also searched dbSNP (<http://www.ncbi.nlm.nih.gov/snp>) and the Exon variant server (EVS, <http://evs.gs.washington.edu/EVS/>) for missense variants that were found in the disease databases, but not in ExAC. The homozygote count, MAF in ExAC and maximal MAF in dbSNP, EVS and subpopulations in ExAC were calculated and used to assign points to the variants according to the decision tree in Supplementary Fig. 1A. The clinical cases reported in PDmutDB and MDSmutDB were evaluated according to the decision tree in Supplementary Fig. 1B-C. The original references were traced back for the indexed families or individuals reported in these databases. Indexed cases reported in both databases cited from the same reference were only evaluated once. Indexed cases reported in a more recent paper showing the same information (same number of family members with same genotype and phenotype) as a case in an older reference were considered as the same family and the older report was used.

For functional evidence, the effects of the variants in the cellular assay were assigned points according to the decision tree in Supplementary Fig. 5. We imposed a 2.5-point cap on functional evidence to ensure that functional data which lacked supporting clinical evidence would not be sufficient on its own to reach the threshold required (3 benign points or 4 pathogenic points; Supplementary Fig. 2) to assign a variant to the *pathogenic* or *benign* categories (9).

476 **Cell culture, cloning and mutagenesis**

477 Human osteosarcoma U2OS cells were a gift from Dr. Robert Screaton (Sunnybrook
478 Research Institute). U2OS cells stably expressing mtKeima (a gift from A. Miyawaki,
479 Laboratory for Cell Function and Dynamics, Brain Science Institute, RIKEN, Japan)
480 were created by transfecting plasmid DNA using jetPRIME (Polyplus), followed by
481 selection with G418 for 2 weeks and sorting using flow cytometry (28). Cells were
482 maintained in DMEM supplemented with 10% fetal bovine serum (FBS), 4 mM
483 L-glutamine and 0.1% Penicillin/Streptomycin, in a 37°C incubator with 5% CO₂. All
484 GFP-Parkin variants were generated using PCR mutagenesis on the GFP-Parkin WT
485 plasmid (addgene#45875) according to the manufacturer's protocol (Agilent
486 Technologies). Constructs were verified by Sanger sequencing.

487 **Mitophagy and GFP-intensity measurement by FACS**

488 U2OS cells stably expressing ecdysone-inducible mt-Keima were induced with 10
489 mM ponasterone A and transiently transfected with WT or variant GFP-Parkin for 24
490 h and treated with DMSO or 20 µM CCCP for 4 h and followed immediately by flow
491 cytometry. To minimize transfection efficiency variation, the same amount of
492 GFP-Parkin WT or variant plasmid was utilized and only the population of
493 GFP-positive cells were analyzed in the subsequent FACS data processing. For flow
494 cytometry, cells were trypsin digested, washed and resuspended in PBS prior to their
495 analysis on an LSR Fortessa (BD Bioscience) equipped with 405 and 561 nm lasers
496 and 610/20 filters (Department of Microbiology and Immunology Flow Cytometry

Facility, McGill University). Measurement of mtKeima was made using a dual-excitation ratiometric pH calculation where pH 7 was detected through the excitation at 405 nm and pH 4 at 561 nm (28). For each untreated sample, 75,000 events were collected and single GFP-Parkin-positive cells were subsequently gated for quantification of the geometric mean of the GFP signal as a measure of steady-state Parkin protein levels. The value for each Parkin missense variant was normalized to that for the WT in each experiment. For each untreated and treated sample, single GFP-Parkin-positive, mtKeima-405 nm-positive cells were subsequently gated. The percentage of cells with an increase in the 405nm:561nm ratio in mtKeima was quantified. The percentage in treated cells minus the percentage in untreated cells was calculated as the induced Parkin-mediated mitophagy. The induced mitophagy for each Parkin missense variant was normalized to that for WT in each repeat. Data was analyzed using FlowJo v10.1 (Tree Star).

Modeling of Parkin Structures, Modifications and Variants

The structures of human Parkin bound to phospho-ubiquitin (PDB 5N2W), rat parkin (PDB 4ZYN), human phospho-parkin bound to phospho-Ub (PDB 6GLC) and fly pParkin-pUb-UbcH7 complex (PDB 6DJX) were analyzed using PyMOL version 1.5 (Schrödinger, New York). Mutations and clashes were simulated using the mutagenesis wizard toolbox. The presence of more than three simulated significant clashes (red disks) was taken to indicate major clashes. One to three significant clashes (red disks) together with other slight clashes (brown and green disks) were

518 considered as minor clashes. Polar contacts within 4 Å distance of the residue were
519 explored for characterizing interactions.

520 **Statistical analysis**

521 For statistical analysis of mitophagy and protein levels, one-way analysis of variance
522 (ANOVA) with Bonferroni post-hoc tests were performed. To determine the ability of
523 hyperactive mutants to rescue *Parkin* variants, two-way analysis of variance (ANOVA)
524 and Bonferroni post-hoc test comparing row factors among the single or double
525 mutations were performed. *P<0.05; **P<0.01; ***P<0.001.

526 **Author contributions**

527 W.Y. performed cloning, genetic analysis, and experiments in cells. E.J.M., M.Y.T.
528 and A.I.K. assisted with cloning and experiments in cells. Z.G-O. assisted with
529 genetic analysis. J.-F.T. assisted with all the structural simulations. W.Y., E.J.M.,
530 M.Y.T., Z.G-O, J.F.T. and E.A.F. participated in the design of experiments, data
531 analysis and preparation of the manuscript.

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539

Table 1. Annotation of *Parkin* missense variants by ACMG terminologies.

Index	Databases	Parkin Variant	Annotation with clinical evidence	Functional group	Annotation with clinical and functional evidence
1	Disease and population	p.R42P	<i>Pathogenic</i>	1	<i>Pathogenic</i>
2	Disease and population	p.V56E	<i>Pathogenic</i>	1	<i>Pathogenic</i>
3	Disease and population	p.K211N	<i>Pathogenic</i>	2	<i>Pathogenic</i>
4	Disease and population	p.C212Y	<i>Pathogenic</i>	1	<i>Pathogenic</i>
5	Disease and population	p.C238W	<i>Pathogenic</i>	1	<i>Pathogenic</i>
6	Disease	p.C253Y	<i>Pathogenic</i>	1	<i>Pathogenic</i>
7	Disease and population	p.G284R	<i>Pathogenic</i>	2	<i>Pathogenic</i>
8	Disease and population	p.T415N	<i>Pathogenic</i>	2	<i>Pathogenic</i>
9	Disease	p.C431F	<i>Pathogenic</i>	2	<i>Pathogenic</i>
10	Disease and population	p.C441R	<i>Pathogenic</i>	1	<i>Pathogenic</i>
11	Disease	p.T240R	<i>Likely Pathogenic</i>	2	<i>Pathogenic</i>
12	Disease and population	p.R275W	<i>Likely pathogenic</i>	1	<i>Pathogenic</i>
13	Disease and population	p.G430D	<i>Likely pathogenic</i>	2	<i>Pathogenic</i>
14	Disease and population	p.A46T	<i>Likely benign</i>	3	<i>Likely benign</i>
15	Disease and population	p.P153R	<i>Likely benign</i>	4	<i>Benign</i>
16	Disease and population	p.M192L	<i>Likely benign</i>	4	<i>Benign</i>
17	Disease and population	p.R402C	<i>Likely benign</i>	4	<i>Benign</i>
18	Disease and population	p.Q34R	<i>Benign</i>	3	<i>Benign</i>
19	Disease and population	p.A82E	<i>Benign</i>	4	<i>Benign</i>
20	Disease and population	p.S167N	<i>Benign</i>	4	<i>Benign</i>
21	Disease and population	p.R334C	<i>Benign</i>	5	<i>Benign</i>
22	Disease and population	p.V380L	<i>Benign</i>	4	<i>Benign</i>
23	Disease and population	p.D394N	<i>Benign</i>	4	<i>Benign</i>
24	Disease and population	p.D18N	<i>Uncertain significance</i>	4	<i>Likely benign</i>
25	Disease and population	p.R33Q	<i>Uncertain significance</i>	4	<i>Likely benign</i>
26	Disease and population	p.P37L	<i>Uncertain significance</i>	5	<i>Likely benign</i>
27	Disease and population	p.R42C	<i>Uncertain significance</i>	4	<i>Likely benign</i>
28	Disease and population	p.R42H	<i>Uncertain significance</i>	4	<i>Likely benign</i>
29	Disease	p.W54R	<i>Uncertain significance</i>	3	<i>Uncertain significance</i>
30	Disease and population	p.R104W	<i>Uncertain significance</i>	3	<i>Uncertain significance</i>
31	Disease	p.K161N	<i>Uncertain significance</i>	2	<i>Pathogenic</i>
32	Disease	p.M192V	<i>Uncertain significance</i>	4	<i>Uncertain significance</i>
33	Population	p.V224A	<i>Uncertain significance</i>	5	<i>Likely benign</i>
34	Disease and population	p.R234Q	<i>Uncertain significance</i>	5	<i>Likely benign</i>
35	Disease and population	p.T240M	<i>Uncertain significance</i>	2	<i>Pathogenic</i>

36	Population	p.V248I	<i>Uncertain significance</i>	4	<i>Likely benign</i>
37	Disease	p.C253F	<i>Uncertain significance</i>	1	<i>Pathogenic</i>
38	Disease and population	p.R256C	<i>Uncertain significance</i>	5	<i>Likely benign</i>
39	Disease and population	p.D280N	<i>Uncertain significance</i>	4	<i>Likely benign</i>
40	Disease	p.C289G	<i>Uncertain significance</i>	1	<i>Pathogenic</i>
41	Disease and population	p.E310R	<i>Uncertain significance</i>	4	<i>Likely benign</i>
42	Disease	p.G328E	<i>Uncertain significance</i>	4	<i>Uncertain significance</i>
43	Disease and population	p.R334H	<i>Uncertain significance</i>	4	<i>Uncertain significance</i>
44	Disease	p.T351P	<i>Uncertain significance</i>	4	<i>Uncertain significance</i>
45	Disease and population	p.G359D	<i>Uncertain significance</i>	3	<i>Uncertain significance</i>
46	Disease and population	p.R366W	<i>Uncertain significance</i>	4	<i>Likely benign</i>
47	Disease and population	p.R396G	<i>Uncertain significance</i>	4	<i>Uncertain significance</i>
48	Disease	p.C418R	<i>Uncertain significance</i>	1	<i>Pathogenic</i>
49	Disease and population	p.P437L	<i>Uncertain significance</i>	2	<i>Pathogenic</i>
50	Disease and population	p.E444Q	<i>Uncertain significance</i>	4	<i>Uncertain significance</i>
51	Disease and population	p.M458L	<i>Uncertain significance</i>	5	<i>Likely benign</i>

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545 References

- 546 1 Poewe, W., Seppi, K., Tanner, C.M., Halliday, G.M., Brundin, P., Volkman, J., Schrag, A.-E. and
547 Lang, A.E. (2017) Parkinson disease. *Nature Reviews Disease Primers*, **3**, 17013.
- 548 2 Koros, C., Simitsi, A. and Stefanis, L. (2017) Genetics of Parkinson's Disease: Genotype-Phenotype
549 Correlations. *International review of neurobiology*, **132**, 197-231.
- 550 3 Nuytemans, K., Theuns, J., Cruts, M. and Van Broeckhoven, C. (2010) Genetic etiology of
551 Parkinson disease associated with mutations in the SNCA, PARK2, PINK1, PARK7, and LRRK2 genes: a
552 mutation update. *Human mutation*, **31**, 763-780.
- 553 4 Cruts, M., Theuns, J. and Van Broeckhoven, C. (2012) Locus-specific mutation databases for
554 neurodegenerative brain diseases. *Human mutation*, **33**, 1340-1344.
- 555 5 Kasten, M., Hartmann, C., Hampf, J., Schaa, S., Westenberger, A., Vollstedt, E.J., Balck, A.,
556 Domingo, A., Vulinovic, F., Dulovic, M. *et al.* (2018) Genotype-phenotype relations for the Parkinson's
557 Disease genes Parkin, PINK1, DJ1: MDSGene Systematic Review. *Movement disorders : official journal*
558 *of the Movement Disorder Society*, **33**, 730-741.
- 559 6 Richards, S., Aziz, N., Bale, S., Bick, D., Das, S., Gastier-Foster, J., Grody, W.W., Hegde, M., Lyon, E.,
560 Spector, E. *et al.* (2015) Standards and guidelines for the interpretation of sequence variants: a joint
561 consensus recommendation of the American College of Medical Genetics and Genomics and the
562 Association for Molecular Pathology. *Genet Med*, **17**, 405-424.
- 563 7 Sherry, S.T., Ward, M.H., Kholodov, M., Baker, J., Phan, L., Smigielski, E.M. and Sirotkin, K. (2001)
564 dbSNP: the NCBI database of genetic variation. *Nucleic acids research*, **29**, 308-311.
- 565 8 Lek, M., Karczewski, K.J., Minikel, E.V., Samocha, K.E., Banks, E., Fennell, T., O'Donnell-Luria, A.H.,

Ware, J.S., Hill, A.J., Cummings, B.B. *et al.* (2016) Analysis of protein-coding genetic variation in 60,706 humans. *Nature*, **536**, 285-291.

9 Nykamp, K., Anderson, M., Powers, M., Garcia, J., Herrera, B., Ho, Y.-Y., Kobayashi, Y., Patil, N.,
10 Thusberg, J., Westbrook, M. *et al.* (2017) Sherloc: a comprehensive refinement of the ACMG-AMP
variant classification criteria. *Genetics In Medicine*, **19**, 1105.

11 MacArthur, D.G., Manolio, T.A., Dimmock, D.P., Rehm, H.L., Shendure, J., Abecasis, G.R., Adams,
D.R., Altman, R.B., Antonarakis, S.E., Ashley, E.A. *et al.* (2014) Guidelines for investigating causality of
sequence variants in human disease. *Nature*, **508**, 469-476.

12 Wenzel, D.M., Lissounov, A., Brzovic, P.S. and Klevit, R.E. (2011) UBC7 reactivity profile reveals
parkin and HHAR1 to be RING/HECT hybrids. *Nature*, **474**, 105-108.

13 Ordureau, A., Sarraf, Shireen A., Duda, David M., Heo, J.-M., Jedrykowski, Mark P., Sviderskiy,
Vladislav O., Olszewski, Jennifer L., Koerber, James T., Xie, T., Beausoleil, Sean A. *et al.* (2014)
Quantitative Proteomics Reveal a Feedforward Mechanism for Mitochondrial PARKIN Translocation
and Ubiquitin Chain Synthesis. *Molecular Cell*, **56**, 360-375.

14 Koyano, F., Okatsu, K., Kosako, H., Tamura, Y., Go, E., Kimura, M., Kimura, Y., Tsuchiya, H.,
Yoshihara, H., Hirokawa, T. *et al.* (2014) Ubiquitin is phosphorylated by PINK1 to activate parkin.
Nature, **510**, 162-166.

15 Kazlauskaitė, A., Kondapalli, C., Gourlay, R., Campbell, D.G., Ritorto, M.S., Hofmann, K., Alessi,
D.R., Knebel, A., Trost, M. and Muqit, M.M. (2014) Parkin is activated by PINK1-dependent
phosphorylation of ubiquitin at Serine65. *The Biochemical journal*, **460**, 127-139.

16 Kane, L.A., Lazarou, M., Fogel, A.I., Li, Y., Yamano, K., Sarraf, S.A., Banerjee, S. and Youle, R.J.
(2014) PINK1 phosphorylates ubiquitin to activate Parkin E3 ubiquitin ligase activity. *The Journal of cell
biology*, **205**, 143-153.

17 Sauve, V., Lilov, A., Seirafi, M., Vranas, M., Rasool, S., Kozlov, G., Sprules, T., Wang, J., Trempe, J.F.
and Gehring, K. (2015) A Ubl/ubiquitin switch in the activation of Parkin. *Embo j*, **34**, 2492-2505.

18 Sauvé, V., Sung, G., Soya, N., Kozlov, G., Blaimschein, N., Miotto, L.S., Trempe, J.-F., Lukacs, G.L.
and Gehring, K. (2018) Mechanism of parkin activation by phosphorylation. *Nature structural &
molecular biology*, **25**, 623-630.

19 Gladkova, C., Maslen, S.L., Skehel, J.M. and Komander, D. (2018) Mechanism of parkin activation
by PINK1. *Nature*, **559**, 410-414.

20 Pickles, S., Vigié, P. and Youle, R.J. (2018) Mitophagy and Quality Control Mechanisms
in Mitochondrial Maintenance. *Current Biology*, **28**, R170-R185.

21 Sriram, S.R., Li, X., Ko, H.S., Chung, K.K.K., Wong, E., Lim, K.L., Dawson, V.L. and Dawson, T.M.
(2005) Familial-associated mutations differentially disrupt the solubility, localization, binding and
ubiquitination properties of parkin. *Human molecular genetics*, **14**, 2571-2586.

22 Wang, C., Tan, J.M., Ho, M.W., Zaiden, N., Wong, S.H., Chew, C.L., Eng, P.W., Lim, T.M., Dawson,
T.M. and Lim, K.L. (2005) Alterations in the solubility and intracellular localization of parkin by several
familial Parkinson's disease-linked point mutations. *Journal of neurochemistry*, **93**, 422-431.

23 Hampe, C., Ardila-Osorio, H., Fournier, M., Brice, A. and Corti, O. (2006) Biochemical analysis of
Parkinson's disease-causing variants of Parkin, an E3 ubiquitin-protein ligase with monoubiquitylation
capacity. *Human molecular genetics*, **15**, 2059-2075.

24 Matsuda, N., Kitami, T., Suzuki, T., Mizuno, Y., Hattori, N. and Tanaka, K. (2006) Diverse Effects of
Pathogenic Mutations of Parkin That Catalyze Multiple Monoubiquitylation in Vitro. *Journal of
Biological Chemistry*, **281**, 3204-3209.

610 24 Narendra, D.P., Jin, S.M., Tanaka, A., Suen, D.-F., Gautier, C.A., Shen, J., Cookson, M.R. and Youle,
611 R.J. (2010) PINK1 Is Selectively Stabilized on Impaired Mitochondria to Activate Parkin. *PLoS Biol*, **8**,
612 e1000298.

613 25 Fiesel, F.C., Caulfield, T.R., Moussaoud-Lamodiere, E.L., Ogaki, K., Dourado, D.F., Flores, S.C., Ross,
614 O.A. and Springer, W. (2015) Structural and Functional Impact of Parkinson Disease-Associated
615 Mutations in the E3 Ubiquitin Ligase Parkin. *Human mutation*, **36**, 774-786.

616 26 Lill, C.M., Mashychev, A., Hartmann, C., Lohmann, K., Marras, C., Lang, A.E., Klein, C. and Bertram,
617 L. (2016) Launching the movement disorders society genetic mutation database (MDSGene).
618 *Movement disorders : official journal of the Movement Disorder Society*, **31**, 607-609.

619 27 Katayama, H., Kogure, T., Mizushima, N., Yoshimori, T. and Miyawaki, A. (2011) A Sensitive and
620 Quantitative Technique for Detecting Autophagic Events Based on Lysosomal Delivery. *Chemistry &*
621 *biology*, **18**, 1042-1052.

622 28 Tang, M.Y., Vranas, M., Krahn, A.I., Pundlik, S., Trempe, J.F. and Fon, E.A. (2017) Structure-guided
623 mutagenesis reveals a hierarchical mechanism of Parkin activation. *Nature communications*, **8**, 14697.

624 29 Trempe, J.-F., Sauvé, V., Grenier, K., Seirafi, M., Tang, M.Y., Ménade, M., Al-Abdul-Wahid, S., Krett,
625 J., Wong, K., Kozlov, G. *et al.* (2013) Structure of Parkin Reveals Mechanisms for Ubiquitin Ligase
626 Activation. *Science*, **340**, 1451-1455.

627 30 Kumar, A., Chaugule, V.K., Condos, T.E.C., Barber, K.R., Johnson, C., Toth, R., Sundaramoorthy, R.,
628 Knebel, A., Shaw, G.S. and Walden, H. (2017) Parkin-phosphoubiquitin complex reveals cryptic
629 ubiquitin-binding site required for RBR ligase activity. *Nature structural & molecular biology*, **24**,
630 475-483.

631 31 Safadi, S.S. and Shaw, G.S. (2007) A disease state mutation unfolds the parkin ubiquitin-like
632 domain. *Biochemistry*, **46**, 14162-14169.

633 32 Spratt, D.E., Martinez-Torres, R.J., Noh, Y.J., Mercier, P., Manczyk, N., Barber, K.R., Aguirre, J.D.,
634 Burchell, L., Purkiss, A., Walden, H. *et al.* (2013) A molecular explanation for the recessive nature of
635 parkin-linked Parkinson's disease. *Nature communications*, **4**, 1983.

636 33 Rasool, S., Soya, N., Truong, L., Croteau, N., Lukacs, G.L. and Trempe, J.F. (2018) PINK1
637 autophosphorylation is required for ubiquitin recognition. *EMBO Rep*, **19**.

638 34 Okatsu, K., Koyano, F., Kimura, M., Kosako, H., Saeki, Y., Tanaka, K. and Matsuda, N. (2015)
639 Phosphorylated ubiquitin chain is the genuine Parkin receptor. *The Journal of cell biology*, **209**,
640 111-128.

641 35 Kitada, T., Asakawa, S., Hattori, N., Matsumine, H., Yamamura, Y., Minoshima, S., Yokochi, M.,
642 Mizuno, Y. and Shimizu, N. (1998) Mutations in the parkin gene cause autosomal recessive juvenile
643 parkinsonism. *Nature*, **392**, 605-608.

644 36 Safadi, S.S., Barber, K.R. and Shaw, G.S. (2011) Impact of Autosomal Recessive Juvenile
645 Parkinson's Disease Mutations on the Structure and Interactions of the Parkin Ubiquitin-like Domain.
646 *Biochemistry*, **50**, 2603-2610.

647 37 Geisler, S., Holmstrom, K.M., Skujat, D., Fiesel, F.C., Rothfuss, O.C., Kahle, P.J. and Springer, W.
648 (2010) PINK1/Parkin-mediated mitophagy is dependent on VDAC1 and p62/SQSTM1. *Nature cell*
649 *biology*, **12**, 119-131.

650 38 Panicker, N., Dawson, V.L. and Dawson, T.M. (2017) Activation mechanisms of the E3 ubiquitin
651 ligase parkin. *The Biochemical journal*, **474**, 3075-3086.

652 39 Pickrell, A.M. and Youle, R.J. (2015) The Roles of PINK1, Parkin, and Mitochondrial Fidelity in
653 Parkinson's Disease. *Neuron*, **85**, 257-273.

654 40 Grenier, K., McLelland, G.L. and Fon, E.A. (2013) Parkin- and PINK1-Dependent Mitophagy in
655 Neurons: Will the Real Pathway Please Stand Up? *Frontiers in neurology*, **4**, 100.
656 41 Whitworth, A.J. and Pallanck, L.J. (2017) PINK1/Parkin mitophagy and neurodegeneration—what
657 do we really know in vivo? *Current opinion in genetics & development*, **44**, 47-53.

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659

660 **Figure legends**

661 **Figure 1. *Parkin* missense variants displayed a wide range of functional**
 662 **alterations. (A)** 75 *Parkin* missense variants were reported in disease specific
 663 databases (PDmutDB, MDSgene), 215 were reported in population databases (ExAC,
 664 dbSNP) and 51 were reported in both. Variants were assigned with 1 of 5 standard
 665 ACMG terminologies: *Pathogenic* (red), *likely pathogenic* (pink), *likely benign*
 666 (green), *benign* (olive) and *uncertain significance* (grey). **(B-C)** Quantification of the
 667 function of *Parkin* missense variants assigned as **(B)** *Pathogenic* (red), *likely*
 668 *pathogenic* (pink), *likely benign* (green), *benign* (olive) or **(C)** *Uncertain significance*
 669 (grey) by clinical evidence. Solid bars show mitophagy after 4h of CCCP treatment
 670 quantified from mtKeima signal in U2OS cells expressing GFP-*Parkin* variants
 671 normalized to wildtype (WT) *Parkin*. Hatched bars show GFP intensity of cells
 672 expressing GFP-*Parkin* variants normalized to WT *Parkin*. * $P < 0.05$, ** $P < 0.01$, in
 673 one-way ANOVA with Dunnett's post-hoc test comparing the function of each variant
 674 with WT. N=3-7.

675 **Figure 2. Integration of clinical and functional evidence refined the classification**
 676 **of *Parkin* variants. (A)** Functional alteration of variants assigned as *pathogenic* (red),
 677 *likely pathogenic* (pink), *likely benign* (green), *benign* (olive), and of *uncertain*
 678 *significance* (grey) based on clinical evidence were plotted for mitophagy activity on
 679 the X axis and GFP intensity on the Y axis. Functional alteration segregated into five
 680 groups, indicated by black boxes. 1. Significantly decreased mitophagy activity and
 681 GFP intensity compared with WT. 2. Severely decreased mitophagy activity with WT

682 GFP intensity. 3. Moderately decreased mitophagy activity with WT GFP intensity. 4.
 683 WT mitophagy activity and GFP intensity. 5. Significantly increased mitophagy
 684 activity with WT GFP intensity. **(B)** Quantification of the variants within each of the
 685 functional groups from (A). **(C)** Quantification of variants within the functional
 686 groups described in (A) according to their segregation or lack of segregation with PD
 687 in families. **(D)** Quantification of variants within the functional groups described in
 688 (A) according to the observation of the variant as more than 1 homozygote (blue), 1
 689 homozygote (light-blue), or no homozygotes (white) in ExAC. **(E)** Quantification of
 690 variants within the functional groups described in (A) according to the observation of
 691 the variant in PD patients. **(F)** Quantification of variants within the functional groups
 692 described in (A) according to their classification based on clinical and functional
 693 evidence.

694 **Figure 3. Steric clashes in structural simulations predicted the dysfunction of**
 695 ***Parkin* variants.** **(A)** Schematic representation of *Parkin* missense variants on *Parkin*
 696 protein 2D structure. Each circle indicates a missense variant. The location of the
 697 variant on the 2D sequence was plotted in the X axis with dotted lines separating the
 698 *Parkin* domains. The functional groups described in Figure 2A, were plotted on the Y
 699 axis. The colors indicate the type of clash introduced by the missense variant from
 700 structural simulation. **(B)** Distribution of the variants from (A) within functional
 701 groups according to the type of clash they introduce. **(C)** Structure of human *Parkin*
 702 bound to phospho-ubiquitin (PDB 5n2w) was used to illustrate the impact of the R42P
 703 mutation. Substitution of the arginine side chain (black) to proline (white) introduced

major clashes (red disks), which would destabilize the β -sheet in the Ubl. **(D)** Substitution of the arginine side chain (black) to histidine (white) introduced mild clashes (red and green disks). **(E)** Substitution of the arginine side chain (black) to cysteine (white) did not introduce any clashes.

Figure 4. Structural analysis of *Parkin* variants revealed various pathogenic mechanisms. **(A)** Pathogenic variants were mapped onto the 3D structure of human Parkin bound to phospho-ubiquitin (PDB 5N2W). The side-chains of the amino acids substituted by the variants were highlighted in black. The blue spheres represent the phosphate of pUb. The grey spheres represent zinc. The color of the text indicates the type of disruption to Parkin caused by the variant. **(B)** Close-up view of pUbl-RING0 interface in the structure of fly pParkin bound to phospho-Ub (PDB 6DJX). Lys161 and Lys211 form ionic interactions with the phosphate on Ser65 of the pUbl. Mutations of these lysine residues would weaken the pUbl-RING0 interaction, preventing activation of Parkin. **(C)** Close-up view of the pUb:RING1 interface in human Parkin bound to pUb (PDB 5N2W). The G284R variant in Parkin RING1 would introduce major clashes with pUb, disrupting the interaction. **(D)** Close-up view of Arg275 in human Parkin bound to pUb (PDB 5N2W). Arg275 interacts with Glu321 in the helix that interacts with pUb. Mutation to a tryptophan (white) would introduce clashes with this helix as well as Ser10 in the Ubl domain.

Figure 5. Structure-guided designer hyperactive *Parkin* mutants can rescue mitophagy in *pathogenic* variants. **(A)** Quantification of induced mitophagy after 4h

725 of CCCP treatment in U2OS cells expressing WT GFP-Parkin, *pathogenic* missense
726 variants, or W403A or F146A *in cis* with WT or *pathogenic* variants. Mitophagy
727 mediated by each Parkin missense variant was normalized to that of WT Parkin in
728 each replicate. * $P < 0.05$, ** $P < 0.01$, in two-way ANOVA with Dunnett's post-hoc
729 test comparing the function of each variant with the variant *in cis* with W403A or
730 F146A. N=3-7. **(B)** The number of families or individuals with PD carrying the
731 *pathogenic* missense variants for which mitophagy was or was not rescued by the
732 designer mutations are shown. **(C)** The sum of the MAF in ExAC of the *pathogenic*
733 missense variants for which mitophagy was or was not rescued by the designer
734 mutations are shown.

735 **Figure 6. Structural basis for the effects of the naturally occurring hyperactive**
736 **Parkin variants.** **(A)** Close-up view of R234Q and R256C variant sites in the
737 structure of human Parkin bound to pUb (PDB 5N2W). Arg256 forms a hydrogen
738 bond with Glu402, and its mutation to a cysteine would destabilize the REP:RING1
739 interaction, similar to W403A. The side-chain of Arg234 also stacks with the indole
740 ring of Trp403. **(B)** Close-up view of M458L variant site in the RING0:RING2
741 interface (PDB 5N2W). M458L introduced major clashes that would destabilize the
742 RING0:RING2 interaction, similarly to F146A. **(C)** Close-up view of V224A variant
743 site (PDB 5N2W). Val224 interacts with pUb and forms van der Waals force
744 interactions with Asn60. Mutation to alanine could modulate the affinity for pUb.

745 **Figure 7. The naturally-occurring *Parkin* p.V224A hyperactive variant rescued**
746 **mitophagy in several *pathogenic* variants.** (A) Quantification of GFP intensity from
747 GFP signal by FACS in untreated cells expressing WT GFP-Parkin, *pathogenic*
748 missense variants, or p.V224A *in cis* with WT or *pathogenic* variants. The GFP
749 intensity for each Parkin missense variant was normalized to that for WT Parkin in
750 each replicate. (B) Quantification of induced mitophagy after 4h of CCCP treatment
751 in cells expressing WT GFP-Parkin, *pathogenic* missense variants, or p.V224A *in cis*
752 with WT or *pathogenic* variants. Mitophagy mediated by each Parkin missense
753 variant was normalized to that of WT Parkin in each replicate. * P<0.05; ** P<0.01,
754 in two-way ANOVA with Dunnett's post-hoc test comparing the function of each
755 variant with variant *in cis* with p.V224A. N=3-7.

Figure 1. Parkin missense variants displayed a wide range of functional alterations.

Figure 2. Integration of clinical and functional evidence refined the classification of *Parkin* variants.

Figure 3. Steric clashes in structural simulations predicted the dysfunction of *Parkin* variants.

Figure 4. Structural analysis of *Parkin* variants revealed various pathogenic mechanisms.

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C

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Figure 5. Structure-guided designer hyperactive Parkin mutants can rescue mitophagy in *pathogenic* variants.

Figure 6. Structural basis for the effects of the naturally occurring hyperactive Parkin variants.

Figure 7. The naturally-occurring *Parkin* p.V224A hyperactive variant rescued mitophagy in several *pathogenic* variants.