

This is a repository copy of *Vitamin B12 modulates Parkinson's disease LRRK2 kinase activity through allosteric regulation and confers neuroprotection*.

White Rose Research Online URL for this paper:

<https://eprints.whiterose.ac.uk/142412/>

Version: Accepted Version

Article:

Schaffner, Adam, Li, Xianting, Gomez-Llorente, Yacob et al. (17 more authors) (2019) Vitamin B12 modulates Parkinson's disease LRRK2 kinase activity through allosteric regulation and confers neuroprotection. *Cell Research*. pp. 313-329. ISSN 1748-7838

<https://doi.org/10.1038/s41422-019-0153-8>

Reuse

Items deposited in White Rose Research Online are protected by copyright, with all rights reserved unless indicated otherwise. They may be downloaded and/or printed for private study, or other acts as permitted by national copyright laws. The publisher or other rights holders may allow further reproduction and re-use of the full text version. This is indicated by the licence information on the White Rose Research Online record for the item.

Takedown

If you consider content in White Rose Research Online to be in breach of UK law, please notify us by emailing eprints@whiterose.ac.uk including the URL of the record and the reason for the withdrawal request.

1 **Title:**

2 **Vitamin B₁₂ modulates Parkinson's disease LRRK2 kinase activity through**
3 **allosteric regulation and confers neuroprotection**

4
5 **(Short title: Vitamin B₁₂ modulates LRRK2 activity and toxicity)**

6
7 Adam Schaffner^{1,2}, Xianting Li¹, Yacob Gomez-Llorente², Emmanouela Leandrou³, Anna
8 Memou³, Nicolina Clemente⁴, Chen Yao⁵, Farinaz Afsari⁶, Lianteng Zhi⁷, Nina Pan¹, Keita
9 Morohashi², Xiaoluan Hua^{1,2}, Ming-Ming Zhou², Chunyu Wang⁴, Hui Zhang⁷, Shu G. Chen⁵,
10 Christopher J. Elliott⁶, Hardy Rideout³, Iban Ubarretxena-Belandia^{2,8}, and Zhenyu Yue¹

11
12 These authors contributed equally: Adam Schaffner, Xianting Li

13 Correspondence: Zhenyu Yue (zhenyu.yue@mssm.edu)

14
15 **Affiliation**

16 ¹Department of Neurology and Neuroscience, Friedman Brain Institute, Icahn School of
17 Medicine at Mount Sinai, New York, NY 10029

18 ²Department of Pharmacological Sciences, Icahn School of Medicine at Mount Sinai, New York,
19 NY 10029

20 ³Division of Basic Neurosciences, Biomedical Research Foundation of the Academy of Athens,
21 Athens, Greece

22 ⁴Department of Biological Sciences, Center for Biotechnology and Interdisciplinary Studies,
23 Rensselaer Polytechnic Institute, Troy, New York 12180, USA

24 ⁵Department of Pathology, Case Western Reserve University, Cleveland, OH 44106, USA

25 ⁶Department of Biology, University of York, York, YO1 5DD, UK

26 ⁷Department of Neuroscience, Thomas Jefferson University, Philadelphia, PA 19107, USA

27 ⁸Biofisika Institute (CSIC, UPV/EHU), University of the Basque Country, Leioa, Spain

28
29 **Classification:** Biological Sciences

39 **Abstract**

40 Missense mutations in *Leucine-Rich Repeat Kinase 2* (LRRK2) cause the majority of familial
41 and some sporadic forms of Parkinson's disease (PD). The hyperactivity of LRRK2 kinase
42 induced by the pathogenic mutations underlies neurotoxicity, promoting the development of
43 LRRK2 kinase inhibitors as therapeutics. Many potent and specific small molecule LRRK2
44 inhibitors have been reported with promise. However, nearly all inhibitors are ATP competitive
45 – some with unwanted side effects and unclear clinical outcome - alternative types of LRRK2
46 inhibitors are lacking. Herein we find 5'-deoxyadenosylcobalamin (AdoCbl), a physiological
47 form of the essential micronutrient vitamin B₁₂ as a mixed-type allosteric inhibitor of LRRK2
48 kinase activity. Multiple assays show that AdoCbl directly binds LRRK2, leading to the
49 alterations of protein conformation and ATP binding in LRRK2. STD-NMR analysis of a
50 LRRK2 homologous kinase reveals the contact sites in AdoCbl that interface with the kinase
51 domain. Furthermore, we provide evidence that AdoCbl modulates LRRK2 activity through
52 disruption of LRRK2 dimerization. Treatment with AdoCbl inhibits LRRK2 kinase activity in
53 cultured cells and brain tissue, and importantly prevents neurotoxicity in primary rodent cultures
54 as well as in transgenic *C. elegans* and *D. melanogaster* expressing LRRK2 disease variants.
55 Finally, AdoCbl alleviates deficits in dopamine release sustainability caused by *LRRK2* disease
56 variants in mouse models. Our study uncovers vitamin B₁₂ as a novel class of LRRK2 kinase
57 modulator with a distinct mechanism, which can be harnessed to develop new LRRK2-based PD
58 therapeutics in the future.

59

60 **Key words:** Vitamin B₁₂, LRRK2, Parkinson's disease, kinase

61 **Introduction**

62 Parkinson's disease (PD) is the most common chronic neurodegenerative movement
63 disorder affecting 1% of the world population over the age of sixty. The pathological hallmarks
64 of PD include the age-dependent loss of dopaminergic neurons in the substantia nigra and the
65 progressive spatiotemporal distribution of Lewy bodies and Lewy neurites¹. There is currently no
66 cure or disease-modifying therapy for PD, and available treatments target only the symptoms of
67 the disease but not its progression². In addition, the pathogenesis of PD remains poorly
68 understood. Discovered over a decade ago, Leucine-Rich Repeat Kinase 2 (LRRK2) has now
69 emerged as a major target not only for understanding the molecular basis of PD pathogenesis but
70 also for therapeutic intervention³.

71 Missense mutations in the *PARK8/LRRK2* gene represent the prevalent cause for
72 autosomal-dominant PD^{4,5}. In addition, *LRRK2* mutations have been implicated in a significant
73 number of sporadic PD cases⁶⁻⁹. PD-linked *LRRK2* variants associate with neuropathologies and
74 clinical symptoms indistinguishable from idiopathic PD cases^{10,11}, suggesting that both inherited
75 and sporadic forms of the disease share a similar pathogenic mechanism. *LRRK2* encodes a
76 286kDa protein containing catalytic GTPase and kinase domains, as well as Armadillo, Ankyrin,
77 LRR and WD40 protein-protein interaction accessory domains (**Fig. 1a**). LRRK2 adopts a highly
78 compact dimer structure with extensive intramolecular interactions¹², and dimerization has been
79 proposed to correlate with LRRK2 kinase activity *in vitro*¹³. Of the six reported pathogenic
80 mutations, the G2019S variant has the highest prevalence¹⁴, accounting for 1% of sporadic and
81 5% of hereditary PD cases worldwide¹⁰, and up to 30–40% of all PD cases among North
82 Africans and Ashkenazi Jews¹⁵. Located in a conserved region of the kinase activation loop, the
83 G2019S variant has been consistently associated with increased LRRK2 kinase activity *in*

84 *vitro*^{13,16-18} and *in vivo*¹⁹⁻²². In addition, the G2019S variant also increases the phosphorylation of
85 a subset of Rab GTPases, recently identified as promising physiological LRRK2 substrates^{23,24}.

86 Multiple lines of evidence demonstrate that LRRK2 kinase hyperactivity caused by PD
87 pathogenic mutations, including G2019S, is causal to neurotoxicity or neuronal dysfunctions.
88 LRRK2 kinase inhibitors attenuate the cell toxicity caused by the G2019S mutation in primary
89 cortical neurons²⁵ and normalize G2019S-mediated postsynaptic abnormal activity in brain slice
90 cultures²⁶. In addition, LRRK2 kinase activity inhibitors prevent G2019S-potentiated α -
91 synuclein accumulation in dopaminergic neurons^{27,28}, and their administration suppressed
92 neurodegeneration in *C. elegans*, *D. melanogaster* and mouse PD models^{25,29-31}. Consequently,
93 extensive effort has been devoted to the development of ATP-competitive small molecule
94 LRRK2 kinase inhibitors. Early generation kinase inhibitors displayed high potency against
95 LRRK2, but lacked the specificity required to be considered for therapeutics^{25,32-34}. Among the
96 next generation, several inhibitors were highly potent and specific, but did not possess the
97 pharmacokinetic properties for effective brain penetration^{35,36}, while others elicited dose toxicity
98 and abnormal lung phenotypes in nonhuman primates³⁷. The current generation of ATP-
99 competitive inhibitors show promise, but will require further modification³⁸ and preclinical
100 testing³⁹ before their therapeutic potential can be fully assessed. Remarkably, LRRK2 kinase
101 activity inhibitors displaying alternative mechanisms of inhibition to these ATP-competitive
102 inhibitors have yet to be reported.

103 Here we discovered that the FDA-approved natural compound 5'-
104 deoxyadenosylcobalamin (AdoCbl), one of two physiological forms of the essential human
105 micronutrient vitamin B₁₂, is a unique mixed-type allosteric modulator of LRRK2 kinase
106 activity. AdoCbl is capable of disturbing LRRK2 protein conformation and dimerization. In

107 addition, we explore the ability of AdoCbl to prevent mutant *LRRK2* induced neurotoxicity and
108 Parkinson-like phenotypes in PD animal models. We conclude that vitamin B₁₂ is a novel type of
109 LRRK2 kinase modulator, which is distinguished from other ATP competitive inhibitors. Future
110 experiment should investigate the structural basis for LRRK2- vitamin B₁₂ interaction that can be
111 harnessed to develop new therapeutics for LRRK2-based PD.

112

113 **Results**

114 **Identification of vitamin B₁₂ as a LRRK2 kinase activity inhibitor**

115 AdoCbl (Supplimentary information, **Fig. S1a**) was identified as a kinase activity inhibitor of
116 FLAG-tagged wild type (WT) LRRK2 purified from BAC transgenic mouse brain¹⁹
117 (Supplimentary information, **Fig. S1b**) from a high-throughput screen (HTS) of a small library of
118 2,080 FDA-approved compounds (Supplimentary information, **Table S1**). To assay LRRK2
119 kinase activity we measured the time-resolved fluorescence resonance energy transfer (TR-
120 FRET) between phosphorylated Fluorescein-LRRKtide peptide and Terbidium-labeled anti-
121 pLRRKtide antibody⁴⁰. In this screen AdoCbl displayed a half-maximal inhibitory concentration
122 (IC₅₀) of 1.2 μM (**Fig. 1b**). Vitamin B₁₂ consists of a central cobalt ion that is equatorially
123 chelated by a tetradentate corrin macrocycle and up to two axially coordinating ligands
124 (Supplimentary information, **Fig. S1a**). The ‘lower’ (α)-coordinating ligand is usually a
125 dimethylbenzimidazole (DMZ) base that connects to the f-side chain of the chelator by an α-
126 ribazole containing backbone⁴¹.

127 Vitamin B₁₂ has additional forms in addition to AdoCbl, including cyanocobalamin
128 (CNCbl), hydroxycobalamin (HOCbl), and methylcobalamin (MeCbl), which are distinguished
129 by their (β)-coordinating ligand⁴² (Supplimentary information, **Fig. S1a**). Only MeCbl and

130 AdoCbl are physiologically active in cells, as coenzymes of MeCbl-dependent methionine
131 synthase and AdoCbl-dependent methylmalonyl coenzyme A mutase⁴². In humans, these
132 enzymatic reactions play a key role in the metabolism of amino acids, nucleotides, and fatty
133 acids, in addition to the normal functioning of the nervous system, and the formation of red
134 blood cells⁴³.

135 Similar to AdoCbl, these other forms of vitamin B₁₂ inhibited the LRRK2 catalyzed
136 phosphorylation of the LRRKtide peptide with an IC₅₀ of ~1 μM (**Fig. 1b**), suggesting that the
137 nature of the (β)-coordinating ligand was not essential for the inhibition. Next, we validated the
138 LRRK2 kinase inhibition by the various forms of vitamin B₁₂ using highly pure strep-tagged
139 LRRK2-WT expressed in HEK293 cells (Supplimentary information, **Fig. S1c**). To this end, we
140 assayed LRRK2 autophosphorylation (**Fig. 1c**) and phosphorylation of the generic substrate
141 myelin basic protein (MBP) (**Fig. 1d**) by measuring the incorporation of radioactive ³²P. In both
142 cases, we determined IC₅₀s in the ~10 μM range for each form of vitamin B₁₂. We note that
143 AdoCbl inhibited LRRK2-G2019S catalyzed phosphorylation of the recently identified LRRK2
144 physiological substrate Rab10^{23,24} also with a ~10 μM IC₅₀ (**Fig. 1e**).

145 Next, we derived Mouse Embryonic Fibroblast (MEF) cells from our LRRK2-G2019S
146 BAC transgenic mice¹⁹, and incubated them with AdoCbl, CNCbl, HOCbl, or MeCbl to measure
147 their effect on LRRK2 autophosphorylation. As a readout we measured autophosphorylation
148 using anti-LRRK2 pS935⁴⁴ and pS1292²⁰ antibodies. In this system both antibodies reported
149 decreased LRRK2 autophosphorylation levels upon treatment with the established LRRK2 GNE-
150 1023²⁰ inhibitor (Supplimentary information, **Figs. S2a-b**). These MEFs constitutively
151 overexpress the pathogenic LRRK2-G2019S variant, which in accordance with literature
152 displayed kinase hyperactivity compared to LRRK2-WT¹⁹⁻²² (Supplimentary information, **Fig.**

153 **S2c**). Interestingly, we found that only AdoCbl, but not the other forms of vitamin B₁₂, exhibited
154 inhibition of LRRK2-G2019S autophosphorylation in MEF cells with an IC₅₀ of ~10 μM, similar
155 to that measured in vitro (**Figs. 1f-g**). We observed a similar inhibition profile for the different
156 forms of vitamin B₁₂ in macrophages derived from the LRRK2-G2019S transgenic mice
157 (Supplementary information, **Fig. S2d**). The lack of inhibition displayed by CNCbl, HOCbl, or
158 MeCbl is not understood at present, but one possibility is that differences in cellular uptake,
159 localization and metabolism in the cells affect their efficacy compared to AdoCbl. Because
160 AdoCbl showed the greatest potential for LRRK2 inhibition in cultured cells we focused our
161 research efforts on this physiological form of vitamin B₁₂.

162

163 **AdoCbl binds directly to LRRK2**

164 We next tested if AdoCbl binds directly to human LRRK2. We first used agarose functionalized
165 with AdoCbl to pull-down purified LRRK2, which could be eluted as a function of AdoCbl
166 concentration (**Fig. 2a** and **Supplementary information, Fig. S3**). In thermal shift assays
167 (TSA)⁴⁵ the melting temperature of LRRK2 increased substantially from 50 to 54°C in the
168 presence of AdoCbl (**Fig. 2b**). This thermostabilization by AdoCbl was comparable to that
169 measured in the presence of the established LRRK2 kinase inhibitor PF-06447475³⁸.
170 Furthermore, we applied microscale thermophoresis (MST)⁴⁶ to measure the binding affinity of
171 AdoCbl for LRRK2 and determined an apparent dissociation equilibrium constant (K_D) of 12.0
172 and 4.1 μM for purified LRRK2-WT and LRRK2-G2019S, respectively (**Fig. 2c**), in agreement
173 with our IC₅₀ values. As a validation of the MST assay, under the same conditions LRRK2-
174 G2019S binds PF-06447475 with a K_D of 70 nM (**Fig. 2d**), in line with the reported IC₅₀ of 11
175 nM for this inhibitor³⁸.

176 We next sought to identify the functional groups in AdoCbl responsible for interacting
177 with LRRK2 by using an extension of the saturation transfer difference (STD) NMR method
178 termed ATP-STD NMR⁴⁷. This method was developed to screen protein kinase inhibitors by
179 recording STD signals in the presence of competing ATP⁴⁸. Due to an unstable and aggregation
180 prone human LRRK2 kinase domain, which makes it intractable for biophysical/structural
181 analysis, we decided to use the humanized kinase domain of the Roco4 protein from
182 *Dictyostelium discoideum*⁴⁹. The kinase domain of Roco4 (amino acids 1018–1292) has a 47%
183 similarity with the kinase domain of human LRRK2 (amino acids 1879–2138), and its
184 humanized variant, where amino acid residues F1107 and F1161 are substituted for Leucine to
185 mimic the residues L1949 and L2001 in LRRK2, is considered a valuable model for the
186 structural characterization of LRRK2 kinase inhibitors^{47,49}. We first verified that AdoCbl
187 inhibited autophosphorylation of the humanized Roco4 kinase (**Fig. 2e**) with an IC₅₀ of 73.6 μM
188 (**Fig. 2f**). In agreement with published data⁴⁷, we obtained a clear STD signal for ATP binding to
189 Roco4 kinase (**Fig. 2g, orange spectrum**). Addition of AdoCbl to the ATP/Roco4 sample in a
190 1:1 AdoCbl:ATP ratio resulted in the emergence of additional STD signals corresponding to
191 AdoCbl protons, confirming a direct binding of AdoCbl to Roco4. At a 10:1 AdoCbl:ATP ratio,
192 the STD peaks corresponding to AdoCbl became much stronger while ATP peaks weakened,
193 demonstrating that AdoCbl diminished ATP binding to Roco4 kinase. Stronger STD signals
194 likely correspond to AdoCbl protons in the vicinity or at the protein binding interface. These
195 protons are distributed around one side of the molecule and are contributed by the adenine,
196 corrin, and DMZ moieties (**Fig. 2h**).

197

198 **AdoCbl is a mixed-type allosteric LRRK2 kinase inhibitor**

199 The current collection of commercially available LRRK2 kinase inhibitors, including the latest
200 generation PF-06447475³⁸ and MLi-2³⁹, are considered to be ATP-competitive, with the
201 exception of FX2149 that is GTP-competitive⁵⁰. To determine the mode of inhibition of vitamin
202 B₁₂ we measured V_{max} and K_m as a function of ATP and in the presence of an increasing
203 concentration of AdoCbl. For our *in vitro* inhibition kinetics assays we used purified full-length
204 LRRK2-WT and LRRK2-G2019S to measure relative velocity based on the quantification of
205 LRRK2 autophosphorylation pS1292 signal (Supplementary information, **Fig. S4**). Consistent
206 with literature^{13,16-18}, the relative velocity of LRRK2-G2019S was two-fold higher than LRRK2-
207 WT (Supplementary information, **Table S2**). Titration of AdoCbl caused a decrease in apparent
208 V_{max} and an increase in apparent K_m (**Figs. 3a-c**), suggesting mixed-type inhibition, as confirmed
209 by reciprocal Lineweaver-Burk plots of the data (**Figs. 3b-d and Supplementary information,**
210 **Table S2**). Mixed-type inhibitors generally bind to an allosteric site and can affect an enzyme's
211 ability to catalyze a reaction and to bind its substrate⁵¹. To further characterize the mode of
212 AdoCbl inhibition of LRRK2 we measured the competition with AMP-PNP using MST (**Fig.**
213 **3e**). Under our assay conditions AMP-PNP displayed a K_D of 0.9 μ M against LRRK2 (**Fig. 3f**).
214 In further support of the mixed-inhibition mode of action, increasing concentrations of AMP-
215 PNP reduced but did not overcome the binding of AdoCbl to LRRK2 (**Fig. 3e**).

216 We further compared AdoCbl with the reported ATP-competitive LRRK2 inhibitor IN-1
217 (LRRK2-IN-1) for its ability to inhibit the human LRRK2 A2016T variant. The A2016T
218 substitution in the ATP-site of LRRK2 results in a normally active enzyme, which is, however,
219 significantly less sensitive to the ATP-competitive inhibitors H-1152 and sunitinib⁵², and up to
220 400-fold less sensitive against LRRK2-IN-1 compared to the WT protein³⁵. Using the G2019S
221 mutation as a background we confirmed that the A2016T substitution confers resistance to

222 LRRK2-IN-1 (Supplimentary information, **Figs. S5a-b**). In contrast, this variant displayed a
223 similar level of inhibition by AdoCbl as the G2019S protein alone. Similarly WT Roco4 from *D.*
224 *discoideum*^{47,49}, which has a 241-fold lower affinity for LRRK2-IN-1 than humanized Roco4⁴⁷,
225 was inhibited by the same degree as the humanized version (Supplimentary information, **Figs.**
226 **S5c-d**). The data suggests distinct mechanisms of LRRK2 binding between LRRK2-IN-1 and
227 AdoCbl.

228

229 **AdoCbl induces conformational changes and disrupts dimerization of LRRK2**

230 Allosteric inhibitor binding normally induces a conformational change in the enzyme that results
231 in reduced affinity for the substrate⁵³. To test the possibility of an AdoCbl-induced
232 conformational change in LRRK2 we conducted limited proteolysis assays in the presence of
233 AdoCbl. The addition of AdoCbl markedly increased the susceptibility of LRRK2 to proteolysis
234 by both trypsin and chymotrypsin (**Fig. 4a**). At a 90-min. interval, we showed that the presence
235 of AdoCbl significantly increases the sensitivity of trypsin degradation of LRRK2 protein in a
236 dose dependent manner (**Fig. 4b**). This observation does not result from an enhancement of the
237 intrinsic activity of these proteases, as demonstrated by the fact that AdoCbl did not affect the
238 proteolysis rate of a control kinase TBK1 (Supplimentary information, **Fig. S6a**). Of note, the
239 well-known ATP-competitive LRRK2 kinase inhibitors GSK2578215A³⁶, GNE-1023²⁰ and PF-
240 06447475³⁸, produced the opposite effect as AdoCbl, i.e. they protected LRRK2 from proteolytic
241 digestion (**Fig. 4b**), whereas AMP-PNP had no effect (**Supplimentary information, Fig. S6b**).
242 For more evidence in support of conformational changes in LRRK2 upon binding to AdoCbl we
243 measured the intrinsic fluorescence of LRRK2 as a function of AdoCbl. With 27 tryptophan
244 amino acid residues, the fluorescence emission spectra of LRRK2 at an excitation wavelength of
245 295 nm displayed an AdoCbl dose-dependent decrease in fluorescence intensity (**Fig. 4c**). Note

246 that AdoCbl absorbs light at 295 nm and 340-360 nm, thus it was necessary to correct for the
247 inner-filter effect (**Supplementary information, Figs. S6c-d**). Such a decrease in fluorescence
248 intensity is consistent with conformational changes in LRRK2 where initially buried tryptophan
249 residues become exposed to the solvent as a function of AdoCbl.

250 We hypothesize that the effect of AdoCbl binding in LRRK2 conformation alters
251 oligomeric state of LRRK2. Ample evidence now supports dimeric LRRK2 as the main
252 oligomeric species of the enzyme *in vitro*¹², and available data indicates that LRRK2
253 dimerization correlates with kinase activity¹³. We quantified cellular LRRK2 dimers using a
254 novel adaptation of the proximity biotinylation approach. We expressed recombinant LRRK2
255 protein fusion to BirA (biotin ligase) or to AP (acceptor peptide) in HEK293T cells in the
256 presence of vehicle or AdoCbl. As a negative control BirA- and AP-LRRK2 expressing cells
257 were lysed without having been given the biotin pulse; and in such samples, the number of
258 labeled LRRK2 dimers purified on streptavidin plates is negligible (**Fig. 4d**). Expression levels
259 of both forms of LRRK2 are comparable as determined by ELISA or Western immunoblot. Cells
260 co-expressing BirA-LRRK2/AP-LRRK2 contain robust levels of biotinylated LRRK2 dimers,
261 normalized to total expression of LRRK2. However, in cells treated with AdoCbl, we detected a
262 dose-dependent reduction in the levels of LRRK2 dimers, both in cells expressing WT LRRK2,
263 as well as in cells expressing the LRRK2-G2019S or LRRK2-I2020T variant proteins (**Fig. 4e**).
264 In contrast to other LRRK2 kinase inhibitors⁵⁴⁻⁵⁶, we did not observe a significant decrease in
265 LRRK2 expression following treatment with AdoCbl (**Figs. 4d-f**), indicating that this compound
266 disrupts LRRK2 dimers without affecting expression.

267

268 **AdoCbl protects dopaminergic neurons from *LRRK2-G2019S* induced neurotoxicity in *C.***
269 ***elegans***

270 The degeneration of dopaminergic (DAergic) neurons is the pathological hallmark of PD².
271 However, recapitulating this phenotype in mammalian models of LRRK2-linked PD has been a
272 challenge, nearly all reported genetic models (either transgenic overexpression or knock-in)
273 failed to display clear neurodegeneration^{19,57-59}. In contrast, invertebrate PD models of LRRK2
274 show a robust degeneration of DAergic neurons⁶⁰⁻⁶³. The DAergic pathway in *C. elegans* is
275 important for the basal slowing response, a behavior by which worms slow their locomotive
276 movement when encountering food⁶⁴. Transgenic *LRRK2-G2019S* nematodes exhibit progressive
277 impairment of the basal slowing response, but this locomotive behavioral deficit can be restored
278 by treatment with the LRRK2 kinase inhibitors LRRK2-IN-1 and TTT-3002³⁰. In this *C. elegans*
279 model of LRRK2 PD, these two inhibitors targeted specifically LRRK2, as they were ineffective
280 against the neurodegenerative phenotype displayed in transgenic *LRRK2-A2016T/G2019S* worms
281 carrying the inhibitor-resistant LRRK2 A2016T mutation³⁰. Therefore, we selected the
282 established human *LRRK2-G2019S C. elegans* model to ask if AdoCbl protects against DAergic
283 neuron degeneration. *LRRK2-G2019S* transgenic worms that were fed up to 1.25 μ M AdoCbl
284 during their larval stage resisted the locomotive behavioral deficit on adult day 3 in a dose-
285 dependent manner with a half-maximal effective concentration (EC₅₀) value of 0.53 μ M (**Fig.**
286 **5a**). Consistent with the lack of an effect of the LRRK2 A2016T mutation on the inhibition by
287 AdoCbl in vitro (**Supplementary information, Figs. S5a-b**), treating transgenic *LRRK2-*
288 *A2016T/G2019S* worms with AdoCbl resulted in a rescued neurodegenerative phenotype
289 (**Supplementary information, Fig. S7a**). The data also supports the distinct mechanism of AdoCbl
290 in blocking LRRK2 activity and LRRK2 associated neurodegeneration from IN-1. Additionally,

291 the AdoCbl-induced rescue was observed in transgenic *LRRK2-R1441C* worms as well, which
292 also display an impaired basal slowing response (Supplimentary information, **Fig. S7b**). *C.*
293 *elegans* possess eight DAergic neurons that can be readily visualized by coupling GFP to a DA-
294 neuron-specific promoter. Four GFP-tagged DAergic neurons of cephalic sensilla (CEP neurons)
295 in the head were examined using fluorescence microscopy. Overexpression of *LRRK2-G2019S*
296 causes age-dependent degeneration of these DAergic neurons, where less than 60% remain on
297 adult day 9, compared to 75% in control worms expressing *GFP* alone. When fed 1.25 μ M
298 AdoCbl during their larval stage, age-synchronized adult *LRRK2-G2019S* worms displayed a
299 robust increase in DAergic neuron survival, nearly back to the levels of the GFP-control worms
300 (**Figs. 5b-c**). As in the case of TTT-3002 and *LRRK2-IN-1*³⁰, treatment of WT worms
301 expressing GFP marker alone with AdoCbl did not result in any significant changes in basal
302 slowing response or DAergic neuron survival, suggesting that the effect of AdoCbl was specific
303 to the transgenic *LRRK2-G2019S* worms.

304

305 **AdoCbl prevents *LRRK2-G2019S* induced neurotoxicity in *D. melanogaster* model of PD**

306 Signal regulation in the human retina depends largely on dopamine⁶⁵, and this process can be
307 affected by the loss of DA that is characteristic in PD patients⁶⁶. In *D. melanogaster*, vision is
308 also regulated by comparable DAergic circuits^{67,68}. Transgenic overexpression of the human
309 *LRRK2-G2019S* gene in *D. melanogaster* has been shown to elicit DA-dependent retinal
310 degeneration and loss of visual response due to an abnormal increase in contrast sensitivity,
311 which can be rescued using *LRRK2* kinase activity inhibitors^{31,69}. After feeding *Drosophila*
312 larvae with AdoCbl at concentrations up to 2.5 μ M, we recorded the visual response to flickering
313 blue light in 1-day-old flies. Our Fast Fourier Transform (FFT) algorithm separates this visual

314 response according to the first three stages of the fly visual system: photoreceptors, lamina
315 neurons, and medulla neurons (**Fig. 6a**). Increasing the contrast of the flickering light resulted in
316 a greater retinal response of the fly, revealing characteristic Contrast Response Functions (CRFs)
317 that are dependent on the combination of genotype and AdoCbl treatment (**Fig. 6b**). In the
318 photoreceptors and lamina neurons, the physiological response increased and plateaued at 70%
319 applied contrast, while the medulla neurons generated a complex response, as indicated by the
320 peak response at 40% applied contrast. In each case, flies with dopaminergic expression of
321 *LRRK2-G2019S* have a much greater response compared to those expressing *LRRK-WT*.
322 Notably, feeding flies with 2.5 μ M AdoCbl throughout larval life rescued the *LRRK2-G2019S*
323 phenotype completely, with the photoreceptor, lamina neurons and medulla neurons all showing
324 a CRF close to that of WT flies (**Fig. 6b**). Furthermore, titration of AdoCbl revealed an EC_{50}
325 between 250 and 500 nM in all three stages of the visual pathway (**Fig. 6c, and Supplementary**
326 **information, Fig. S8**). To determine the specificity of AdoCbl toward LRRK2 kinase activity,
327 we tested a 2.5 μ M concentration against flies with a kinase-dead background (*LRRK2-G2019S-*
328 *K1906M*) and found no significant change in any of the three measured regions (**Fig. 6d**). In
329 contrast to the results from *C. elegans*, treating wild-type *drosophila* with a 2.5 μ M
330 concentration of AdoCbl significantly rescues the visual response (**Fig. 6e**). Finally, we tested for
331 off-target effects by feeding 2.5 μ M of AdoCbl to flies with little expression of the LRRK2
332 homolog (*dLRRK⁻*) and found no statistically significant difference between any of the
333 photoreceptor, lamina and medulla neurons (**Fig. 6f**).

334

335 **AdoCbl prevents *LRRK2-G2019S* induced neurotoxicity and rescues deficits in dopamine**
336 **transmission in LRRK2-PD mouse models**

337 Previous studies have demonstrated that transient overexpression of *LRRK2-G2019S*, but
338 not *LRRK2-WT*, leads to toxicity in primary cortical neuron cultures^{70,71}. We employed the same
339 approach and found that transfection of *LRRK2-G2019S* indeed caused neurotoxicity in
340 dissociated cortical neurons, as evidenced by apoptotic nuclear features. Treatment of the
341 transfected neurons with AdoCbl, however, suppressed the frequency of apoptotic neurons in a
342 dose-dependent manner (**Fig. 7a**). As a positive control in this assay, the most recent generation
343 LRRK2 inhibitor MLI-2³⁹ showed potent protection at a 10 nM concentration.

344 The lack of frank neurodegeneration in nearly all LRRK2 transgenic mouse models
345 prevents us from testing AdoCbl neuroprotection *in vivo*. However, a common pathological
346 feature for reported LRRK2 models is the deficit in DA transmission^{19,72}. To investigate if
347 AdoCbl prevents such a defect, we first tested the inhibition of neuronal LRRK2 by AdoCbl
348 using striatal brain slices from *LRRK2-G2019S* BAC transgenic mice under *ex vivo* conditions.
349 Striatal slices were incubated in oxygenated artificial cerebrospinal fluid (ACSF) for two hours
350 in the presence of LRRK2 inhibitor or vehicle. Administration of AdoCbl in the ACSF caused
351 dose-dependent inhibition of LRRK2 autophosphorylation in slice lysates (**Fig. 7b**). Similarly,
352 GNE-1023 exhibited dose dependent inhibition of LRRK2 under the same conditions (*SI*
353 *Supplementary information, Figs. S9a-b*). Compared to WT controls, *LRRK2-G2019S* BAC
354 transgenic mice were reported to have decreased sustainability of evoked DA release at the age
355 of 12 months old¹⁹. Therefore, we measured single-pulse evoked DA release sustainability in
356 striatal slices from *LRRK2-G2019S* BAC transgenic mice and WT littermates at the age of 12-15
357 months using Fast Scan Cyclic Voltammetry (FSCV)⁷³ (**Figs. 7c-d**). A bipolar stimulating
358 electrode was placed in the dorsal striatum ~150 μ m from the recording microelectrode and
359 depolarizing currents were applied at 2-minute intervals for 20 minutes. In control slices, the

360 amplitude of DA release at a given site evoked by single pulses decreased with the first few
361 stimulations and declined by 20% by the end of the 20-minute period. Consistent with a previous
362 report¹⁹, this decline was much more profound in the brain slices of the G2019S mice.
363 Remarkably, AdoCbl alleviated this deficit and restored the sustainability to the level of WT
364 control slices.

365 In addition, we examined the effects of AdoCbl using striatal slices from another preclinical
366 *LRRK2-R1441G* BAC transgenic mouse model. The declined DA release evoked by 2-minute
367 intervals during a 20-minute period was robust in slices from LRRK2-R1441G mice compared to
368 WT controls, and again, AdoCbl alleviated this deficit, restoring the sustainability to the level of
369 WT control slices (**Figs. 7e-f**). Taken together, our data demonstrates that AdoCbl is capable of
370 rescuing the impairment of DAergic neurons in evoked DA release caused by multiple LRRK2
371 PD mutants.

372 **Discussion**

373 LRRK2 has emerged as a most promising drug target for the treatment of PD. Although
374 extensive research has yielded potent and selective LRRK2 kinase inhibitors, they are ATP
375 competitors, some of which are associated with unwanted side effect and unclear clinical
376 outcome³⁷. Thus, an alternative class of inhibitors should be considered. Herein, we present
377 evidence that AdoCbl, one of two physiologically active forms of vitamin B₁₂, inhibits LRRK2
378 kinase activity with a distinct mechanism. Despite the less potent nature in LRRK2 inhibition
379 compared to many industrially produced compounds, AdoCbl displays a unique feature of
380 LRRK2 binding and kinase activity modulation mechanism by disturbing LRRK2 protein
381 conformation or dimerization, which may serve as a base for the development of novel allosteric
382 inhibitors of LRRK2. Moreover, AdoCbl prevents neurotoxicity and dopamine deficits in animal

383 models carrying LRRK2 disease variants. Therefore, our study identifies a novel class of LRRK2
384 kinase modulator that can be used to probe LRRK2 structure and function relationship and
385 develop new allosteric LRRK2 inhibitors in the future.

386 Our studies demonstrated the ability of AdoCbl to bind directly human LRRK2 through
387 multiple methods including kinetics, TSA, MST and intrinsic fluorescence (**Figs. 1-3**). Our data
388 indicates that AdoCbl acts as a mixed-type allosteric inhibitor capable of affecting ATP binding
389 to LRRK2. To date, the majority of reported LRRK2 kinase inhibitors has been known as ATP-
390 competitors. Although the structure details of the binding between LRRK2 kinase domain and
391 the inhibitors are unavailable, insight based on Roco4 kinase studies suggests they are type I and
392 II inhibitors^{47,49}. These types of inhibitors target the kinase active site; but while Type I bind to
393 the active conformation, type II bind to the inactive conformation⁷⁴. Vitamin B₁₂ was shown to
394 suppress the activity of nitric oxide synthase⁷⁵ and HIV-1 integrase⁷⁶, while the ability of B₁₂ to
395 inhibit kinase activity has never been documented. Indeed, the structure of Vitamin B₁₂ does not
396 resemble any known kinase inhibitor and no Vitamin B₁₂ mediated kinase inhibition has ever
397 been reported. In the absence of detailed structural information, our STD-NMR analysis revealed
398 extensive contacts between vitamin B₁₂ and the Roco4 kinase domain involving the adenosyl
399 moiety, the bulky corrin ring of cobalamin, and the DMZ base (**Fig. 2h**). Although ATP and
400 AdoCbl share an adenosyl moiety, the fact that HOCbl, MeCbl and CNCbl could inhibit LRRK2
401 kinase activity *in vitro* with comparable IC₅₀s (**Fig. 1**) suggests that the (β)-coordinating ligand
402 in vitamin B₁₂ is not essential for binding to LRRK2. In addition, the ATP-site LRRK2 variant
403 A2016T, which displays resistance against several ATP-competitive inhibitors such as LRRK2-
404 IN-1 did not have an effect on AdoCbl inhibition *in vitro*, or in the rescuing by AdoCbl of
405 behavioral abnormality and DA neuron degeneration in *C. elegans* expressing LRRK2-

406 A2016T/G2019S. Thus, our study suggests that vitamin B₁₂ modulates LRRK2 activity by
407 binding at distinct sites in kinase domain than those for other known LRRK2 inhibitors.

408 Mechanistically AdoCbl distinguishes itself from other LRRK2 inhibitors by being
409 capable of altering LRRK2 protein conformation and disturbing LRRK2 dimer status. Several
410 groups including ours have previously demonstrated that LRRK2 can form dimers^{12,17,18}, which
411 is thought to represent the kinase active form of LRRK2 in detriment of LRRK2 monomers¹³.
412 Since AdoCbl did not affect total LRRK2 levels in cells, it is likely that AdoCbl inhibits LRRK2
413 kinase activity by shifting the equilibrium from LRRK2 dimers to the kinase inactive monomeric
414 form (**Fig. 4**). This hypothesis is consistent with our observation that in contrast to ATP-
415 competitive inhibitors, AdoCbl renders LRRK2 susceptible to proteolysis, as the monomeric
416 species might be structurally more accessible to proteases than the dimer. Our study also raises a
417 possibility that AdoCbl prefers dimeric to monomeric LRRK2¹² for binding. While this idea is
418 under investigation, our data suggests that the ability of AdoCbl to disrupt dimerization might
419 offer advantages over known ATP-competitive inhibitors as an allosteric inhibitor to modulate
420 LRRK2 kinase function. A better understanding of the mode of LRRK2 - vitamin B₁₂ interaction
421 underlying the mechanism of inhibition of LRRK2 activity will depend on future efforts to solve
422 the structures of LRRK2-vitamin B₁₂ complexes.

423 Compared to the nM inhibition efficacy displayed by the second generation ATP-
424 competitive LRRK2 kinase inhibitors PF-06447475³⁸ and MLI-2³⁹, vitamin B₁₂ showed a modest
425 μM inhibition *in vitro*. However, it is surprising that the efficacy of AdoCbl in animal models
426 (**Figs. 5-6**) was comparable to those high-affinity inhibitors^{25,30,31}. While the unusual increase in
427 efficacy of AdoCbl inside the cells relative to *in vitro* condition is surprising and not understood
428 at present, we speculate that a couple of factors may contribute to the unexpected efficacy of

429 AdoCbl in the cells. First, vitamin B₁₂ enters the cells through active transport mediated by
430 **specific proteins** transcobalamin and its receptor (**CD320**), which ubiquitously located on the cell
431 surface, rather than diffusion (e.g. small compounds)^{77,78}. This mechanism may enhance
432 intracellular AdoCbl bioavailability, especially in the mitochondria **where AdoCbl is normally**
433 **located**. Second, **a potential cell non-autonomous mechanism, where a variety of cells (including**
434 **glial cells) produce and secrete transcobalamin that could facilitate the uptake of cobalamins in**
435 **neurons, may help explain the observation**⁷⁹. Third, vitamin B₁₂ may provide benefits through
436 acting on additional targets. Thus, the increased efficacy could result from a combination of
437 multiple target effects including LRRK2 inhibition. Vitamin B₁₂ is capable of crossing the blood
438 brain barrier (BBB), and it plays a key role in the regulation of excitotoxic homocysteine levels
439 in the brain⁸⁰, as well as in the synthesis of fatty acids incorporated into neuronal lipids and
440 myelin sheaths⁸¹. Indeed, vitamin B₁₂ deficiency in humans is known to contribute to a variety of
441 neurological conditions^{80,81}. Low vitamin B₁₂ levels have been described in patients with
442 idiopathic PD⁸²⁻⁸⁴, and there is also evidence that chronic L-3,4-Dihydroxyphenylalanine (L-
443 dopa) intake decreases vitamin B₁₂ plasma levels⁸⁵. A recent study showed that low levels of
444 vitamin B₁₂ predicts worse motor symptom in early PD⁸⁶, however, the mechanism is unknown.
445 It is likely that vitamin B₁₂ supplement provides some benefit in PD^{84,88}, but the lack of
446 knowledge of bioavailability of specific forms of vitamin B₁₂ (particularly in CNS) due to the
447 limitation of measurements hinders the understanding of the beneficial effect of vitamin B₁₂ in
448 human tissues.

449 Although it is challenging to understand the benefit or therapeutic potential of vitamin
450 B₁₂ in PD due to above obstacles, our study implies inhibition of LRRK2 through vitamin B₁₂ as
451 a potential mechanism. Our observation raises a possibility that tissue levels of AdoCbl

452 (including CNS and peripheral tissues) might modulate disease penetrance or progression of
453 LRRK2 variants in light of current reports showing the involvement of peripheral tissue/cells in
454 LRRK2 related pathogenesis⁸⁷⁻⁹⁰. Related epidemiologic studies should be performed in the near
455 future to address the possible association⁹¹. Indeed, future studies should investigate biochemical
456 and structural basis underlying the modulation of LRRK2 activity by vitamin B₁₂ as well as
457 vitamin B₁₂ efficacy and bioavailability in tissues in order to understand the therapeutic potential
458 of vitamin B₁₂ in PD. Nonetheless, AdoCbl represents a starting point for the development of a
459 new class of LRRK2 activity modulators (e.g. allosteric inhibitor) for the much-needed treatment
460 of LRRK2-linked pathological conditions such as PD and inflammatory bowel disease (IBD)⁹².

461

462

463

464

465

466

467 **Materials and Methods**

468 *Chemicals*

469 The FDA-approved chemical library used for the primary screen at the Mount Sinai Integrated
470 Screening Core was purchased from Microsource Discovery (Gaylordsville, CT, USA) and
471 contained 2,080 bioactive compounds approved for use in humans or animals. Trypsin, ATP,
472 AMP-PNP, and all forms of cobalamin were purchased from Sigma Aldrich (St. Louis, MO,
473 USA). [All experiments involving AdoCbl were performed under light-protected conditions to](#)
474 [reduce photolability.](#) GNE-1023 was kindly gifted to us by Genentech (South San Francisco, CA,

475 USA). The inhibitors GSK2578215A, PF-06447475, and MLI-2 were purchased from Tocris
476 (Bristol, UK). Bovine purified myelin basic protein was obtained from EMD Millipore
477 (Darmstadt, Germany) and purified Rab10 was purchased from Origene (Rockville, MD, USA).
478 Purified full-length flag-tagged LRRK2 was purchased from Invitrogen (Carlsbad, CA, USA).

479

480 ***Antibodies***

481 Anti-LRRK2 N241A/34 NeuroMab clone was obtained from the Michael J. Fox Foundation,
482 anti-pS935⁴⁴ LRRK2 (ab133450) was from Abcam, and anti-pS1292²⁰ was kindly gifted by
483 Genentech. Anti-Rab10 (8127) was purchased from Cell Signaling and anti-pRab10 pT73 was
484 obtained from the University of Dundee, UK.

485

486 ***Protein Purification***

487 BAC-transgenic mouse brain overexpressing flag-tagged LRRK2 was homogenized in
488 homogenization buffer (20 mM HEPES at pH 7.4, 0.32 M Sucrose, 1 mM NaHCO₃, 0.25 mM
489 CaCl₂, 1 mM MgCl₂, 1 mM PMSF, and complete protease inhibitor cocktail), then Triton X-100
490 was added to a final concentration of 1% and incubated at 4°C on a rotator for 30 min.
491 Homogenized brain was clarified at 12,000 x g for 10 min at 4°C and the FLAG-LRRK2 protein
492 were purified using Anti-FLAG Affinity Gel (Sigma, A220) with extensive wash before elution.
493 The protein was eluted using 150 ng/μL FLAG-peptide (Sigma, F4799) and stored at 80°C until
494 use.

495

496 The human *LRRK2* pDEST-NSF-tandem affinity plasmid was kindly gifted by Dr. Christian
497 Johannes Gloeckner (University of Tübingen, Germany). Strep-tagged LRRK2 was expressed in

498 HEK293T cells through transient transfection for 48 hours, as described previously¹². Cells were
499 harvested and incubated in lysis buffer (50 mM Tris pH 7.5, 100 mM NaCl, 2 mM DTT, 5 mM
500 MgCl₂, 0.5 mM EGTA, 1% Triton X-100, 10% Glycerol, Roche protease inhibitors
501 (11836170001)) for 30 minutes at 4°C. Lysate was centrifuged at 13,000 x g for 15 minutes.
502 Supernatant was mixed with Strep-Tactin Sepharose (2-1206-002) from Iba Life Sciences for 2
503 hours and washed extensively with buffer above, substituting 0.02% Triton X-100 for 1%. Strep-
504 Flag LRRK2 was eluted using 10mM desthiobiotin and stored at 80°C until use.

505
506 The 6xHis-GST-tagged *Roco4 kinase* domain plasmid, kindly gifted by Dr. Andy West
507 (University of Alabama Birmingham, AL, USA), was transformed into BL21 (DE3) cells
508 (Agilent Technologies, 230132) and grown at 37°C in 2xYT broth until reaching an OD600 of
509 0.6. Overexpression was induced for 16 hours at 18°C with 300 μM IPTG. Cells were pelleted
510 and resuspended in 50 mM Tris pH 7.5, 150 mM NaCl, 1 mM TCEP, 0.02% Reduced TX100, 1
511 mM PMSF, 25 μg/mL lysozyme, 4 ug/mL DNase. Protein was bound to Ni-NTA resin and
512 washed and eluted with increasing concentrations of imidazole. Protein was concentrated and
513 treated with TEV protease to cleave the 6xHis-GST tag, while dialyzing in buffer overnight to
514 remove imidazole. 6xHis-GST tag was separated using Ni-NTA resin and pure Roco4 kinase
515 domain was collected in the flow through.

516 The pGEX-6p-1 construct was expressed as above using BL21 (DE3) cells. Crude lysate was
517 bound to Glutathione Sepharose 4B (17-0618-01, GE Healthcare) for 1 hour before beads were
518 washed 3x with PBS. GST protein was eluted with reduced glutathione.

519

520 ***Compound Screen and TR-FRET Kinase Assay***

521 Compounds from the FDA-Approved library were tested against flag-tagged LRRK2 purified
522 from BAC transgenic mouse brain. LRRK2 kinase activity was monitored by measuring time
523 resolved fluorescence resonance energy transfer (TR-FRET) emission ratio upon the
524 phosphorylation of Fluorescein-LRRKtide (PV4901, Invitrogen) and subsequent binding of
525 Terbidium-pLRRKtide antibody (PV4898, Invitrogen). This TR-FRET-based assay was used in
526 a high-throughput screen (HTS) of small molecule chemical compounds for LRRK2 using the
527 TECAN (Mannedorf, Switzerland) Freedom EVO 200 liquid handling system, and relative
528 fluorescence was measured using the TECAN Safire 2 fluorescence spectrometer. To determine
529 the TR-FRET ratio between Fluorescein-LRRKtide and Terbidium-pLRRKtide antibody,
530 fluorescent intensity was measured at wavelengths 515 nm and 485 nm.

531 Prior to the HTS, each compound was prepared at 4x final concentration (1.6 or 4%
532 residual DMSO volume) in Kinase Buffer S, containing 50 mM Tris pH 8.5, 10 mM MgCl₂,
533 0.01% Brij-35, 1 mM EGTA in 96-well polypropylene non-treated plates (Thermo Scientific,
534 #12-565-436). The kinase reaction was performed in 10 µl total volume in a low-volume white
535 384-well plate (Corning, #3673), with 20 nM LRRK2, 400 nM Fluorescein-ERM (LRRKtide),
536 and small molecules in Kinase Buffer S supplemented with 2 mM DTT on the day of the
537 experiment. Small molecules were screened at 3.3 or 4 µM with 0.4 or 1% residual DMSO in
538 duplicate. The assay plates were prepared by adding 2.5 µl of 4x compound solutions, 2.5 µl of
539 4x LRRK2, and 5 µl of 2x LRRKtide and ATP mixture. After incubation at room temperature
540 for 4 hours, the kinase reaction was terminated by the addition of 10 µl of 2x EDTA and 2x Tb-
541 anti-pERM antibody in the detection buffer, containing 20 mM Tris-HCl, 0.01% NP40. EDTA
542 was mixed with the antibody right before the addition of the mixture to wells, as the antibody
543 was stable in EDTA only for several hours. The final concentration of EDTA and Tb-anti-pERM

544 were 5 mM and 2.5 nM, respectively. After 1-hour incubation at room temperature, TR-FRET
545 measurements were obtained according to the parameters described in instrument settings.
546 During the incubation, 384-well plates were covered by aluminum sealing tapes (Corning,
547 #6570) to reduce evaporation and exposure to light. All the liquid handling was carried out using
548 fixed 8-tip LiHa arms on TECAN EVO200 workstation. HTS was performed with 0.4 or 1%
549 DMSO as negative control and no LRRK2 as positive control. Hit compounds were selected
550 based on the normalized percent inhibition by first computing emission ratio (emission intensity
551 of acceptor divided by donor) and then computing percent inhibition of kinase activity relative to
552 the DMSO treated control as 0% inhibition and the control in the absence of LRRK2 as 100%
553 inhibition. Compounds in wells showing greater than 30% inhibition in either of the duplicates
554 were selected as hits.

555

556 *In Vitro Kinase Assays*

557 Kinase reactions were performed in 30 uL kinase buffer (20 mM Tris pH 7.5, 1 mM DTT, 15
558 mM MnCl₂, 20 mM β-glycerophosphate) at 37°C for 30 minutes in the presence of [γ -³²P] ATP
559 (3000 Ci/mmol; BLU502H250UC, PerkinElmer Life Sciences) and 50 μM cold ATP.
560 Beforehand, LRRK2 or Roco4 Kinase was loaded with inhibitor and/or substrate, followed by 30
561 min incubation on ice. Reactions were stopped by addition of Laemmli buffer and boiling at
562 95°C for 10 minutes. Samples were resolved on 4-12% SDS-PAGE pre-cast gels (NP0323BOX,
563 Invitrogen). Radioactive signal was captured onto a phosphor-screen (S0230, GE Lifesciences)
564 and was digitally collected using a Typhoon scanner. ImageQuant densitometry was used to
565 quantify the phosphor-signal.

566

567 ***GTP Hydrolysis Assay***

568 GTPase activity of strep-tagged LRRK2 was measured in 30 uL GTPase buffer (20 mM Tris pH
569 7.5, 150 mM NaCl, 1 mM DTT, 5 mM MgCl₂, 1 mM EDTA) at 30°C for 90 minutes. LRRK2
570 was incubated with inhibitor for 30 min on ice before reactions were initiated with the addition
571 of 50 μM cold GTP and [α -³²P] GTP (3000Ci/mmol; BLU006H250UC, PerkinElmer Life
572 Sciences). Reactions were terminated with the addition of 0.5 M EDTA. 2 uL of the reaction
573 mixture were dotted onto TLC plates (M1055790001, EMD Millipore). GDP and GTP were
574 separated by TLC using 0.5 M KH₂PO₄ pH 3.5 for 60 minutes. The TLC plate was dried for 15
575 minutes and radioactive signal was captured and using a phosphor-screen and a Typhoon
576 scanner. ImageQuant densitometry was used to quantify the phosphor-signal.

577

578 ***B12-Agarose Binding Assay***

579 500 μL of 40 nM strep-tagged LRRK2 or 500 uL of 100 nM GST, pre-loaded with AdoCbl or
580 buffer for 30 min on ice, were incubated with 50 uL B12-Agarose (V3254, Sigma Aldrich). B12-
581 Agarose was washed 3x with buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM TCEP,
582 0.02% Triton X-100, 1% Ficoll 400) and bound protein was eluted with Laemmli buffer and
583 boiling at 95°C. Bound LRRK2 or GST was analyzed by western blot.

584

585 ***Thermal Shift Assay***

586 300 nM strep-tagged LRRK2 was incubated with Sypro Orange and inhibitor or buffer to a final
587 volume of 30 uL. Using a Stratagene Mx3000 Real-time PCR machine, samples were heated to
588 95°C and fluorescence intensity of Sypro Orange was measured at every 0.5°C increment. For
589 each experiment, data was normalized to the maximum fluorescent intensity.

590

591 *Microscale Thermophoresis*

592 Microscale Thermophoresis (MST) measurements were obtained using a Monolith NT.115
593 (NanoTemper Technologies). Purified strep-tagged LRRK2 was labeled with NT-647 dye
594 (NanoTemper Technologies) and experiments were performed using a 2 nM final concentration.
595 A 12-point dilution series of AdoCbl, ranging from 200 nM to 100 μ M, was added to labeled
596 LRRK2. After 30 min incubation on ice, the binding reaction was loaded onto Standard
597 capillaries (NanoTemper Technologies) and measurements were taken using 30% LED power
598 and 50% MST power. Laser on-time was set to 30 seconds and laser off-time was set to 5
599 seconds. Data was processed using GraphPad Prism 6.0 and a K_D was derived from three
600 independent thermophoresis experiments by fitting a curve based on the law of mass action.
601 Quality of each MST run was assessed by performing a capillary scan before and after each data
602 collection to check that the fluorescence between samples stays within $\pm 10\%$. Furthermore, each
603 time-trace showed a smooth decrease in normalized fluorescence, suggesting that no
604 precipitation occurred during the experiments.

605

606 *STD-NMR*

607 Saturation Transfer Difference (STD) NMR was carried out with 1024 scans on a Bruker
608 800MHz NMR spectrometer equipped with a cryogenic probe at 277 K according to Mayer et
609 al⁹³. Saturation on and off frequencies were set to -1 and -20 ppm, respectively, with saturation
610 achieved using a 2 s train of 50 ms Gaussian pulses at 86 Hz. 1024 scans were used. STD-NMR
611 samples contained 50 mM Tris, 150 mM NaCl, 1 mM TCEP and 2 mM MgCl₂ at a pH of 7.5,

612 20-30 μ M Roco4 protein, with vitamin B₁₂ at 100-fold excess over Roco4 in concentration.
613 Vitamin B₁₂ resonances were assigned in D₂O according to Summers et al⁹⁴.

614

615 *Intrinsic Fluorescence*

616 Strep-tagged LRRK2 at a concentration of 150 nM was incubated with AdoCbl or buffer in a
617 total volume of 80 μ L. Samples were loaded onto a 96-well black-walled plate and fluorescence
618 was measured using a Tecan Safire microplate reader. For these experiments, a correction
619 equation must be applied to the fluorescent measurements to compensate for the inner filter
620 effect. AdoCbl absorbs light at 295 nm, which affects the excitation strength of the incident light,
621 and from 340-360 nm, which affects the amount of light reaching the detector after tryptophan
622 emission. Therefore, we calculated a corrected fluorescence value (**Supplementary**
623 **information, Figs. S5c-d**) for each sample by measuring the absorbance at the excitation and
624 emission wavelengths and multiplying by the original fluorescence value⁹⁵.

625

626 *Limited Proteolysis Assays*

627 Strep-tagged LRRK2 or TBK1 was subjected to limited proteolysis by trypsin or chymotrypsin
628 with a 10:1 molar ratio of LRRK2 to protease at 37°C. LRRK2 was loaded with inhibitor for 30
629 min on ice before protease was added. Reactions were stopped with the addition of Laemmli
630 buffer and boiling at 95°C.

631

632 *In situ labeling and detection of LRRK2 dimers.*

633 For the biochemical detection and purification of biotinylated LRRK2 dimers, we used an
634 adaptation of the proximity biotinylation approach⁹⁶. A manuscript describing the full
635 characterization of this assay is currently under review (Leandrou et al.; submitted to Biochem
636 J). HEK293T cells, maintained throughout the duration of the experiment in biotin-depleted
637 media, were co-transfected with plasmids encoding WT LRRK2, fused to biotin ligase (BirA) or
638 an acceptor peptide (AP). The following day, the growth media was replaced and the indicated
639 concentrations of AdoCbl, diluted in media, were added, and the cultures maintained for an
640 additional 48h. Prior to cell lysis, the cells were washed in pre-warmed PBS and given a brief (5
641 min) pulse with 50 μ M biotin, followed extensive washing with PBS. Cytoplasmic extracts were
642 prepared in lysis buffer (20mM HEPES, pH 7.4; 150 mM NaCl; 0.5% NP-40; 2mM EGTA; 2
643 mM MgCl₂; 10% glycerol; pH 7.2). Following lysis, 5 μ g of total clarified cell extract was bound
644 to streptavidin-coated ELISA plates for 1h at 37°C under constant agitation. The supernatant was
645 removed and retained, and the wells were washed, and the amount of biotinylated LRRK2
646 present in each sample was quantified using HRP-conjugated (in house) anti-LRRK2 (75-253,
647 NeuroMab/Antibodies Incorporated; clone N241A/B34). Duplicate samples were incubated in
648 the parallel ELISA plates pre-coated with anti-LRRK2 (ab195024, Abcam) in order to quantify
649 the total amount of LRRK2 present in each sample. In each experiment, control samples were
650 prepared from cells co-expressing AP-LRRK2 together with Flag-LRRK2 (without the BirA
651 biotin ligase). To visualize expression of both LRRK2 constructs, parallel extracts were
652 separated by SDS-PAGE (6%), and membranes probed with anti-LRRK2 (clone N241A/B34).

653

654 ***Generation of Mouse Embryonic Fibroblasts (MEFs)***

655 LRRK2-G2019S and wild type control MEFs were isolated from mouse embryos at day E13.5
656 resulting from crosses between heterozygous LRRK2-G2019S and wild type C57/BL/6J mice.
657 All the MEFs were cultured for at least 30 passages to immortalize the cells. All cells were
658 cultured in DMEM containing 10% FBS, 2mM L-glutamine and 100 units/ml Penicillin-
659 Streptomycin, and were maintained at 37°C with 5% CO₂. All cells lines were confirmed by
660 PCR genotyping and western blot, but were not tested for mycoplasma contamination. To test
661 inhibition, cells were treated with inhibitor or DMSO for 24 hours in DMEM with 3% FBS
662 before being lysed and subject to western blot for analysis.

663

664 ***Treatment of C. elegans with AdoCbl***

665 *C. elegans* strains were cultured on standard nematode growth medium (NGM) agar plates
666 seeded with *E. coli* OP50 as a food source. Mixed stage animals were maintained as bulk culture
667 on NGM agar at room temperature (22°C). Prior to each experiment, animals were age-
668 synchronized by standard bleaching and washing protocol to obtain embryos, from which
669 developmental stages were followed. The following transgenic *C. elegans* lines expressing green
670 fluorescent protein (GFP) either alone (SGC730: Pdat-1::GFP) or together with human *LRRK2*-
671 *G2019S* (SGC856: Pdat-1::LRRK2-G2019S; Pdat-1::GFP) or human *LRRK2-R1441C* (SGC851:
672 Pdat-1::LRRK2-R1441C Pdat-1::GFP) in dopaminergic neurons were used⁶³.

673

674 Treatment with AdoCbl was done in liquid culture to ensure adequate drug exposure using the
675 published protocol as described³⁰. Briefly, worms were age-synchronized to generate L1 larva in
676 M9 buffer [For 1 liter: KH₂PO₄, 3 g; Na₂HPO₄, 6 g; NaCl, 5 g; MgSO₄ (1 M), 1 ml], which

677 were distributed into a 12-well microtiter plate seeded with *E. coli* OP50 with roughly 50 L1
678 worms in a total volume of 900 ml. AdoCbl stock made in water was added to achieve the
679 desired concentrations. The 12-well plate was covered in aluminum foil to protect from light,
680 maintained in a humidified chamber at room temperature and shaken at 100 rpm. Worms were
681 monitored every day and placed onto agar plates with OP50 when most of them reached L4
682 larval stage (about 3 days). L4 worms were grown on NGM agar plates seeded with *E. coli* OP50
683 for 3 days for behavioral assay or 9 days for neuronal assessment as described below.

684

685 *C. elegans* Basal Slowing Assay

686 Well-fed worms with intact dopaminergic neural circuitry move slower in the presence of
687 bacterial food than in its absence⁶⁴. This basal slowing response was assayed as described
688 previously^{63,64}. Briefly, a set of NGM assay plates were seeded with bacterial food, *E. coli* OP50,
689 in a ring shape, and another set of NGM assay plates were uncoated. Age-synchronized worms
690 (about 10 worms of each strain) were washed twice in S basal buffer (100 mM NaCl, 10 mg/ml
691 cholesterol, 50 mM potassium phosphate, pH 6.0). Worms were then transferred to the center of
692 the NGM plates coated with or without *E. coli* OP50 as described above, settled for 5 min, and
693 their locomotion were recorded with a digital camera in 20 s intervals. Body bends were
694 examined using an unbiased machine-vision analysis system (WormLab, MBF Bioscience,
695 Williston, VT). Basal slowing was calculated as the percent slowing in body bends per 20 s in
696 the presence vs. the absence of bacterial lawn.

697

698 *Assessment of Dopaminergic Neuron Survival in C. elegans*

699 Dopaminergic neurons in live *C. elegans* were examined essentially as described⁶³. Briefly,
700 worms were immobilized in the presence of 3 mM levamisole and were mounted on glass slides.
701 The dopaminergic neurons in the head regions [four cephalic neurons (CEPs)] were visualized
702 for GFP fluorescence under a Zeiss Axiovert 200M microscope. The total numbers of CEPs with
703 the intact cell body (survived) as well as those missing most of the cell body and neurites
704 (degenerated) were counted. For each strain, about 30 worms were analyzed in at least three
705 independent experiments. The percent of dopaminergic neuron survival was calculated as the
706 number of intact CEPs observed in all animals divided by total number of CEPs expected if no
707 degeneration occurred (four in each animal times the number of animals tested), times 100.
708 Fluorescent images of DA neurons in the head region of worms were taken with a Zeiss Axiovert
709 200M microscope using 1 s exposure time at 20× magnification.

710

711 Sample size was determined according to the Statistical Solutions LLC calculator
712 (http://www.statisticalsolutions.net/pssTtest_calc.php). Assuming alpha value of 0.05, to detect
713 difference of 10% between 100% mean for the control group and 90% mean for an experimental
714 group, 5% expected standard deviation (two-sided t-test), and a power of 0.8, a sample size of 4
715 animals is obtained. In Fig. 4, more than 20 worms were used for each group. All live and age-
716 synchronized worms were included in the experiments. Only dead worms, if any, were excluded.
717 The number of worms available for experiments was in general five times more than the number
718 of worms being assayed (e.g., 20 worms were randomly picked for from a culture of 100
719 worms). For these experiments, the investigator was not blinded to group allocations. Data is
720 normally distributed, and the variance was similar between the groups that are being statistically
721 compared.

722

723 ***Treatment of Drosophila with AdoCbl***

724 Flies were used and raised as described recently³¹. Briefly, the *TH* (*tyrosine hydroxylase*) GAL4
725 was crossed with either UAS-*hLRRK2-wildtype*, UAS-*hLRRK2-G2019S* or the kinase-dead
726 UAS-*hLRRK2-G2019S-K1906M* line to produce progeny dopaminergic expression of the
727 transgene (*DA* → *hLRRK2*, *DA* → *G2019S*, *DA* → *KD*). The crosses were allowed to lay eggs
728 onto instant fly food (Carolina) or onto instant food supplemented with AdoCbl. The final
729 concentration of AdoCbl in the fly food ranged from 100 to 2500 nM. Females were collected on
730 the day of emergence and transferred to new vials (no AdoCbl) for 24 hours. In control
731 experiments, low expression dLRRK flies (dLRRK^{e03680}) were fed instant fly food or food
732 supplemented with AdoCbl.

733

734 **Physiological recordings:** 18-24 hour old females were aspirated in a pipette tip, restrained with
735 nail polish, and allowed to recover for >20 minutes. A recording electrode was placed in the
736 center of the eye, and a reference electrode in the mouthparts. After 2 minutes in the dark, the fly
737 was illuminated with light from a blue flickering LED and the resulting electroretinogram signal
738 amplified and stored for off-line analysis. The response was analyzed by the Fast Fourier
739 Transform (FFT), generating components corresponding to the photoreceptors, second order
740 lamina neurons, and third/fourth order medulla neurons. Stimulus generation, recording and
741 analysis were accomplished in Matlab, as described recently³¹; Matlab code available at
742 <https://github.com/wadelab/flyCode>). The number of flies used was sufficient according to
743 previously published data³¹. All data from tested flies were included. Male and female flies of
744 the required genotype were placed in randomly chosen vials (+/- drug) and allowed to mate and

745 lay eggs. Offspring were harvested daily and flies were sampled at random. The investigators
746 were blinded to the genotype while the experiments were in progress. For statistical analysis,
747 estimates of variation were made and are similar between groups being compared.

748

749 *Determination of Survival of Primary Cortical Neurons*

750 Primary rat embryonic cortical neurons were prepared and cultured as described^{70,97}. Briefly,
751 embryonic day 17 rat cortices were dissociated and plated on poly-d-lysine coated 12mm
752 diameter glass coverslips in Neurobasal medium (12348017, Invitrogen-ThermoScientific) with
753 B-27 serum free supplements (17504044, Invitrogen-ThermoScientific) at a density of 125,000
754 neurons per cm². On day four following plating, neurons were transiently co-transfected with
755 LRRK2-WT or LRRK2-G2019S and pcms-EGFP at a ratio of 4:1 using Lipofectamine 2000
756 according to the manufacturer's instructions. The indicated concentrations of AdoCbl or MLi-2
757 in Neurobasal/B-27 medium was added to the neurons on the morning following transfection and
758 supplemented one additional time at the mid-point (36 h) of the total duration of the experiment.
759 Following a period of 72 h of expression, the coverslips were fixed in 4% formaldehyde and
760 stained with anti-GFP antibodies (ab13970, Abcam) and DAPI. We had determined in parallel
761 neurons double stained with GFP and anti-LRRK2 antibodies (ab133474, Abcam) that the
762 percentage of GFP-positive neurons over-expressing LRRK2 was approximately 90% (not
763 shown). To simplify quantification of apoptotic degenerating neurons, GFP-positive neurons
764 were visualized and determine to be apoptotic or viable. For quantification, apoptotic neurons
765 were defined as those having condensed fragmented chromatin comprised of two or more
766 apoptotic bodies. More than 100 neurons per coverslip were assessed in triplicate coverslips in a

767 blinded fashion, from two to three independent cultures. The data are presented as the percentage
768 of GFP-positive neurons containing apoptotic nuclear features.

769

770 *Animals and Brain Slice Preparation*

771 The use of the animals followed the National Institutes of Health guidelines and was approved
772 by the Institutional Animal Care and Use Committee at Thomas Jefferson University. All efforts
773 were made to minimize the number of animals used. BAC LRRK2(hR1441G) transgenic (TG)
774 mice were obtained from Chenjian Li's laboratory at Weill Medical College of Cornell
775 University and maintained on Taconic FVB/N background and BAC LRRK2(G2019S) TG mice
776 previously described¹⁹ maintained on C57/NJ background.

777 Three- to 5-month-old male transgenic LRRK2-G2019S mice and their non-transgenic
778 littermates were used for LRRK2 kinase inhibition in striatal brain slices. For preparing striatal
779 slices, mice were decapitated without anesthesia after cervical dislocation and brains were
780 immediately dissected out. Coronal striatal brain slices at 250 μm were prepared on a vibratome
781 (VT1200, Leica, Solms, Germany). The striatal slices were allowed to recover for 0.5 to 1 hour
782 at 36°C in a holding chamber containing oxygenated artificial CSF (ACSF: 125 mM NaCl, 2.5
783 mM KCl, 26 mM NaHCO₃, 2.4 mM CaCl₂, 1.3 mM MgSO₄, 0.3 mM KH₂PO₄, and 10 mM
784 glucose, pH 7.3-7.4). To examine the effects of the LRRK2 inhibitors, slices were incubated for
785 2 h in oxygenated ACSF containing LRRK2 inhibitors. For the incubation treatment, striatal
786 slices were bisected, and one striatum was exposed to LRRK2 inhibitor (2 h) while the other was
787 exposed to vehicle (DMSO or water). After treatment, the slices were collected and rapidly
788 frozen in dry ice and stored in -80°C until assayed.

789

790 ***Slice Preparation for Evoked DA Transmission***

791 Twelve- to 15-month-old male LRRK2-G2019S as well as 6- to 14-month-old male
792 LRRK2-R1441G BAC transgenic mice and their age-matched non-transgenic littermates were
793 used. For preparing striatal slices, mice were decapitated without anesthesia after cervical
794 dislocation and brains were immediately dissected out. Coronal striatal brain slices at 250 μm
795 were prepared on a vibratome (VT1200, Leica, Solms, Germany) for electrophysiological
796 recording. The striatal slices were allowed to recover for at least 1 hour at 36°C in a holding
797 chamber containing oxygenated artificial CSF (ACSF) and then placed in a recording chamber
798 superfused (1.5 ml/min) with ACSF at 36°C. The pH of all ACSF solutions were adjusted to 7.3–
799 7.4 with concentrated hydrochloric acid and ACSF solutions were saturated with carbogen (95%
800 O₂/5% CO₂) prior to use to ensure stable pH buffering and adequate oxygenation.

801 Striatal slices were bisected, and one striatum was incubated for 2 hours in ACSF at 36°C
802 containing 300 μM AdoCbl while the other was exposed to vehicle (water) as the control. Slices
803 were washed with ACSF for 20 min after treatment before fast scan cyclic voltammetry (FSCV)
804 recording.

805

806 ***Fast scan cyclic voltammetry recording (FSCV)***

807 FSCV was used to measure evoked DA release in the dorsal striatum (dSTR). Electrochemical
808 recordings and electrical stimulation were performed as previously described⁷³. Briefly, freshly
809 cut carbon fiber electrodes $\sim 5 \mu\text{m}$ in diameter were inserted $\sim 50 \mu\text{m}$ into the dSTR slice. For
810 FSCV, a triangular voltage wave (-400 to 900 mV at 280 V/sec versus Ag/AgCl) was applied to
811 the electrode every 100 msec. Current was recorded with an Axopatch 200B amplifier (Axon
812 Instruments, Foster City, CA), with a low-pass Bessel filter set at 10 kHz, digitized at 25 kHz
813 (ITC-18 board; InstruTech, Great Neck, NY). Triangular wave generation and data acquisition

814 were controlled by a personal computer running a locally written (Dr. E. Mosharov, Columbia
815 University, New York, NY) IGOR program (WaveMetrics, Lake Oswego, OR). Striatal slices
816 were electrically stimulated (400 μ A x 1 ms pulse duration) by an Iso-Flex stimulus isolator
817 triggered by a Master-8 pulse generator (AMPI, Jerusalem, Israel) using a bipolar stimulating
818 electrode placed at a distance of \sim 150 μ m from the recording electrode. The slices were
819 stimulated every 2 min. Background-subtracted cyclic voltammograms served for electrode
820 calibration and to identify the released substance. DA oxidation current was converted to
821 concentration based upon a calibration of 5 μ M DA in ACSF after the experiment. For each
822 experimental condition, at least three slices from at least three different mice were examined
823 unless specified otherwise. The number of the recording sites was determined according to
824 previously published experiments¹⁹ and SSD sample size power analysis. No randomization and
825 no blinding were used for experimental groupings. All recorded data was included. Statistical
826 tests were justified as appropriate, as data meets test assumptions, with a similar estimated
827 variance between groups that are statistically compared.

828

829

830

831

832

833

834

835

836

837

838

839

840

841

842

843 **Acknowledgements**

844

845 This work was supported in part by awards from the Michael J. Fox Foundation to MMZ, IU-B
846 ZY, HJR. ZY was also supported by NIH R01NS060809. ZY and IU-B were supported by NIH
847 R01GM115844. HZ was supported by NIH R01NS097530. SGC was supported by NIH
848 R21NS073170. CE was supported by an award from The Wellcome Trust [ref: 097829] through
849 the Centre for Chronic Diseases and Disorders (C2D2) at the University of York. We thank Dr.
850 Insup Choi, Dr. Kerry Purtell, and Yuanxi Zhang from Yue's lab for technical support.

851

852

853 **Author contributions:** X.L., N.P., K.M., M-M.Z, and Z.Y. performed the HTS and identified
854 vitamin B₁₂ as a LRRK2 inhibitor. X.L. and Z.Y. performed brain LRRK2 purification. A.S.,
855 X.H., Y.G.-Ll. and I.U.-B. performed HEK293T LRRK2, Roco4 kinase, and GST purifications.
856 A.S., Z.Y., and I.U.-B. performed in vitro kinase and GTP hydrolysis assays. A.S. and I.U.-B.
857 performed B12-agarose binding, Microscale Thermophoresis, Thermal Shift Assays, Intrinsic
858 Fluorescence, and Limited proteolysis assays. X.L. and Z.Y. performed Michaelis-Menten
859 kinetics assays. N.C. and C.W. performed STD-NMR. X.L. and Z.Y. generated LRRK2 MEFs.
860 A.S., X.L. and Z.Y. performed cellular inhibition assays. L.Z. and H.Z. prepared mouse brain
861 slices, inhibitor incubation, and performed FSCV recording experiments. E.L., A.M., and H.R.
862 performed apoptotic analysis of primary cortical neurons. C.Y. and S.C. performed all *C. elegans*
863 experiments. F.A. and C.E. performed all *D. melanogaster* experiments. A.S. and I.U.-B. wrote
864 manuscript drafts. A.S., I.U.-B., and Z.Y. edited the manuscript. I.U.-B. and Z.Y. designed the
865 research.

866

867 **Glossary of Terms**

868 ACSF: artificial cerebrospinal fluid
869 AdoCbl: 5'-deoxyadenosylcobalamin, or adenosylcobalamin
870 AP: acceptor peptide
871 a.u.: arbitrary unit
872 BirA: biotin ligase
873 BAC: bacterial artificial chromosome
874 CEP: cephalic neurons
875 CNCbl: cyanocobalamin
876 CRF: contrast response function
877 DAergic: dopaminergic
878 DMZ: dimethylbenzimidazole
879 FFT: fast Fourier transform
880
881 FSCV: fast scan cyclic voltammetry

882 HOCbl: hydroxycobalamin
883 HTS: High-throughput Screen
884 LRRK2: Leucine-Riche Repeat Kinase 2
885 MBP: myelin basic protein
886 MeCbl: methylcobalamin
887 MEF: mouse embryonic fibroblast
888 MST: microscale thermophoresis
889 PD: Parkinson's Disease
890 STD-NMR: saturation transfer difference nuclear magnetic resonance
891 TR-FRET: time-resolved fluorescence resonance energy transfer
892 TSA: thermal stability assay
893 WT: wild-type

894

895

896

897

898

899

900

901

902

903

904

905

906

907

908

909

910

911 **References**

- 912 1 Dickson, D. W. *et al.* Neuropathology of non-motor features of Parkinson disease. *Parkinsonism*
913 *& Related Disorders* **15**, **Supplement 3**, S1-S5, doi:[http://dx.doi.org/10.1016/S1353-](http://dx.doi.org/10.1016/S1353-8020(09)70769-2)
914 [8020\(09\)70769-2](http://dx.doi.org/10.1016/S1353-8020(09)70769-2) (2009).
- 915 2 Lees, A. J., Hardy, J. & Revesz, T. Parkinson's disease. *The Lancet* **373**, 2055-2066,
916 doi:10.1016/S0140-6736(09)60492-X (2009).
- 917 3 Funayama, M. *et al.* A new locus for Parkinson's disease (PARK8) maps to chromosome 12p11.2-
918 q13.1. *Ann Neurol* **51**, 296-301, doi:10.1002/ana.10113 [pii] (2002).
- 919 4 Paisán-Ruíz, C. *et al.* Cloning of the gene containing mutations that cause PARK8-linked
920 Parkinson's disease. *Neuron* **44**, 595-600, doi:10.1016/j.neuron.2004.10.023 (2004).
- 921 5 Zimprich, A. *et al.* Mutations in LRRK2 cause autosomal-dominant parkinsonism with
922 pleomorphic pathology. *Neuron* **44**, 601-607, doi:S0896627304007202 [pii]
923 10.1016/j.neuron.2004.11.005 (2004).
- 924 6 Satake, W. *et al.* Genome-wide association study identifies common variants at four loci as
925 genetic risk factors for Parkinson's disease. *Nature Genet.* **41**, 1303-U1361, doi:10.1038/ng.485
926 (2009).
- 927 7 Simon-Sanchez, J. *et al.* Genome-wide association study reveals genetic risk underlying
928 Parkinson's disease. *Nature Genet.* **41**, 1308-U1368, doi:10.1038/ng.487 (2009).
- 929 8 Do, C. B. *et al.* Web-based genome-wide association study identifies two novel loci and a
930 substantial genetic component for Parkinson's disease. *PLoS genetics* **7**, e1002141,
931 doi:10.1371/journal.pgen.1002141 (2011).
- 932 9 Lill, C. M. *et al.* Comprehensive research synopsis and systematic meta-analyses in Parkinson's
933 disease genetics: The PDGene database. *PLoS genetics* **8**, e1002548,
934 doi:10.1371/journal.pgen.1002548 (2012).
- 935 10 Healy, D. G. *et al.* Phenotype, genotype, and worldwide genetic penetrance of LRRK2-associated
936 Parkinson's disease: a case-control study. *The Lancet Neurology* **7**, 583-590, doi:10.1016/S1474-
937 4422(08)70117-0 (2008).
- 938 11 Tomiyama, H. *et al.* Clinicogenetic study of mutations in LRRK2 exon 41 in Parkinson's disease
939 patients from 18 countries. *Movement Disorders* **21**, 1102-1108, doi:10.1002/mds.20886 (2006).
- 940 12 Guaitoli, G. *et al.* Structural model of the dimeric Parkinson's protein LRRK2 reveals a compact
941 architecture involving distant interdomain contacts. *Proceedings of the National Academy of*
942 *Sciences of the United States of America*, 201523708-201523708, doi:10.1073/pnas.1523708113
943 (2016).
- 944 13 Sen, S., Webber, P. J. & West, A. B. Dependence of leucine-rich repeat kinase 2 (LRRK2) kinase
945 activity on dimerization. *Journal of Biological Chemistry* **284**, 36346-36356,
946 doi:10.1074/jbc.M109.025437 (2009).
- 947 14 Bardien, S., Lesage, S., Brice, A. & Carr, J. Genetic characteristics of leucine-rich repeat kinase 2
948 (LRRK2) associated Parkinson's disease. *Parkinsonism and Related Disorders* **17**, 501-508,
949 doi:10.1016/j.parkreldis.2010.11.008 (2011).
- 950 15 Benamer, H. T. S. & De Silva, R. LRRK2 G2019S in the North African population: A review.
951 *European Neurology* **63**, 321-325, doi:10.1159/000279653 (2010).
- 952 16 West, A. B. *et al.* Parkinson's disease-associated mutations in leucine-rich repeat kinase 2
953 augment kinase activity. *Proceedings of the National Academy of Sciences of the United States of*
954 *America* **102**, 16842-16847, doi:10.1073/pnas.0507360102 (2005).
- 955 17 Greggio, E. *et al.* The Parkinson disease-associated leucine-rich repeat kinase 2 (LRRK2) is a
956 dimer that undergoes intramolecular autophosphorylation. *Journal of Biological Chemistry* **283**,
957 16906-16914, doi:10.1074/jbc.M708718200 (2008).

958 18 Berger, Z., Smith, K. A. & Lavoie, M. J. Membrane localization of LRRK2 is associated with
959 increased formation of the highly active Lrrk2 dimer and changes in its phosphorylation.
960 *Biochemistry* **49**, 5511-5523, doi:10.1021/bi100157u (2010).

961 19 Li, X. *et al.* Enhanced striatal dopamine transmission and motor performance with LRRK2
962 overexpression in mice is eliminated by familial Parkinson's disease mutation G2019S. *The*
963 *Journal of Neuroscience: The Official Journal of the Society for Neuroscience* **30**, 1788-1797,
964 doi:10.1523/JNEUROSCI.5604-09.2010 (2010).

965 20 Sheng, Z. *et al.* Ser1292 autophosphorylation is an indicator of LRRK2 kinase activity and
966 contributes to the cellular effects of PD mutations. *Science translational medicine* **4**, 164ra161-
967 164ra161, doi:10.1126/scitranslmed.3004485 (2012).

968 21 Yue, M. *et al.* Progressive dopaminergic alterations and mitochondrial abnormalities in LRRK2
969 G2019S knock-in mice. *Neurobiology of Disease* **78**, 172-195,
970 doi:<http://dx.doi.org/10.1016/j.nbd.2015.02.031> (2015).

971 22 Fraser, K. B., Moehle, M. S., Alcalay, R. N. & West, A. B. Urinary LRRK2 phosphorylation predicts
972 parkinsonian phenotypes in G2019S LRRK2 carriers. *Neurology* **86**, 994-999,
973 doi:10.1212/wnl.0000000000002436 (2016).

974 23 Steger, M. *et al.* Phosphoproteomics reveals that Parkinson's disease kinase LRRK2 regulates a
975 subset of Rab GTPases. *eLife* **5**, doi:10.7554/eLife.12813.001 (2016).

976 24 Ito, G. *et al.* Phos-tag analysis of Rab10 phosphorylation by LRRK2: a powerful assay for
977 assessing kinase function and inhibitors. *Biochemical Journal* **473**, 2671 (2016).

978 25 Lee, B. D. *et al.* Inhibitors of leucine-rich repeat kinase-2 protect against models of Parkinson's
979 disease. *Nature medicine* **16**, 998-1000, doi:10.1038/nm.2199 (2010).

980 26 Sweet, E. S., Saunier-Rebori, B., Yue, Z. & Blitzer, R. D. The Parkinson's Disease-Associated
981 Mutation LRRK2-G2019S Impairs Synaptic Plasticity in Mouse Hippocampus. *The Journal of*
982 *Neuroscience* **35**, 11190 (2015).

983 27 Daher, J. P. L. *et al.* Leucine-rich Repeat Kinase 2 (LRRK2) Pharmacological Inhibition Abates α -
984 Synuclein Gene-induced Neurodegeneration. *Journal of Biological Chemistry* **290**, 19433-19444
985 (2015).

986 28 Volpicelli-Daley, L. A. *et al.* G2019S-LRRK2 Expression Augments α -Synuclein Sequestration into
987 Inclusions in Neurons. *The Journal of Neuroscience* **36**, 7415 (2016).

988 29 Liu, Z. *et al.* Inhibitors of LRRK2 kinase attenuate neurodegeneration and Parkinson-like
989 phenotypes in *Caenorhabditis elegans* and *Drosophila* Parkinson's disease models. *Human*
990 *Molecular Genetics* **20**, 3933-3942, doi:10.1093/hmg/ddr312 (2011).

991 30 Yao, C. *et al.* Kinase inhibitors arrest neurodegeneration in cell and *C. elegans* models of LRRK2
992 toxicity. *Human Molecular Genetics* **22**, 328-344, doi:10.1093/hmg/ddc431 (2013).

993 31 Afsari, F. *et al.* Abnormal visual gain control in a Parkinson's disease model. *Human Molecular*
994 *Genetics* **23**, 4465-4478, doi:10.1093/hmg/ddu159 (2014).

995 32 Dzamko, N. *et al.* Inhibition of LRRK2 kinase activity leads to dephosphorylation of
996 Ser(910)/Ser(935), disruption of 14-3-3 binding and altered cytoplasmic localization. *The*
997 *Biochemical journal* **430**, 405-413, doi:10.1042/BJ20100784 (2010).

998 33 Ramsden, N. *et al.* Chemoproteomics-based design of potent LRRK2-selective lead compounds
999 that attenuate Parkinson's disease-related toxicity in human neurons. *ACS Chemical Biology* **6**,
1000 1021-1028, doi:10.1021/cb2002413 (2011).

1001 34 Zhang, J., Deng, X., Geun, H., Alessi, D. R. & Gray, N. S. Characterization of TAE684 as a potent
1002 LRRK2 kinase inhibitor. *Bioorganic & Medicinal Chemistry Letters* **22**, 1864-1869,
1003 doi:10.1016/j.bmcl.2012.01.084 (2012).

1004 35 Deng, X. *et al.* Characterization of a selective inhibitor of the Parkinson's disease kinase LRRK2.
1005 *Nature chemical biology* **7**, 203-205, doi:10.1038/nchembio.538 (2011).

1006 36 Reith, A. D. *et al.* GSK2578215A ; A potent and highly selective 2-arylmethoxy-5-substituent- N
1007 -arylbenzamide LRRK2 kinase inhibitor. *Bioorganic & Medicinal Chemistry Letters* **22**, 5625-5629,
1008 doi:10.1016/j.bmcl.2012.06.104 (2012).

1009 37 Fujii, R. N. *et al.* Effect of selective LRRK2 kinase inhibition on nonhuman primate lung. *Science*
1010 *translational medicine* **7**, 273ra215-273ra215, doi:10.1126/scitranslmed.aaa3634 (2015).

1011 38 Henderson, J. L. *et al.* Discovery and preclinical profiling of 3-[4-(morpholin-4-yl)-7H-pyrrolo[2,3-
1012 d]pyrimidin-5-yl]benzotrile (PF-06447475), a highly potent, selective, brain penetrant, and in
1013 vivo active LRRK2 kinase inhibitor. *Journal of Medicinal Chemistry* **58**, 419-432,
1014 doi:10.1021/jm5014055 (2015).

1015 39 Fell, M. J. *et al.* MLi-2, a Potent, Selective, and Centrally Active Compound for Exploring the
1016 Therapeutic Potential and Safety of LRRK2 Kinase Inhibition. *The Journal of pharmacology and*
1017 *experimental therapeutics* **355**, 397-409, doi:10.1124/jpet.115.227587 (2015).

1018 40 Anand, V. S. *et al.* Investigation of leucine-rich repeat kinase 2: Enzymological properties and
1019 novel assays. *FEBS Journal* **276**, 466-478, doi:10.1111/j.1742-4658.2008.06789.x (2009).

1020 41 Randaccio, L., Geremia, S., Demitri, N. & Wuerges, J. Vol. 15 3228-3259 (2010).

1021 42 Banerjee, R., Gherasim, C. & Padovani, D. The tinker , tailor , soldier in intracellular B 12
1022 trafficking. *Current Opinion in Chemical Biology* **13**, 484-491, doi:10.1016/j.cbpa.2009.07.007
1023 (2009).

1024 43 Green, R. *et al.* Vitamin B12 deficiency. *Nature Reviews Disease Primers* **3**, 17040,
1025 doi:10.1038/nrdp.2017.40 (2017).

1026 44 Li, X. *et al.* Phosphorylation-dependent 14-3-3 binding to LRRK2 is impaired by common
1027 mutations of familial parkinson's disease. *PLoS ONE* **6**, 1-13, doi:10.1371/journal.pone.0017153
1028 (2011).

1029 45 Semisotnov, G. V. *et al.* Study of the "molten globule" intermediate state in protein folding by a
1030 hydrophobic fluorescent probe. *Biopolymers* **31**, 119-128, doi:10.1002/bip.360310111 (1991).

1031 46 Wienken, C. J., Baaske, P., Rothbauer, U., Braun, D. & Duhr, S. Protein-binding assays in
1032 biological liquids using microscale thermophoresis. *Nature Communications* **1**, 1-7,
1033 doi:10.1038/ncomms1093 (2010).

1034 47 Gilsbach, B. K. *et al.* Structural characterization of LRRK2 inhibitors. *Journal of Medicinal*
1035 *Chemistry* **58**, 3751-3756, doi:10.1021/jm5018779 (2015).

1036 48 McCoy, M. A., Senior, M. M. & Wyss, D. F. Screening of Protein Kinases by ATP-STD NMR
1037 Spectroscopy. *Journal of the American Chemical Society* **127**, 7978-7979, doi:10.1021/ja0425942
1038 (2005).

1039 49 Gilsbach, B. K. *et al.* Roco kinase structures give insights into the mechanism of Parkinson
1040 disease-related leucine-rich-repeat kinase 2 mutations. *Proceedings of the National Academy of*
1041 *Sciences of the United States of America* **109**, 10322-10327, doi:10.1073/pnas.1203223109
1042 (2012).

1043 50 Li, T. *et al.* A novel GTP-binding inhibitor, FX2149, attenuates LRRK2 toxicity in Parkinson's
1044 disease models. *PLoS ONE* **10**, 1-15, doi:10.1371/journal.pone.0122461 (2015).

1045 51 Copeland, R. A. in *Evaluation of Enzyme Inhibitors in Drug Discovery* 57-121 (John Wiley &
1046 Sons, Inc., 2013).

1047 52 Nichols, R. J. *et al.* Substrate specificity and inhibitors of LRRK2, a protein kinase mutated in
1048 Parkinson's disease. *The Biochemical journal* **424**, 47-60, doi:10.1042/BJ20091035 (2009).

1049 53 Copeland, R. A., Harpel, M. R. & Tummino, P. J. Targeting enzyme inhibitors in drug discovery.
1050 *Expert Opinion on Therapeutic Targets* **11**, 967-978, doi:10.1517/14728222.11.7.967 (2007).

1051 54 Lobbestael, E. *et al.* Pharmacological LRRK2 kinase inhibition induces LRRK2 protein
1052 destabilization and proteasomal degradation. *Scientific Reports* **6**, 33897,
1053 doi:10.1038/srep33897 (2016).

1054 55 Zhao, J., Molitor, T. P., Langston, J. W. & Nichols, R. J. LRRK2 dephosphorylation increases its
1055 ubiquitination. *Biochemical Journal* **469**, 107-120 (2015).

1056 56 Skibinski, G., Nakamura, K., Cookson, M. R. & Finkbeiner, S. Mutant LRRK2 toxicity in neurons
1057 depends on LRRK2 levels and synuclein but not kinase activity or inclusion bodies. *Journal of*
1058 *Neuroscience* **34**, 418-433 (2014).

1059 57 Li, X. *et al.* Leucine-rich repeat kinase 2 (LRRK2)/PARK8 possesses GTPase activity that is altered
1060 in familial Parkinson's disease R1441C/G mutants. *Journal of Neurochemistry* **103**, 238-247,
1061 doi:10.1111/j.1471-4159.2007.04743.x (2007).

1062 58 Melrose, H. L. *et al.* Impaired dopaminergic neurotransmission and microtubule-associated
1063 protein tau alterations in human LRRK2 transgenic mice. *Neurobiology of Disease* **40**, 503-517,
1064 doi:10.1016/j.nbd.2010.07.010 (2010).

1065 59 Tong, Y. *et al.* R1441C mutation in LRRK2 impairs dopaminergic neurotransmission in mice.
1066 *Proceedings of the National Academy of Sciences of the United States of America* **106**, 14622-
1067 14627, doi:10.1073/pnas.0906334106 (2009).

1068 60 Imai, Y. *et al.* Phosphorylation of 4E-BP by LRRK2 affects the maintenance of dopaminergic
1069 neurons in *Drosophila*. *The EMBO journal* **27**, 2432-2443, doi:10.1038/emboj.2008.163 (2008).

1070 61 Liu, Z. *et al.* A *Drosophila* model for LRRK2-linked parkinsonism. *Proceedings of the National*
1071 *Academy of Sciences of the United States of America* **105**, 2693-2698,
1072 doi:10.1073/pnas.0708452105 (2008).

1073 62 Saha, S. *et al.* LRRK2 modulates vulnerability to mitochondrial dysfunction in *Caenorhabditis*
1074 *elegans*. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **29**,
1075 9210-9218, doi:10.1523/JNEUROSCI.2281-09.2009 (2009).

1076 63 Yao, C. *et al.* LRRK2-mediated neurodegeneration and dysfunction of dopaminergic neurons in a
1077 *Caenorhabditis elegans* model of Parkinson ' s disease. *Neurobiology of Disease* **40**, 73-81,
1078 doi:10.1016/j.nbd.2010.04.002 (2010).

1079 64 Sawin, E. R., Ranganathan, R. & Horvitz, H. R. C. *elegans* Locomotory Rate Is Modulated by the
1080 Environment through a Dopaminergic Pathway and by Experience through a Serotonergic
1081 Pathway. *Neuron* **26**, 619-631, doi:10.1016/S0896-6273(00)81199-X (2000).

1082 65 Jackson, C. R. *et al.* Retinal Dopamine Mediates Multiple Dimensions of Light-Adapted Vision.
1083 *Journal of Neuroscience* **32**, 9359-9368, doi:10.1523/JNEUROSCI.0711-12.2012 (2012).

1084 66 Harnois, C. & Di Paolo, T. Decreased dopamine in the retinas of patients with Parkinson's
1085 disease. *Investigative Ophthalmology and Visual Science* **31**, 2473-2475 (1990).

1086 67 Hindle, S. *et al.* Dopaminergic expression of the Parkinsonian gene LRRK2-G2019S leads to non-
1087 autonomous visual neurodegeneration, accelerated by increased neural demands for energy.
1088 *Human Molecular Genetics* **22**, 2129-2140, doi:10.1093/hmg/ddt061 (2013).

1089 68 Chyb, S. *et al.* Modulation of the light response by cAMP in *Drosophila* photoreceptors. *The*
1090 *Journal of neuroscience : the official journal of the Society for Neuroscience* **19**, 8799-8807
1091 (1999).

1092 69 West, R. J. H., Furrmston, R., Williams, C. A. C. & Elliott, C. J. H. Neurophysiology of *Drosophila*
1093 Models of Parkinson's Disease. *Parkinson's Disease* **2015**, 11, doi:10.1155/2015/381281 (2015).

1094 70 Ho, C. C.-Y., Rideout, H. J., Ribe, E., Troy, C. M. & Dauer, W. T. The Parkinson disease protein
1095 leucine-rich repeat kinase 2 transduces death signals via Fas-associated protein with death
1096 domain and caspase-8 in a cellular model of neurodegeneration. *The Journal of neuroscience :*
1097 *the official journal of the Society for Neuroscience* **29**, 1011-1016, doi:10.1523/JNEUROSCI.5175-
1098 08.2009 (2009).

1099 71 Xiong, Y., Yuan, C., Chen, R., Dawson, T. M. & Dawson, V. L. ArfGAP1 is a GTPase activating
1100 protein for LRRK2: reciprocal regulation of ArfGAP1 by LRRK2. *The Journal of neuroscience : the*

1101 *official journal of the Society for Neuroscience* **32**, 3877-3886, doi:10.1523/JNEUROSCI.4566-
1102 11.2012 (2012).

1103 72 Li, Y. *et al.* Mutant LRRK2(R1441G) BAC transgenic mice recapitulate cardinal features of
1104 Parkinson's disease. *Nature neuroscience* **12**, 826-828, doi:10.1038/nn.2349 (2009).

1105 73 Zhang, H. & Sulzer, D. Glutamate Spillover in the Striatum Depresses Dopaminergic Transmission
1106 by Activating Group I Metabotropic Glutamate Receptors. *The Journal of Neuroscience* **23**, 10585
1107 (2003).

1108 74 Dar, A. C. & Shokat, K. M. The Evolution of Protein Kinase Inhibitors from Antagonists to
1109 Agonists of Cellular Signaling. *Annual Review of Biochemistry* **80**, 769-795, doi:10.1146/annurev-
1110 biochem-090308-173656 (2011).

1111 75 Weinberg, J. B. *et al.* Free Radical Biology & Medicine Inhibition of nitric oxide synthase by
1112 cobalamins and cobinamides. *Free Radical Biology and Medicine* **46**, 1626-1632,
1113 doi:10.1016/j.freeradbiomed.2009.03.017 (2009).

1114 76 Weinberg, J. B., Shugars, D. C., Sherman, P. A., Sauls, D. L. & Fyfe, J. A. Cobalamin Inhibition of
1115 HIV-1 Integrase and Integration of HIV-1 DNA into Cellular DNA. *BIOCHEMICAL AND*
1116 *BIOPHYSICAL RESEARCH COMMUNICATIONS* **397**, 393-397, doi:10.1006/bbrc.1998.8629 (1998).

1117 77 Carkeet, C. *et al.* Human vitamin B(12) absorption measurement by accelerator mass
1118 spectrometry using specifically labeled (14)C-cobalamin. *Proceedings of the National Academy*
1119 *of Sciences of the United States of America* **103**, 5694-5699, doi:10.1073/pnas.0601251103
1120 (2006).

1121 78 Wuerges, J. *et al.* Structural basis for mammalian vitamin B12 transport by transcobalamin.
1122 *Proceedings of the National Academy of Sciences of the United States of America* **103**, 4386-
1123 4391, doi:10.1073/pnas.0509099103 (2006).

1124 79 Pezacka, E. H., Jacobsen, D. W., Luce, K. & Green, R. Glial cells as a model for the role of
1125 cobalamin in the nervous system: Impaired synthesis of cobalamin coenzymes in cultured
1126 human astrocytes following short-term cobalamin-deprivation. *Biochemical and Biophysical*
1127 *Research Communications* **184**, 832-839, doi:[https://doi.org/10.1016/0006-291X\(92\)90665-8](https://doi.org/10.1016/0006-291X(92)90665-8)
1128 (1992).

1129 80 Bhatia, P. & Singh, N. Homocysteine excess: Delineating the possible mechanism of
1130 neurotoxicity and depression. *Fundamental and Clinical Pharmacology* **29**, 522-528,
1131 doi:10.1111/fcp.12145 (2015).

1132 81 Miller, A., Korem, M., Almog, R. & Galboiz, Y. Vitamin B12, demyelination, remyelination and
1133 repair in multiple sclerosis. *Journal of the Neurological Sciences* **233**, 93-97,
1134 doi:10.1016/j.jns.2005.03.009 (2005).

1135 82 Shen, L. Associations between B vitamins and Parkinson's disease. *Nutrients* **7**, 7197-7208,
1136 doi:10.3390/nu7095333 (2015).

1137 83 Triantafyllou, N. I. *et al.* Folate and vitamin B12 levels in levodopa-treated Parkinson's disease
1138 patients: Their relationship to clinical manifestations, mood and cognition. *Parkinsonism &*
1139 *Related Disorders* **14**, 321-325, doi:<http://dx.doi.org/10.1016/j.parkreldis.2007.10.002> (2008).

1140 84 Toth, C. *et al.* Levodopa, methylmalonic acid, and neuropathy in idiopathic Parkinson disease.
1141 *Annals of Neurology* **68**, 28-36, doi:10.1002/ana.22021 (2010).

1142 85 Muller, T., Renger, K. & Kuhn, W. Levodopa-associated increase of homocysteine levels and sural
1143 axonal neurodegeneration. *Archives of neurology* **61**, 657-660, doi:10.1001/archneur.61.5.657
1144 (2004).

1145 86 Christine, C. A., Peggy; Joslin, Amelia; Yelapaala, Yuora; Green, Ralph. Vitamin B12 and
1146 Homocysteine Levels Predict Different Outcomes in Early Parkinson's Disease. *Movement*
1147 *Disorders* **33**, 762-770, doi:10.1002/mds.27301 (2018).

1148 87 Baptista, M. A. S. *et al.* Loss of Leucine-Rich Repeat Kinase 2 (LRRK2) in Rats Leads to Progressive
1149 Abnormal Phenotypes in Peripheral Organs. *PLOS ONE* **8**, e80705,
1150 doi:10.1371/journal.pone.0080705 (2013).

1151 88 Perera, G., Ranola, M., Rowe, D. B., Halliday, G. M. & Dzamko, N. Inhibitor treatment of
1152 peripheral mononuclear cells from Parkinson's disease patients further validates LRRK2
1153 dephosphorylation as a pharmacodynamic biomarker. *Scientific Reports* **6**, 31391,
1154 doi:10.1038/srep31391
1155 <https://www.nature.com/articles/srep31391-supplementary-information> (2016).

1156 89 Fan, Y. *et al.* Interrogating Parkinson's disease LRRK2 kinase pathway activity by assessing Rab10
1157 phosphorylation in human neutrophils. *Biochemical Journal* **475**, 23 (2018).

1158 90 Fraser, K. R., Ashlee; Clark, Rachel; Alcalay, Roy, Standaert, David; Liu, Nianjun; West, Andrew.
1159 Ser(P)-1292 LRRK2 in urinary exosomes is elevated in idiopathic Parkinson's disease. *Movement*
1160 *Disorders* **31**, 1543-1550, doi:10.1002/mds.26686 (2016).

1161 91 Santos-garcía, D. *et al.* COPPADIS-2015 (COhort of Patients with PARkinson ' s Disease in Spain ,
1162 2015), a global – clinical evaluations , serum biomarkers , genetic studies and neuroimaging –
1163 prospective , multicenter , non-interventional , long-term study on Parkinson ' s dise. *BMC*
1164 *Neurology* **2015**, 1-14, doi:10.1186/s12883-016-0548-9 (2016).

1165 92 Hui, K. Y. *et al.* Functional variants in the LRRK2 gene confer shared effects on risk for Crohn's
1166 disease and Parkinson's disease. *Science Translational Medicine* **10** (2018).

1167 93 Mayer, M. & Meyer, B. Group Epitope Mapping by Saturation Transfer Difference NMR To
1168 Identify Segments of a Ligand in Direct Contact with a Protein Receptor. *Journal of the American*
1169 *Chemical Society* **123**, 6108-6117, doi:10.1021/ja0100120 (2001).

1170 94 Summers, M. F., Marzilli, L. G. & Bax, A. Complete proton and carbon-13 assignments of
1171 coenzyme B12 through the use of new two-dimensional NMR experiments. *Journal of the*
1172 *American Chemical Society* **108**, 4285-4294, doi:10.1021/ja00275a008 (1986).

1173 95 Zhang, J., Tian, Z., Liang, L., Subirade, M. & Chen, L. Binding interactions of beta-conglycinin and
1174 glycinin with vitamin B12. *J Phys Chem B* **117**, 14018-14028, doi:10.1021/jp408578m (2013).

1175 96 Fernández-Suárez, M., Chen, T. S. & Ting, A. Y. Protein-Protein Interaction Detection in Vitro and
1176 in Cells by Proximity Biotinylation. *Journal of the American Chemical Society* **130**, 9251-9253,
1177 doi:10.1021/ja801445p (2008).

1178 97 Melachroinou, K. *et al.* Activation of FADD-Dependent Neuronal Death Pathways as a Predictor
1179 of Pathogenicity for LRRK2 Mutations. *PLOS ONE* **11**, e0166053,
1180 doi:10.1371/journal.pone.0166053 (2016).

1181

1182 Supplementary information is available at *Cell Research's* website

1183
1184

1185

1186

1187 **Fig. 1. AdoCbl inhibits LRRK2 kinase activity.** (a) Domain structure of LRRK2. (b) Dose-
1188 response curves of brain-purified flag-tagged LRRK2 kinase as a function of different forms of

1189 cobalamin. Phosphorylation is quantified by measuring TR-FRET emission ratios of fluorescein-
1190 LRRKtide and a Terbium-labeled pLRRKtide antibody. (c) Dose-response curves of strep-
1191 tagged LRRK2 autophosphorylation or (d) phosphorylation of myelin basic protein as a function
1192 of different forms of cobalamin. (e) Dose-response curve of strep-tagged LRRK2-G2019S
1193 phosphorylation of purified Rab10 as a function of AdoCbl. (f) Dose-response curves of
1194 pS935/Total LRRK2 and (g) pS1292/Total LRRK2 after treatment with different forms of
1195 cobalamin in MEF cells derived from LRRK2-G2019S BAC transgenic mice. Data from each
1196 replicate was normalized to LRRK2 phosphorylation without cobalamin treatment. All data
1197 points represent the mean (\pm s.d.) of three biological replicates.

1198

1199

1200

1201

1202

1203

1204

1205

1206

1207

1208

1209

1210 **Fig. 2. Direct binding of AdoCbl to LRRK2 protein.** (a) Binding of strep-tagged LRRK2 to
1211 AdoCbl-agarose in the presence of AdoCbl. Input represents the amount of protein that was

1212 added to beads, while pull-down denotes the amount of protein left on the beads after washes.
1213 Significance was calculated by one-way ANOVA using the mean (\pm s.d.) of three biological
1214 replicates. * $p \leq 0.05$, ** $p \leq 0.005$ (b) Thermal shift assays showing melting temperatures of
1215 strep-LRRK2 in the presence of AdoCbl or PF-06447475. (c) Microscale thermophoretic
1216 analysis of the interaction between AdoCbl or (d) PF-06447475 with strep-tagged LRRK2. (e)
1217 Coomassie stained SDS-PAGE of the Roco4 kinase domain purified from E. coli. (f) Dose-
1218 response curve of Roco4 kinase activity as a function of AdoCbl. (g) ATP STD-NMR shows
1219 direct binding of AdoCbl to the Roco4 kinase domain and competition with ATP. From top to
1220 bottom, the spectra are as follows: 1D ^1H NMR of ATP (blue), AdoCbl (red), STD negative
1221 control with ATP + AdoCbl only (green), STD positive control with ATP and Roco4 kinase
1222 domain (orange), STD of AdoCbl and Roco4 kinase domain with 1:1 ratio of AdoCbl to ATP
1223 (purple), and STD of AdoCbl and Roco4 kinase domain with 10:1 ratio of AdoCbl to ATP
1224 (yellow). AdoCbl protons showing strong STD signals are labeled with assignment. All
1225 experiments were collected at 4°C on a Bruker 800MHz spectrometer equipped with a
1226 cryoprobe. (h) Protons with strong STD signals (highlighted in red) mapped onto the structure of
1227 AdoCbl. The NMR assignment and nomenclature of vitamin B12 is from Summers et al.⁹⁴ Data
1228 points in (a,c,d,f) represent the mean (\pm s.d.) of three biological replicates.

1229

1230

1231

1232

1233 **Fig. 3. AdoCbl exhibits a mixed-mode of inhibition.** (a) Michaelis-Menten kinetics curves of
1234 full-length Invitrogen flag-tagged LRRK2-WT and (c) LRRK2-G2019S as a function of AdoCbl.

1235 Relative velocity represents the value of pS1292/Total LRRK2 after 20 minutes of reaction time
1236 at 30°C (during the linear reaction rate), as detected by western blot and quantified by
1237 densitometry. (b) Lineweaver-Burk plots of LRRK2-WT and (d) LRRK2-G2019S kinetics data.
1238 (e) Microscale thermophoretic analysis of the interaction between AdoCbl and strep-tagged
1239 LRRK2 in the presence of increasing concentrations of AMP-PNP. Fluorescently-labeled strep-
1240 tagged LRRK2 was pre-incubated with AMP-PNP before binding between LRRK2 and AdoCbl
1241 was measured by MST. (f) Microscale thermophoretic analysis of the interaction between AMP-
1242 PNP and LRRK2, showing a K_D of 0.9 μ M. Data points represent the mean (\pm s.d.) of three
1243 biological replicates.

1244

1245

1246

1247

1248

1249

1250

1251

1252

1253

1254

1255

1256 **Fig. 4. AdoCbl causes a LRRK2 conformational change and destabilizes LRRK2 dimers.**

1257 (a) Coomassie stained SDS-PAGE showing limited proteolysis analysis using a 10:1 molar ratio

1258 of LRRK2: Trypsin (left panel) and LRRK2: Chymotrypsin (right panel). Proteolysis was
1259 performed at 30°C with or without 50 μ M AdoCbl and reactions were quenched at the indicated
1260 times by the addition of sample loading buffer. The observed data was consistent across three
1261 biological replicates. **(b)** Limited proteolysis of LRRK2-WT by trypsin in the presence of
1262 increasing concentrations of AdoCbl, or 1 μ M LRRK2 kinase inhibitor. Proteolysis was
1263 performed for 90 minutes at 30°C. Shown is a representative SDS-PAGE of full-length LRRK2,
1264 in which bands were quantified and values were normalized to LRRK2 proteolysis without
1265 AdoCbl. **(c)** The peak intrinsic fluorescence of LRRK2 (339 nm) was measured as a function of
1266 AdoCbl. Strep-tagged LRRK2 was incubated with indicated concentrations of AdoCbl for 30
1267 minutes prior to fluorescence measurements. Significance was measured by one-way ANOVA. *
1268 $p \leq 0.05$, ** $p \leq 0.005$. **(d)** HEK293T cells co-expressing BirA-WT (biotin ligase) and AP-WT
1269 LRRK2 (acceptor peptide) were lysed following a biotin pulse to label dimeric LRRK2, and
1270 extracts bound to streptavidin-coated ELISA plates. LRRK2 was detected using anti-LRRK2
1271 conjugated to HRP (clone N241A/B34) and expressed as a ratio of total LRRK2 levels detected
1272 by ELISA in parallel plates coated with total LRRK2 antibodies (clone c41-2). In the plot,
1273 “WT/WT NP” refers to cells expressing WT LRRK2 dimers that were harvested without
1274 receiving a biotin pulse (“no pulse”). AdoCbl significantly reduced levels of dimeric WT-
1275 LRRK2. Sub-panel shows representative immunoblot of parallel extracts detected with anti-
1276 LRRK2 (clone N241A/B34). BirA-LRRK2 represents the top band, and AP-LRRK2 the bottom
1277 band. **(e)** HEK293T cells expressing BirA- G2019S or I2020T mutant LRRK2 together with AP-
1278 G2019S or I2020T LRRK2, and dimeric LRRK2 quantified by ELISA. Treatment with AdoCbl
1279 significantly reduces dimeric mutant LRRK2. * $p < 0.05$ compared to WT/WT-LRRK2; *** $p <$
1280 0.001 compared to G2019S-LRRK2 dimers or I2020T-LRRK2 dimers alone. **(f)** Representative

1281 immunoblot of parallel extracts detected with anti-LRRK2 (clone N241A/B34). BirA-LRRK2
1282 represents the top band, and AP-LRRK2 the bottom band.

1283

1284

1285

1286

1287

1288

1289

1290

1291

1292

1293

1294

1295

1296

1297

1298

1299

1300

1301

1302 **Fig. 5. AdoCbl rescues mutant human LRRK2-induced behavioral defects and**
1303 **dopaminergic neurodegeneration in *C. elegans*. (a) AdoCbl dose dependently rescues the loss**

1304 of basal slowing response in transgenic *hLRRK2-G2019S C. elegans*. Age-synchronized
1305 nematodes expressing GFP marker only or additionally *hLRRK2-G2019S* in dopaminergic
1306 neurons were treated with either vehicle or AdoCbl in liquid culture during the larval stage L1 to
1307 L4 (3 days), followed by growth on NGM plates for 3 days prior to behavior assay. Basal
1308 slowing response was assayed on NGM plates using an unbiased machine-vision analysis system
1309 (WormLab) as the percent slowing in body bends per 20 s in the presence vs. the absence of
1310 bacterial lawn. Data represent the mean (\pm s.d.) of three biological replicates, each with 20-25
1311 worms per treatment condition. **(b)** AdoCbl treatment attenuated the loss of dopaminergic
1312 neurons induced by *hLRRK2-G2019S* in *C. elegans*. Representative fluorescence images of
1313 dopaminergic neurons (CEP neurons within the outlined head region) in transgenic *C. elegans*
1314 expressing GFP marker only or additionally *hLRRK2-G2019S* following treatment with either
1315 vehicle or 1.25 μ M AdoCbl. Age-synchronized nematodes were treated with either vehicle or
1316 AdoCbl in liquid culture during the larval stage L1 to L4 (3 days), followed by growth on NGM
1317 plates for 9 days. GFP-tagged dopaminergic neurons in live animals were counted under a
1318 fluorescence microscope. **(c)** Quantification of percent dopaminergic neurons survived. Data are
1319 presented as the mean (\pm s.d.) of three biological replicates, each with approximately 30-50
1320 worms per treatment condition. $P < 0.01$, Student's t-test. n.s., not statistically significant.

1321

1322

1323

1324 **Fig. 6. AdoCbl rescues deficits in *Drosophila* visual physiology induced by the**
1325 **dopaminergic expression of human *LRRK2-G2019S*.** **(a)** Outline of the retinal neural network
1326 of *Drosophila*, with three main neuronal layers: photoreceptors, lamina neurons and medulla

1327 neurons (Modified after Afsari et al³¹). **(b)** Contrast response functions (CRFs) for the
1328 photoreceptors, lamina neurons and medulla neurons show that the dopaminergic expression of
1329 *hLRRK2-G2019S* (*DA* → *G2019S*) flies have a much bigger response than either the *DA* →
1330 *hLRRK2* or the *DA* → *G2019S* which have been fed 2.5 μM AdoCbl. **(c)** Dose-response curve
1331 for the effect of AdoCbl on the *DA* → *G2019S* flies, shows a 50% reduction in phenotypes by
1332 250-500 nM AdoCbl, with almost complete rescue by 2.5 μM AdoCbl. **(d)** There is no effect of
1333 2.5 μM AdoCbl on flies with dopaminergic expression of kinase-dead *hLRRK2-G2019S*-
1334 *K1906M* (*DA* → *KD*). **(e)** The visual response of flies with wild-type *dLRRK2* is reduced by
1335 2.5μM AdoCbl. **(f)** Applying 2.5 μM AdoCbl to *dLRRK*⁻ transheterozygote flies (in which the
1336 *drosophila* LRRK2 homolog has been knocked out) has no statistically significant effect. Data
1337 represents the mean (± s.d.) and the numbers in brackets are the number of flies tested. In **(c)**,
1338 statistical analysis from Tukey Post-hoc tests on the first principal component of a PCA, which
1339 accounted for 88% of the variance (Supplementary information, **Fig. S9**). **(d-f)**, analysis by
1340 MANOVA. n.s. not significant; ***p < 0.001). Boxes correspond to the median +/- quartiles.
1341 Dots indicate data from individual flies. *dLRRK*⁻ genotype: *dLRRK*^{e03680}/*dLRRK*^{ex1}; *wild*
1342 *type* genotype: *w*^a/*w*¹¹¹⁸.

1343

1344

1345

1346

1347

1348 **Fig. 7. AdoCbl prevents LRRK2-G2019S induced neurotoxicity and rescues deficits in**

1349 **dopamine transmission in LRRK2-PD mouse models. (a)** Quantification of percent apoptotic

1350 neurons after LRRK2 overexpression and treatment with MLI-2 or AdoCbl. Cortical neurons
1351 were co-transfected with LRRK2 and a GFP reporter. Transfected neurons displaying apoptotic
1352 nuclear morphology were counted 48 h after transfection using DAPI. Apoptotic neurons were
1353 defined as those having condensed fragmented chromatin comprised of two or more apoptotic
1354 bodies. Data represents the mean (\pm s.d.) from $n = 3$ biological replicates of triplicate coverslips.
1355 Significance was measured by one-way ANOVA. **(b)** Quantification of pS1292/Total LRRK2
1356 after brain slice tissue from LRRK2-G2019S BAC-transgenic mice were treated with AdoCbl.
1357 One mouse brain provided enough slices to test each treatment condition one time. Three mouse
1358 brains were used in total, resulting in three biological replicates. Data are the mean (\pm s.d.) and
1359 significance was measured by one-way ANOVA **(c)** Voltammetric traces of striatal DA release
1360 evoked at 2-min intervals from G2019S, or **(e)** R1441G, and WT controls after 2 h treatment
1361 with control vehicle (water), or with 300 μ M AdoCbl. **(d)** Summary of DA release sustainability
1362 for G2019S mice ($n = 9$ sites) or **(f)** R1441G ($n = 10$ sites) compared to WT controls. Data are
1363 expressed as the mean (\pm s.d.) and were analyzed by two-way ANOVA with *Bonferroni's post*
1364 *hoc* analysis. For all figures, * $p \leq 0.01$, ** $p \leq 0.001$, *** $p \leq 0.0001$.













