An Iron-Calcium-Miro Axis Influences Parkinson's Risk and Neurodegeneration

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Summary

Genetic backgrounds and risk factors among individuals with Parkinson's disease (PD) are highly heterogenous, limiting our ability to effectively detect and treat PD. Here we connect several potential PD risk genes and elements to one biological pathway. Elevation of Fe^{2+}-levels causes Ca^{2+}-overflow into the mitochondria, through an interaction of Fe^{2+} with mitochondrial calcium uniporter (MCU), the Ca^{2+}-import channel in the inner mitochondrial membrane, and resultant MCU oligomerization. This mechanism acts in PD neuron models and postmortem brains. Miro, a Ca^{2+}-binding protein, functions downstream of Ca^{2+}-dysregulation, and holds promise to classify PD status and monitor drug efficacy in human blood cells. Polygenetic enrichment of rare, non-synonymous variants in this iron-calcium-Miro axis influences PD risk. This axis can be targeted by multiple ways to prevent neurodegeneration in PD models. Our results show a linear pathway linking several PD risk factors, which can be leveraged for genetic counseling, risk evaluation, and therapeutic strategies.

Main Text

Introduction

Parkinson's disease (PD) is a leading cause of disability, afflicting the aging population. The dopamine (DA)-producing neurons in the substantia nigra are the first to die in PD patients. A bottleneck that hinders our ability to effectively detect and treat PD may be the presence of highly heterogenous genetic backgrounds and risk factors among different patients. More than 90% of the PD cases are considered sporadic with no known causal mutations. Genome-wide association studies (GWAS) have identified over 90 risk loci (Diaz-Ortiz et al., 2022). Functional studies on known causal genes of familial patients and from cellular and animal PD models have pointed to multiple "cellular risk elements", such as mitochondrial damage, lysosomal dysfunction, immune system activation, neuronal calcium mishandling,

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46 and iron accumulation (Angelova et al., 2020; Apicco et al., 2021; Belaidi and Bush, 2016; Buttner et al.,

47 2013; Kim et al., 2020; Lee et al., 2018; Surmeier et al., 2017; Tabata et al., 2018; Verma et al., 2017;

48 Vuuren et al., 2020). These distinct genetic and cellular risk factors may confer individual heterogeneity

49 in disease onset, but also suggest that there are networks and pathways linking these "hubs" in disease

50 pathogenesis. Identifying their connections could be crucial for finding a cure for PD.

51

52 Mitochondria are the center of cellular metabolism and communication. Ions such as calcium and iron,

53 are not only essential for diverse mitochondrial functions but can be stored inside the mitochondria to

54 maintain cellular ionic homeostasis. Ion channels in the plasma and mitochondrial membranes coordinate

55 for ion uptake, transport, and storage. For example, calcium ions enter the cell via voltage- or ligand-gated

56 calcium channels across the cell surface. Inside the cell, they are taken up by mitochondria through the

57 outer mitochondrial membrane (OMM) channel, VDAC, and the inner mitochondrial membrane (IMM)

58 channel, mitochondrial calcium uniporter (MCU) (Baughman et al., 2011), and extruded into the cytosol

59 through the IMM transporter, NCLX (Palty et al., 2010). MCU is a multimeric holocomplex consisting of

60 additional regulatory subunits, such as essential MCU regulator (EMRE), mitochondrial calcium uptake

61 1 (MICU1), MICU2, and MCUb (Fan et al., 2018; Fan et al., 2020; Lambert et al., 2019). Channels

62 complementary to these major mitochondrial calcium channels also exist (Patron et al., 2022). It remains

63 a mystery regarding the relation of calcium and iron ions in PD mechanisms and their contribution to

64 disease susceptibility.

65

66 Identifying the cellular causes to neuron death will not only provide more effective disease management

67 but also shed light on molecular signatures shared by a subset of people affected by the disease. A

68 convenient, cost-effective method to spot the vulnerable population, even before the symptom onset, will

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69 be extremely valuable for early intervention and preventive medicine. It will improve the efficacy of

70 clinical trials for testing experimental drugs, by facilitating patient stratification and serving as a

71 pharmacodynamic marker.

72

73 Understanding disease-causing cellular paths will also help us zoom in on rare genetic variants that

74 contribute to disease etiology but otherwise are difficult to discover through GWAS. Integrating studies

75 of risk variants with disease models and human tissues could establish a causal link of a biological pathway

76 to a disease with complex traits such as PD, with the promise of identifying more effective druggable

77 targets and biomarkers. In this work, we harness the power of combining human genetics, cellular and in

78 vivo models, and patient's tissues, and identify an iron-calcium-Miro axis in PD. Iron accumulation causes

79 mitochondrial calcium overload via promoting MCU oligomerization and its channel activity, which may

80 consequently disturb cellular calcium homeostasis. Miro, a calcium-binding protein, acts downstream of

81 calcium dysregulation in PD models. Functional and genetic impairments in this axis may increase PD

82 risk and indicate PD status.

83

84 **Results**

85 **A high-content Miro1 screening assay identifies a network of Ca^{2+}-related drug hits for PD**

86 Miro is an OMM protein essential for mediating mitochondrial motility and safeguarding their quality.

87 Human Miro1 and Miro2 are paralogs with high sequence similarity. We have previously shown that in

88 fibroblasts or neurons derived from sporadic and familial PD patients, Miro1 degradation upon

89 mitochondrial depolarization is delayed, consequently slowing mitophagy and increasing neuronal

90 sensitivity to stressors (Hsieh et al., 2019; Hsieh et al., 2016; Shaltouki et al., 2018). This Miro1 phenotype

91 would serve as an excellent readout for screening small molecules that promote Miro1 degradation

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92   following depolarization. Many compounds in the commonly used screening libraries have well-defined

93   roles and targets, and some show efficacy to treat certain human diseases. This rich information may allow

94   us to reveal cellular pathways underlying the Miro1 phenotype in PD. To this end, we established a

95   sensitive immunocytochemistry (ICC)-based assay that was suitable for high-throughput screening

96   (Figures 1A, S1, S2, S3A, more details in Method). We performed the primary screens at the Stanford

97   High-Throughput Bioscience Center (HTBC) using 3 drug libraries in a sporadic PD fibroblast line.

98   Overall, we identified 35 actives (1.92% primary hit rate) that reduced Miro1 following mitochondrial

99   depolarization (Figure S1, Table S1A-B). To validate the results of the high-content assays, we retested

100   34 out of the 35 positive Miro1 reducers identified at the Stanford HTBC in our own laboratory using

101   fresh compounds and our confocal microscope. We confirmed that 15 compounds reliably reduced Miro1

102   protein levels following mitochondrial depolarization in PD fibroblasts (Figure S2, Table S1C). Next, we

103   performed a pathway analysis using a knowledge graph-based tool to reveal the potential cellular pathways

104   connecting Miro1 to each hit compound. Strikingly, we discovered intracellular Ca^{2+} as a primary shared

105   factor in the hit drug-Miro1 network (Figure 1B, Table S2). Two drugs, Benidipine and Tranilast, could

106   directly inhibit plasma membrane Ca^{2+}-channels. Benidipine is a blocker of voltage-gated Ca^{2+}-channels

107   (L-, N-, T-type), and Tranilast has been proposed to inhibit ligand-gated Ca^{2+}-channels (TRPV2)

108   (Darakhshan and Pour, 2015). Dysregulation of Ca^{2+} homeostasis has been widely reported in PD models

109   (Angelova et al., 2020; Apicco et al., 2021; Buttner et al., 2013; Kim et al., 2020; Lee et al., 2018; Surmeier

110   et al., 2017; Tabata et al., 2018; Verma et al., 2017). Our results suggest a direct link of the Ca^{2+}-binding

111   protein, Miro, to Ca^{2+}-mediated abnormality in PD.

112

113   **Validation of a role for Benidipine in Miro degradation**

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A

B

Figure 1. HTP Screens Identify Calcium-Related Drug Hits for PD. (A) Schematic representation of a custom-designed drug screen for Miro1 in PD fibroblasts. (B) Pathway analysis identified calcium as a shared factor in the primary hit-Miro1 network. Each individual pathway is generated using a primary hit and Miro1 as search query and the resulting subnetwork is visualized and curated using docs2graph–a knowledge-graph browser. The visualization shows a subgraph generated by docs2graph from the collection of curated supporting documents for each pathway.

114 To understand in detail the relation of Miro with the Ca^{2+}-pathway in Parkinson's pathogenesis, we further

115 examined Benidipine's role in Miro protein stability. Using the same ICC method as in Figure S2, we

116 found that Benidipine reduced Miro1 in a dose-dependent manner in PD fibroblasts treated with carbonyl

117 cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP) (Figure S3B). To exclude the possibility of any

118 artifacts caused by our ICC method, we verified our results using an entirely different approach to detect

119 Miro1 response to depolarization. We measured Miro1 and additional mitochondrial proteins by Western

120 blotting. We also depolarized mitochondria with a different uncoupler, carbonyl cyanide *m*-chlorophenyl

121 hydrazone (CCCP) (Hsieh et al., 2019), instead of FCCP. We detected Miro1 and mitochondrial markers

122 at 6 and 14 hours after CCCP treatment. We have previously demonstrated that in healthy control

123 fibroblasts following CCCP treatment, Miro1 is degraded earlier (6 hours) than multiple other

124 mitochondrial markers (14 hours) (Figure S3C) (Hsieh et al., 2019; Hsieh et al., 2016), consistent with the

125 observation of proteasomal degradation of Miro1 prior to mitophagy (Chan et al., 2011; Hsieh et al., 2019;

126 Hsieh et al., 2016; Wang et al., 2011). Using this alternative method, we confirmed that both Miro1

127 degradation and damaged mitochondrial clearance were impaired in the PD cell line we used for screens.

128 Importantly, Benidipine promoted Miro1 degradation after 6 hours following CCCP treatment without

129 affecting the matrix protein ATP5β and facilitated mitochondrial clearance as was evidenced by the

130 degradation of both Miro1 and ATP5β at 14 hours post-treatment (Figure S3C). Interestingly, Miro2 was

131 also resistant to depolarization-induced degradation in PD cells (Hsieh et al., 2019) and Benidipine

132 rescued its phenotype (Figure S3C). This result suggests that Miro1 and Miro2, which share the Ca^{2+}-

133 binding EF-hands, are functionally redundant in the Ca^{2+}-dependent regulation. We confirmed that

134 Benidipine did not affect *Miro1* messenger RNA (mRNA) expression detected by reverse transcription

135 quantitative real-time PCR (RT-qPCR) under basal and depolarized conditions in PD cells (Figure S3D).

136 Neither did Benidipine alter the basal ATP levels (Figure S3E), nor the mitochondrial membrane potential

137 measured by TMRM staining (Figure S3F). Collectively, we have demonstrated that Benidipine, a Ca^{2+}-

138 channel blocker, specifically promotes Miro degradation upon depolarization in PD fibroblasts using

139 multiple methods.

140

141 **Benidipine rescues Parkinson's phenotypes in human neuron and fly models of PD**

142 We have previously shown that reducing Miro rescues Parkinson's phenotypes in cellular and in vivo

143 models (Hsieh et al., 2019; Hsieh et al., 2016; Li et al., 2021; Shaltouki et al., 2018), suggesting that small

144 molecules that lower Miro protein levels could represent an effective therapeutic approach for PD. From

145 our high-throughput screens we have discovered that Benidipine, which targets the Ca^{2+} pathway,

146 promotes Miro degradation upon depolarization in skin cells of a PD patient (Figures 1, S1-3). We next

147 tested whether Benidipine was useful for alleviating Parkinson's phenotypes in two independent models:

148 the human neuron and fly models. We examined Benidipine using induced pluripotent stem cells (iPSCs)

149 from one familial patient with the A53T mutation in *SNCA* (encodes α-synuclein) and its isogenic wild-

150 type control (Hsieh et al., 2019; Hsieh et al., 2016; Li et al., 2021; Shaltouki et al., 2018). We differentiated

151 iPSCs to neurons expressing tyrosine hydroxylase (TH), the rate-limiting enzyme for DA synthesis as

152 previously described (Figure S4A) (Hsieh et al., 2019; Hsieh et al., 2016; Li et al., 2021; Shaltouki et al.,

153 2018). These patient-derived neurons display increased expression of endogenous α-synuclein (Shaltouki

154 et al., 2018). We identified DA neurons by TH-immunostaining and cell death by terminal

155 deoxynucleotidyl transferase dUTP nick end labeling (TUNEL)-staining (Li et al., 2021; Shaltouki et al.,

156 2018) (Figures 2A-B, S4A). We have previously shown that iPSC-derived DA neurons from PD patients

157 are more vulnerable to stressors than those from healthy controls (Hsieh et al., 2019; Hsieh et al., 2016;

158 Li et al., 2021; Shaltouki et al., 2018). The treatment of the complex III inhibitor, Antimycin A, at 10 μM

159 for 6 hours caused acute neuronal cell death leading to the loss of TH and the increase of TUNEL signals
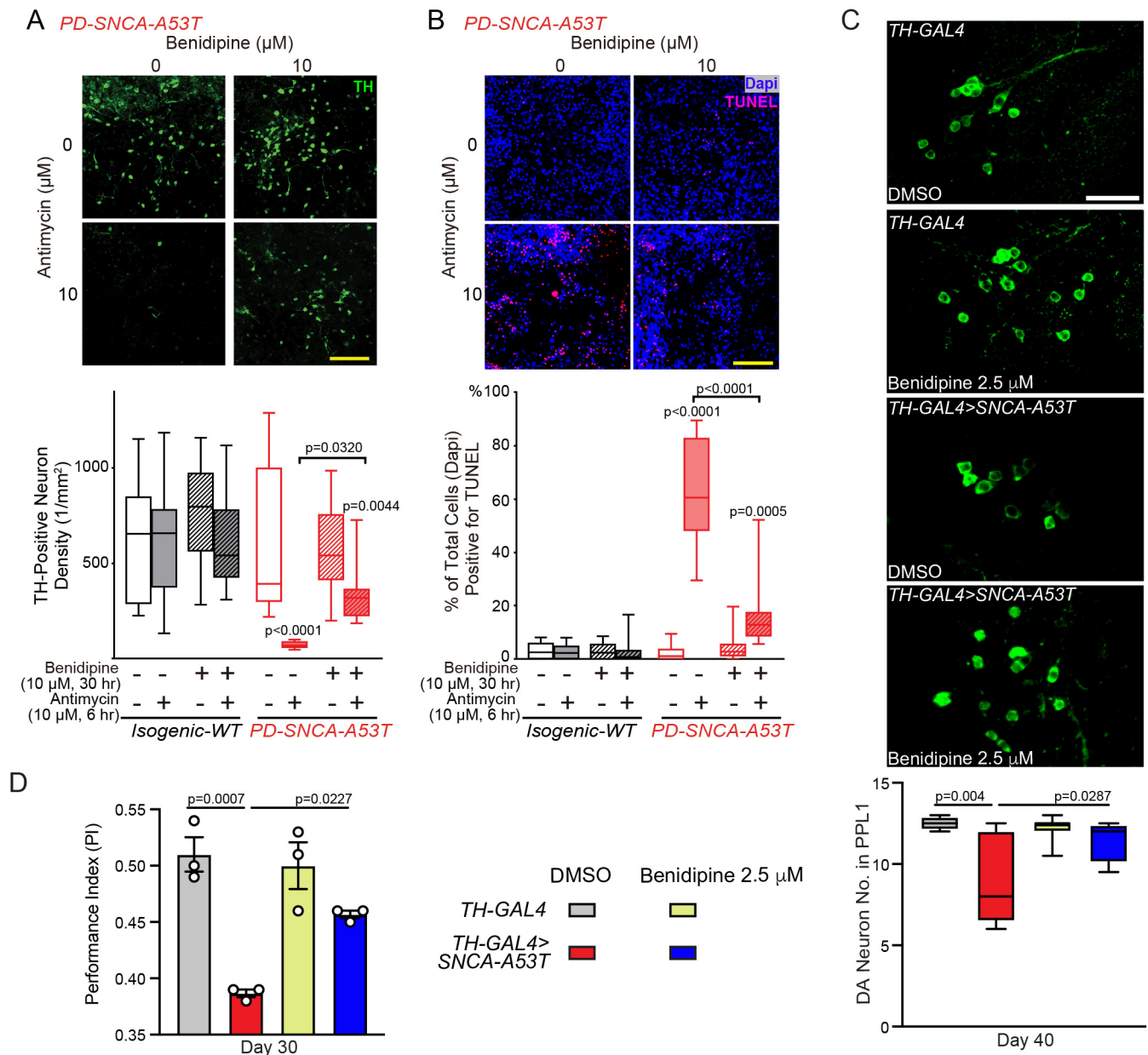
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Figure 2. Benidipine Rescues PD Relevant Phenotypes. (A-B) iPSC-derived neurons from a PD patient with SNCA-A53T and the isogenic control, treated as indicated, were immunostained with anti-TH (A) or TUNEL and Dapi (B), and imaged under a confocal microscope. Scale bars: 100 um. Below: Quantifications of the density of TH-positive neurons (A) or the percentage of TUNEL-positive neurons (B). n=20 images from 3 independent coverslips. P values are compared with the far-left bar, except indicated otherwise. (C) 40-day-old fly brains were immunostained with anti-TH and the DA neuron number was counted in the PPL1 cluster. Scale bar: 20 um. n=4, 7, 7, 4 (from left to right). (D) The Performance Index was measured in 30-day-old flies, fed as indicated. n=59, 57, 54, 57 flies (from left to right), 3 independent experiments. (C-D) Drug treatment was started from adulthood (day 1). One-Way Anova Post Hoc Tukey Test for all panels.

160   in neurons derived from the PD patient (Hsieh et al., 2019; Li et al., 2021; Shaltouki et al., 2018) (Figure

161   2A-B). Notably, Benidipine treatment at 10 μM for 30 hours significantly rescued this stressor-induced

162   neuron death (Figure 2A-B).

163   In order to cross-validate the neuroprotective effect of Benidipine in vivo, we fed Benidipine to a fly

164   model of PD, which expressed the pathogenic human α-synuclein protein with the A53T mutation (α-

165   syn-A53T) in DA neurons driven by *TH-GAL4* (Hsieh et al., 2019; Li et al., 2021; Shaltouki et al., 2018).

166   These flies exhibit PD-relevant phenotypes such as age-dependent locomotor decline and DA neuron loss

167   (Hsieh et al., 2019; Li et al., 2021; Shaltouki et al., 2018). Importantly, feeding PD flies with 2.5 μM

168   Benidipine from adulthood prevented DA neuron loss in aged flies (Figure 2C) and improved their

169   locomotor ability (Figure 2D). Taken together, Benidipine, which eliminates the Miro1 defect in PD

170   fibroblasts, rescues PD-related phenotypes in human neuron and fly models.

171

172   **The EF-hands of Miro play a role in causing Parkinson's-relevant phenotypes**

173   Having demonstrated a link between Miro and Ca^{2+} in multiple models of PD, we next determined whether

174   Ca^{2+} directly interacted with Miro to contribute to phenotypes in these models. To achieve this goal, we

175   made GFP-tagged human Miro1 protein in both the wild-type (Miro1-WT) form and in a mutant form

176   where two point mutations were introduced in the two EF-hands of Miro1 (Miro1-KK) to block Ca^{2+}-

177   binding (Wang and Schwarz, 2009). We expressed GFP-tagged Miro1 (WT or KK) and Mito-dsRed that

178   labeled mitochondria in iPSC-derived neurons from the PD patient and isogenic control, described earlier.

179   We chose the distal segment of the axon for analysis (Hsieh et al., 2016). We have previously shown that

180   following 100 μM Antimycin A treatment that triggers mitophagy, Miro1 and mitochondria are

181   sequentially degraded in wild-type neurons (Hsieh et al., 2019; Hsieh et al., 2016; Shaltouki et al., 2018).

182   We observed the same mitochondrial events in isogenic control axons transfected with GFP-Miro1-WT.

8

183   Within 25 min GFP-Miro1-WT was partially degraded, and within 58 min mitochondrial clearance was

184   induced (Figure 3A-C). In contrast, in PD neuron axons transfected with GFP-Miro1-WT, the degradation

185   rates of both Miro1 and damaged mitochondria upon Antimycin A treatment were slowed (Figure 3A-C),

186   consistent with our previous studies (Hsieh et al., 2019; Shaltouki et al., 2018). Notably, GFP-Miro1-KK

187   significantly rescued these phenotypes in PD axons: it expedited the degradation rates to the control level

188   (Figure 3A-C). These data suggest that Miro1 directly binds to Ca^{2+} to mediate mitochondrial phenotypes

189   in PD neurons, at least in part.

190   To confirm the Miro-Ca^{2+} relation in vivo, we generated transgenic flies carrying T7-tagged fly Miro

191   (DMiro)-WT or DMiro-KK. DMiro is an ortholog of human Miro1 and Miro2. Both DMiro-WT and

192   DMiro-KK were expressed at comparable levels when the transgenes were driven by the ubiquitous driver

193   *Actin-GAL4* (Figure S4B). We next crossed these transgenic flies to a fly PD model described earlier that

194   expressed human α-syn-A53T in DA neurons driven by *TH-GAL4*. Consistent with the results from

195   human neurons, DMiro-KK significantly rescued the PD-relevant phenotypes including the age-

196   dependent DA neuron loss and locomotor decline, as compared to DMiro-WT (Figure 3D-E). Altogether,

197   we have provided evidence showing that the Ca^{2+}-binding domain of Miro plays a key role in causing

198   phenotypes in human neuron and fly models of PD.

199

200   **The Ca^{2+}-uptake ability of mitochondria is enhanced in PD neurons**

201   Our results, showing that either blocking Ca^{2+}-entry into the cell or inhibiting Ca^{2+}-binding to Miro rescues

202   the Miro and neurodegenerative phenotypes in PD models (Figures 1-3), placed Ca^{2+} dysregulation

203   upstream of Miro. We next dissected how Ca^{2+}-handling was mis-regulated in neurons derived from the

204   PD patient (Figures 2-3). We stimulated these neurons with the G-protein-coupled receptor (GPCR)

205   agonist, thrombin, and measured cytosolic and mitochondrial Ca^{2+} levels with live Calcium Green and
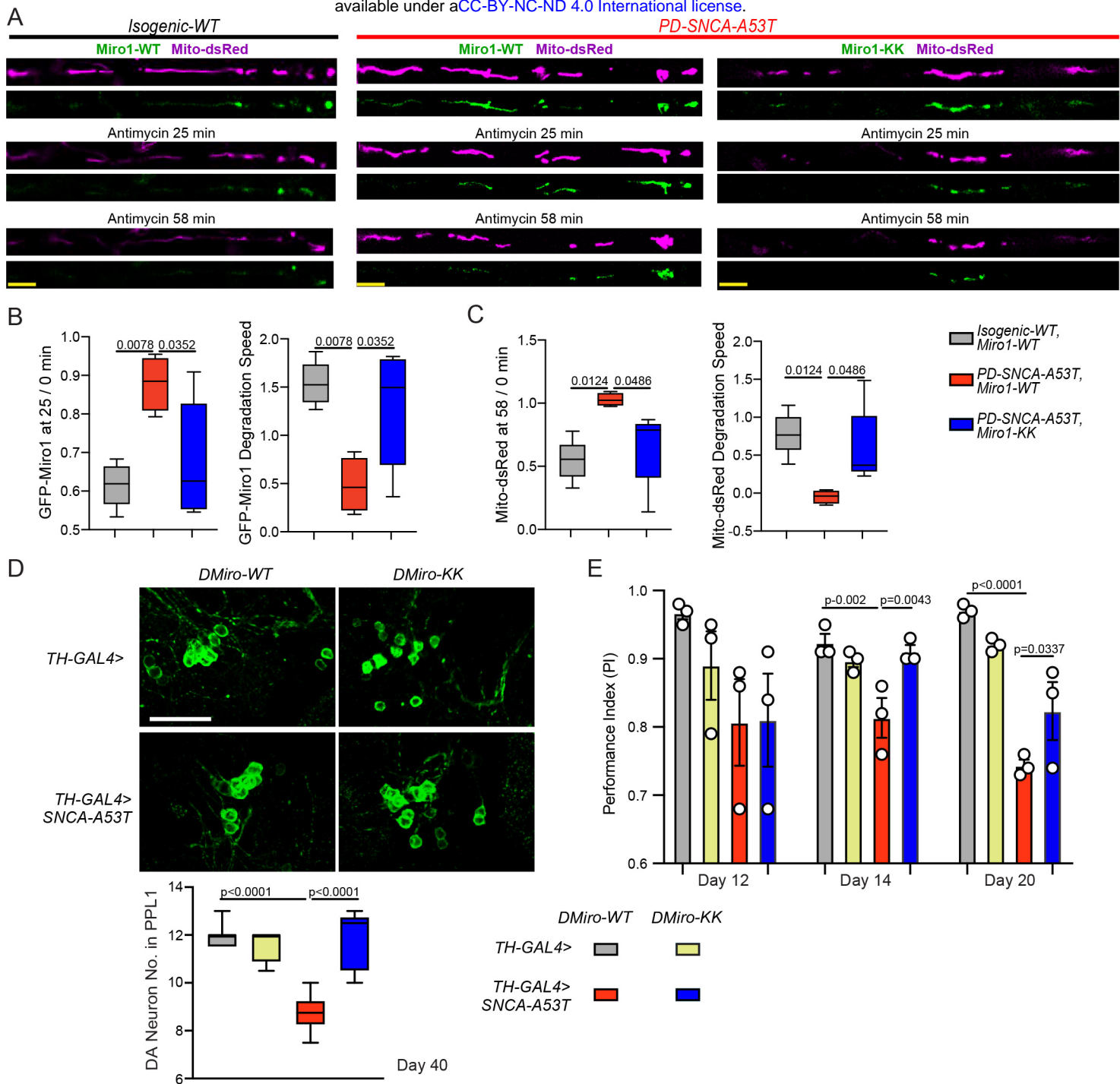
Figure 3. The EF-hands of Miro Play a Role in Causing PD Relevant Phenotypes. (A) Representative still images from live Mito-dsRed and GFP-Miro1 imaging movies of axons of indicated genotypes, following 100 uM Antimycin A treatment. Scale bar: 10 um. (B) Left: Quantification of the GFP-Miro1 intensity at 25 minutes divided by that at 0 minute following 100 uM Antimycin A treatment from the same axonal region. Right: Quantification of the reduction speed of the GFP-Miro1 intensity within 25 minutes following 100 uM Antimycin A treatment. (C) Left: Quantification of the Mito-dsRed intensity at 58 minutes divided by that at 0 minute following 100 uM Antimycin A treatment from the same axonal region. Right: Quantification of the Mito-dsRed intensity reduction speed within 58 minutes following 100 uM Antimycin A treatment. (B-C) n=5, 4, 5 (from left to right) axons (one axon per coverslip). (D) The DA neuron number was counted in the PPL1 cluster of flies with indicated genotypes. Scale bar: 20 um. n=7, 4, 6, 5 (from left to right). (E) The Performance Index was measured in flies with indicated genotypes and age. n (from left to right)=49, 47, 40, 47 flies (day 12); 49, 47, 39, 47 (day 14); 48, 45, 37, 44 (day 20); 3 independent experiments. One-Way Anova Post Hoc Tukey Test for all panels.
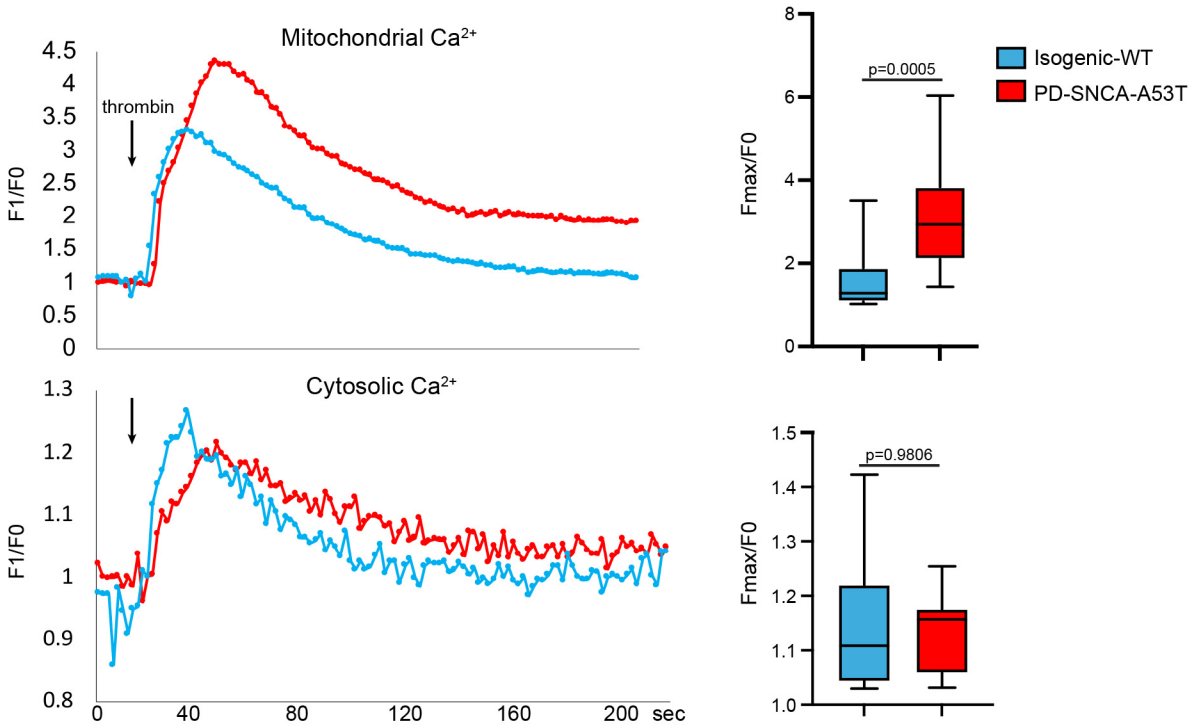
Figure 4. PD Mitochondria Import More Ca2+. iPSC-derived neurons from a PD patient with SNCA-A53T and the isogenic control were stimulated with thrombin and mitochondrial (Rhod-2) and cytosolic Ca2+ levels (Calcium Green) were measured. Left: Representative traces of Ca2+ ions in neurons. Right: Quantifications of the peak fluorescent intensity normalized to baseline. n=15 cell bodies from 3 independent coverslips. Two-tailed Welch's T Test.

206   Rhod-2 staining, respectively. We found that thrombin triggered intracellular Ca^{2+} mobilization and

207   elevation, which was comparable between PD and isogenic control neurons (Figure 4). However,

208   mitochondria in PD neurons sustained significantly larger Ca^{2+}-elevation after thrombin stimulation, as

209   compared to control (Figure 4). These results indicate that the mitochondrial Ca^{2+}-import ability is boosted

210   in PD neurons.

211

212   **Iron promotes the assembly of MCU oligomers**

213   The enhanced mitochondrial Ca^{2+}-uptake observed in PD neurons (Figure 4) led us to investigate the Ca^{2+}-

214   uptake channel in the IMM–MCU. The oligomerization of MCU is essential for MCU's function to import

215   Ca^{2+} into the mitochondria (Dong et al., 2017; Fan et al., 2018; Fan et al., 2020). We hypothesized that

216   MCU oligomerization could be affected by small molecules in the mitochondrial microenvironment,

217   particularly those with a role in PD, including Ca^{2+} (Figure 4), reactive oxygen species (ROS), and iron

218   (Belaidi and Bush, 2016). To explore this possibility, we performed size exclusion chromatography (SEC),

219   where protein complexes with higher molecular weight (MW) are eluted faster than those with lower MW,

220   using lysates of HEK cells treated with Fe^{2+}, Ca^{2+}, or H_2O_2. Detecting MCU from cell lysates using SEC

221   has been successfully shown (Dong et al., 2017; Lambert et al., 2019; Tomar et al., 2016). We found that

222   Fe^{2+}, Ca^{2+}, and H_2O_2 treatment all shifted the MCU elution peaks to the earlier fractions of higher-order

223   oligomers compared to control (Figure 5A, S4C-G; anti-MCU was validated in Figure S4C). In contrast,

224   the elution pattern of Miro1 was largely unaltered by any of these treatments (Figure S4D-G). These

225   results show that intracellular small molecules including iron, Ca^{2+} (Fan et al., 2020), and H_2O_2 (Dong et

226   al., 2017) can influence the equilibrium of MCU oligomeric complexes and may consequently alter the
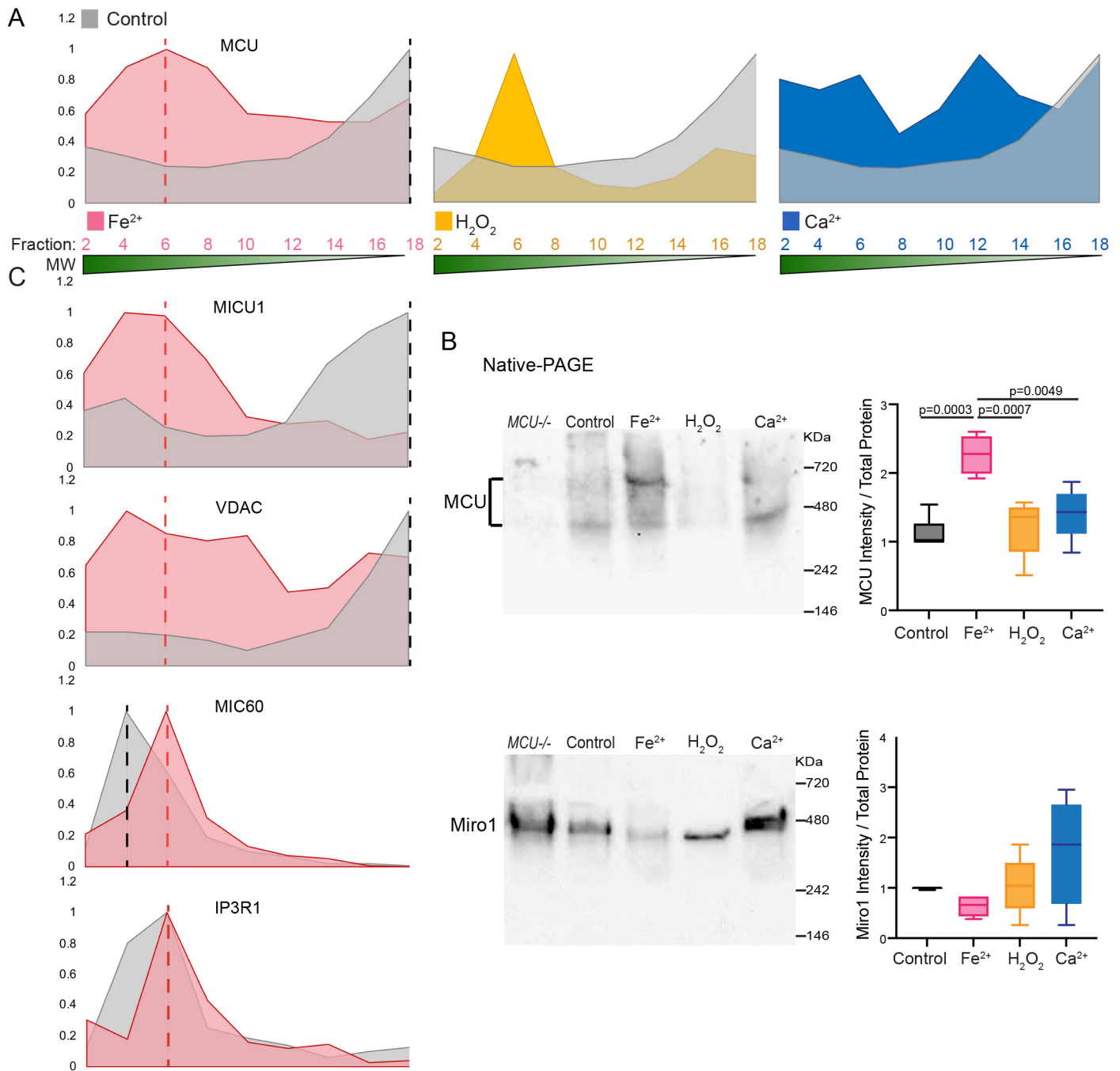
227   MCU channel activity.

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Figure 5. Iron Promotes MCU Oligomerization. (A) Elution profiles of MCU from SEC samples. (B) HEK cells were treated similarly as above, run in Native-PAGE, and blotted. Right: Qualifications of the band intensities normalized to the total protein amount measured by BCA. n=5 independent experiments. One-Way Anova Post Hoc Tukey Test. (C) Elution profiles of additional proteins from SEC samples.

228   SEC is a sensitive method to detect protein composition dynamics, while Native-PAGE can determine

229   the overall form and amount of a multimeric native protein. The human MCU oligomer bands from HEK

230   cells migrated between 400-700 KDa in Native-PAGE (Figure 5B; the negative control in the left lane,

231   *MCU-/-,* showed no signal) (Baughman et al., 2011; Ghosh et al., 2020; Tomar et al., 2016). Importantly,

232   we found that Fe^{2+} treatment, but not Ca^{2+} or H_2O_2, resulted in an increase in the total intensity of the

233   MCU oligomer bands (Figure 5B). Miro1 protein also oligomerized and migrated as a single band around

234   480 KDa in Native-PAGE, which was not significantly affected by any of these treatments (Figure 5B).

235   These data indicate that iron not only shifts the MCU complexes to higher-order oligomers but also

236   enlarges the total number of these complexes, and thus may have a more profound impact on the MCU

237   activity than Ca^{2+} and H_2O_2.

238   We next examined additional membrane proteins that may assist the MCU function using HEK cells.

239   By detecting total protein levels using Western blotting, we found when Fe^{2+} was added in media and lysis

240   buffer, MCUb and NCLX were lowered, but not any other proteins examined (Figure S5). MCUb is an

241   inhibitor of MCU (Lambert et al., 2019), and NCLX is an IMM exchanger believed for mitochondrial

242   Ca^2-extrusion (Lee et al., 2016; Palty et al., 2010). The reduction of both proteins could exacerbate the

243   phenotype of mitochondrial Ca^{2+}-overload. By evaluating oligomeric dynamic changes using SEC, we

244   found that the OMM channel, VDAC, and MCU's gating regulator, MICU1, showed a matching elution

245   pattern with MCU, and the treatment of Fe^{2+} shifted all 3 proteins to the similar earlier fractions of higher

246   order oligomers, which coincided with the elution peak of MIC60, a core structural protein at the crista

247   junctions and contact sites (Zerbes et al., 2012), and IP3R1, the major ER Ca^{2+}-channel that delivers Ca^{2+}

248   to the OMM (Katona et al., 2022) (Figure 5A, C). These data are consistent with VDAC, MICU1, and

249   MCU being associated in the same super-complexes and suggest a possible spatial reorganization of the

250   MCU super-complexes upon iron elevation, thus allowing easier access to ER Ca^{2+} supply. Taken together,

11

251 our results show that iron promotes MCU oligomerization and may cause Ca^{2+}-accumulation inside the

252 mitochondria.

253

254 **Fe^{2+} binds to the MCU complex and acts on its Ca^{2+}-import ability**

255 We probed the mechanism underlying the observed impact of iron on MCU oligomerization (Figure 5A-

256 B). One hypothesis was that the MCU complex bound to iron leading to conformational changes. To

257 explore this possibility, we immunoprecipitated (IP) endogenous MCU from HEK cells and detected the

258 iron concentrations in the IP samples. We found significantly more Fe^{2+} ions pulled down with MCU

259 when HEK cells were treated with Fe^{2+}, compared with other controls (Figure 6A). This result shows that

260 the MCU complex interacts with Fe^{2+}. To determine whether MCU directly bound to Fe^{2+}, we switched

261 our experiments to an in vitro setting. To circumvent the problem of precipitation caused by a fast speed

262 of Fe^{2+} oxidation in vitro, we used an Fe^{2+} mimic, Co^{2+} ion (Billesbolle et al., 2020). We first confirmed

263 that Co^{2+} behaved similarly as Fe^{2+} in our functional assays in HEK cells: Co^{2+} treatment increased MCU

264 oligomerization detected by Native-PAGE (Figure 6B), just like Fe^{2+} (Figures 5B, 6B), and both Fe^{2+} and

265 Co^{2+} treatment enhanced the mitochondrial Ca^{2+}-uptake ability following thrombin application (Figure

266 6C). Using fluorescence-detection SEC on purified human MCU protein (Fan et al., 2020), we found that

267 Co^{2+} caused the formation of higher-order oligomers of MCU, and decreased MCU protein stability with

268 increased temperature (Figure 6D-E). These results demonstrate that the Fe^{2+} mimic, Co^{2+}, directly binds

269 to MCU, increasing its oligomerization.

270 We next searched for amino acid residues in the matrix domain of MCU (PDB: 5KUE) predicted to

271 bind to Fe^{2+} using an in-silico program (http://bioinfo.cmu.edu.tw/MIB/) (Lin et al., 2016; Lu et al., 2012),

272 and found 3 amino acids: 74D, 148D, and 159H. The latter 2 residues were also predicted to bind to Co^{2+}.

273 We mutated these 3 sites to Alanine (named "MCU-3A"). Indeed, we detected significantly less Fe^{2+}
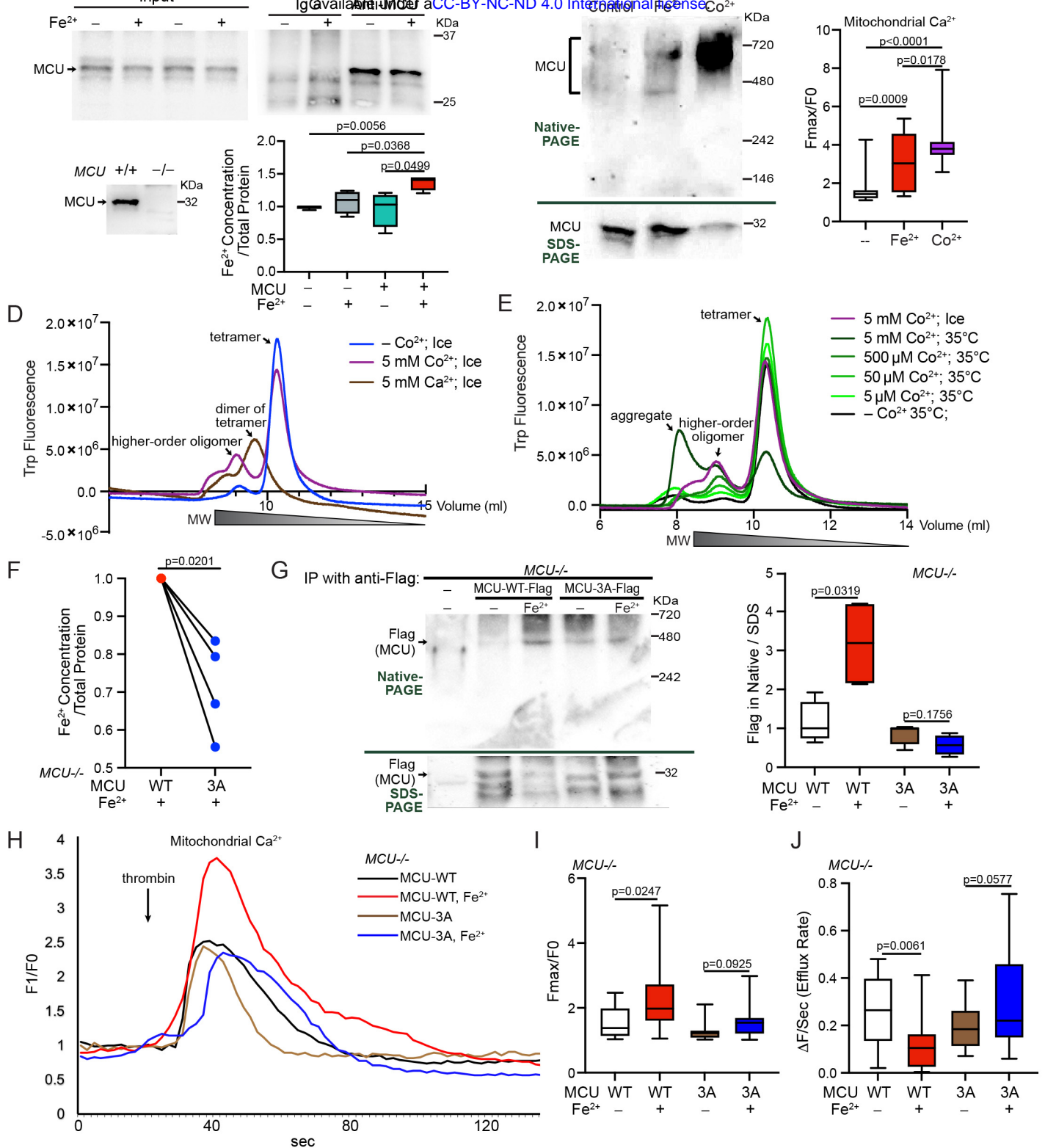
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Figure 6. MCU Binds to Fe2+. (A) HEK cells were treated with or without 5 mM Fe2+ for 21 hours, then IPed with rabbit IgG or anti-MCU, and Fe2+ concentrations in the IP samples were detected. n=4 independent experiments. Top western blots demonstrated the success of IP. Anti-MCU was validated using MCU-/- HEK cell lysate (bottom panel). Arrow indicates the MCU band. (B) HEK cells were treated with 5 mM Fe2+ or Co2+ for 22 hours and lysed in buffer containing the same metal. Lysates were run in Native- or SDS-PAGE and blotted with anti-MCU. Similar results were seen for at least 3 times. (C) HEK cells treated with 5 mM Fe2+ or 500 μM Co2+ for 22 hours were stimulated with thrombin and mitochondrial Ca2+ levels (Rhod-2) were measured. The peak fluorescent intensity normalized to baseline is quantified. n=20 cells from 4 independent coverslips. (D-E) Fluorescence-detection SEC profiles of purified human MCU. The annotation of the peaks is based on (Fan et al., 2018; Fan et al., 2020). (F) MCU-/- HEK cells transfected as indicated were treated with 5 mM Fe2+ for 20 hours, then IPed with anti-Flag, and Fe2+ concentrations in the IP samples were detected. Two-tailed paired T Test. (G) Left: Representative blots of IP with anti-Flag using cell lysates as indicated, run in Native- or SDS-PAGE. Right: Quantification of the band intensity of MCU-Flag in Native-PAGE normalized to that in SDS-PAGE from the same experiment. n=4 independent experiments. (H) HEK cells treated as indicated were stimulated with thrombin and mitochondrial Ca2+ levels (Rhod-2) were measured. Representative traces of Ca2+ ions. (I-J) Based on traces like in (H), the peak fluorescent intensity normalized to baseline (I) or efflux rate (J) is quantified. n=17 cells from 4 independent coverslips. Two-tailed Welch's T Test for all panels except (F).

274   bound to Flag-tagged MCU-3A, as compared to MCU-WT, produced from HEK cells without endogenous

275   MCU (*MCU-/-*) (Figure 6F). To determine whether these mutations were sufficient to eliminate the Fe^{2+}-

276   triggered oligomerization of MCU, we expressed MCU-WT or MCU-3A in *MCU-/-* HEK cells, treated

277   these cells with Fe^{2+}, and ran the IPed proteins in Native-PAGE. As expected, MCU-3A abolished MCU's

278   response to Fe^{2+} treatment: the MCU oligomer band intensity was no longer increased (Figure 6G). We

279   then live imaged mitochondrial Ca^{2+}-dynamics, as described in Figure 4, in these cells. We consistently

280   observed a larger mitochondrial Ca^{2+}-elevation following thrombin stimulation in MCU-WT-transfected

281   HEK cells treated with Fe^{2+} as compared to no Fe^{2+}-treatment, and MCU-3A blunted the peak increase

282   (Figure 6H-I). Because Fe^{2+} also lowered NCLX levels (Figure S5) which could affect mitochondrial Ca^{2+}-

283   extrusion, we measured the mitochondrial Ca^{2+}-efflux rate. Indeed, Fe^{2+} treatment slowed the efflux rate,

284   which was prevented by MCU-3A (Figure 6J), suggesting that the Fe^{2+}-triggered efflux delay might

285   depend on Ca^{2+}-overload. Altogether, our results show that Fe^{2+} binds to the MCU complex, promoting

286   MCU oligomerization and its channel activity.

287

288   **Iron functions upstream of calcium to mediate phenotypes of PD neurons**

289   Our discovery of the action of Fe^{2+} on the MCU activity (Figures 5-6) suggested that in PD neurons, the

290   phenotype of mitochondrial Ca^{2+}-overload (Figure 4) might depend on iron. To confirm their causal

291   relation, we treated PD neurons with deferiprone (DFP), an iron chelator (Munson et al., 2021). Indeed,

292   DFP significantly reduced mitochondrial Ca^{2+}-accumulation following thrombin stimulation (Figure 7A)

293   and prevented cell death triggered by Antimycin A treatment (Figure 7B) in iPSC-derived neurons from

294   the familial PD patient described earlier. We treated neurons from a second, sporadic patient with DFP

295   and saw a similar neuroprotective effect (Figure 7B). In vivo, feeding the fly model of PD as shown earlier

296   (Figures 2-3) with DFP consistently rescued the PD-relevant phenotypes, including age-dependent DA

297   neuron loss and locomotor decline (Figure 7C-D). Collectively, our results show that iron functions

298   upstream of calcium to mediate neurodegeneration in PD models.

299

300   **MCU, MCUb, and NCLX are affected in PD postmortem brain**

301   Our finding showing that iron impacts mitochondrial Ca^{2+}-channels and transporters (Figure 5, 6B, S5)

302   prompted us to examine these proteins in postmortem brains of people with PD, diffuse Lewy body disease

303   (DLBD), or Alzheimer's disease (AD) (Table S3). We homogenized the frontal cortex and ran the brain

304   lysate in Native- or SDS-PAGE. We focused on MCU oligomers, NCLX, and MCUb, which were shown

305   earlier specifically altered by Fe^{2+} treatment in HEK cells (Figure 5B, 6B, S5). We found the PD group

306   clustered and separated from the healthy control group, with higher intensity of the MCU oligomer bands

307   and lower intensity of both the NCLX and MCUb bands (Figure 7E), similar to the observations in HEK

308   cells treated with Fe^{2+} (Figure 5B, 6B, S5). This unique clustering was not observed in the AD or DLBD

309   group (Figure 7E, S6). Together, our data suggest that the combined functional impairment of MCU,

310   MCUb, and NCLX may be one of the molecular signatures shared by people with PD.

311

312   **Miro1 in blood cells reflects PD status and responds to drug treatment**

313   Now we have demonstrated a functional axis of iron-calcium-Miro in neurons of our PD models and

314   patients. We next sought evidence of its impairment in peripheral tissues of PD patients, which could

315   serve as an excellent candidate for biomarker and pharmacodynamic marker development. Measuring the

316   combined impairment of MCU oligomerization, MCUb, and NCLX requires a large amount of protein

317   and Native-PAGE, which is not applicable for high-throughput screening and clinical practice. We then

318   explored Miro1, whose slower degradation following mitochondrial depolarization was downstream of

319   Ca^{2+}-dysregulation in our PD models, as shown earlier (Figures 1-3). Notably, we have previously found

14

320   that in skin fibroblasts and iPSCs the delay of Miro1 degradation upon depolarization distinguishes PD

321   patients and genetic carriers from healthy controls (Hsieh et al., 2019; Hsieh et al., 2016; Nguyen et al.,

322   2021). Although fibroblasts can be readily obtained by a skin biopsy, a blood test remains the most

323   convenient method. We investigated whether Miro1 could be detected in peripheral blood mononuclear

324   cells (PBMCs) acquired from a blood draw. We cultured PBMCs from a healthy donor from the Stanford

325   Blood Center (SBC, Table S4) and depolarized the mitochondrial membrane potential using two different

326   methods: Antimycin A plus Oligomycin (Ordureau et al., 2020), or CCCP. We found that both

327   depolarizing approaches caused the degradation of Miro1 and additional mitochondrial markers in a time-

328   dependent manner, detected by Western blotting (Figure S7A-B), consistent with other cell types (Hsieh

329   et al., 2019; Hsieh et al., 2016; Nguyen et al., 2021).

330   To enable high-content screening, we applied an enzyme-linked immunosorbent assay (ELISA) of

331   Miro1 (Figure S7C-D) to PBMCs from the same donor with 6-hour CCCP treatment. We saw a similar

332   Miro1 response to CCCP using ELISA (SBC, Table S4). We then used this ELISA to screen a total of 80

333   healthy controls and 107 PD patients (Table S4). Miro1 Ratio (Miro1 protein value with CCCP divided

334   by that with DMSO from the same person) was significantly higher in PD patients compared to healthy

335   controls (Figure 7F, Table S4), indicating that Miro1 is more stable upon depolarization in PD patients.

336   To determine whether our method could be used to classify an individual into a PD or healthy group,

337   we employed machine learning approaches using our dataset. We trained a logistic regression model to

338   assess the impact of Miro1 Ratio on PD diagnosis, solely on its own or combined with additional

339   demographic and clinical parameters (Method). Unified Parkinson's Disease Rating Scale (UPDRS) is a

340   tool to measure motor and non-motor symptoms of PD which may reflect disease severity and progression.

341   Using UPDRS, our model yielded an accuracy (an individual was correctly classified as with PD or

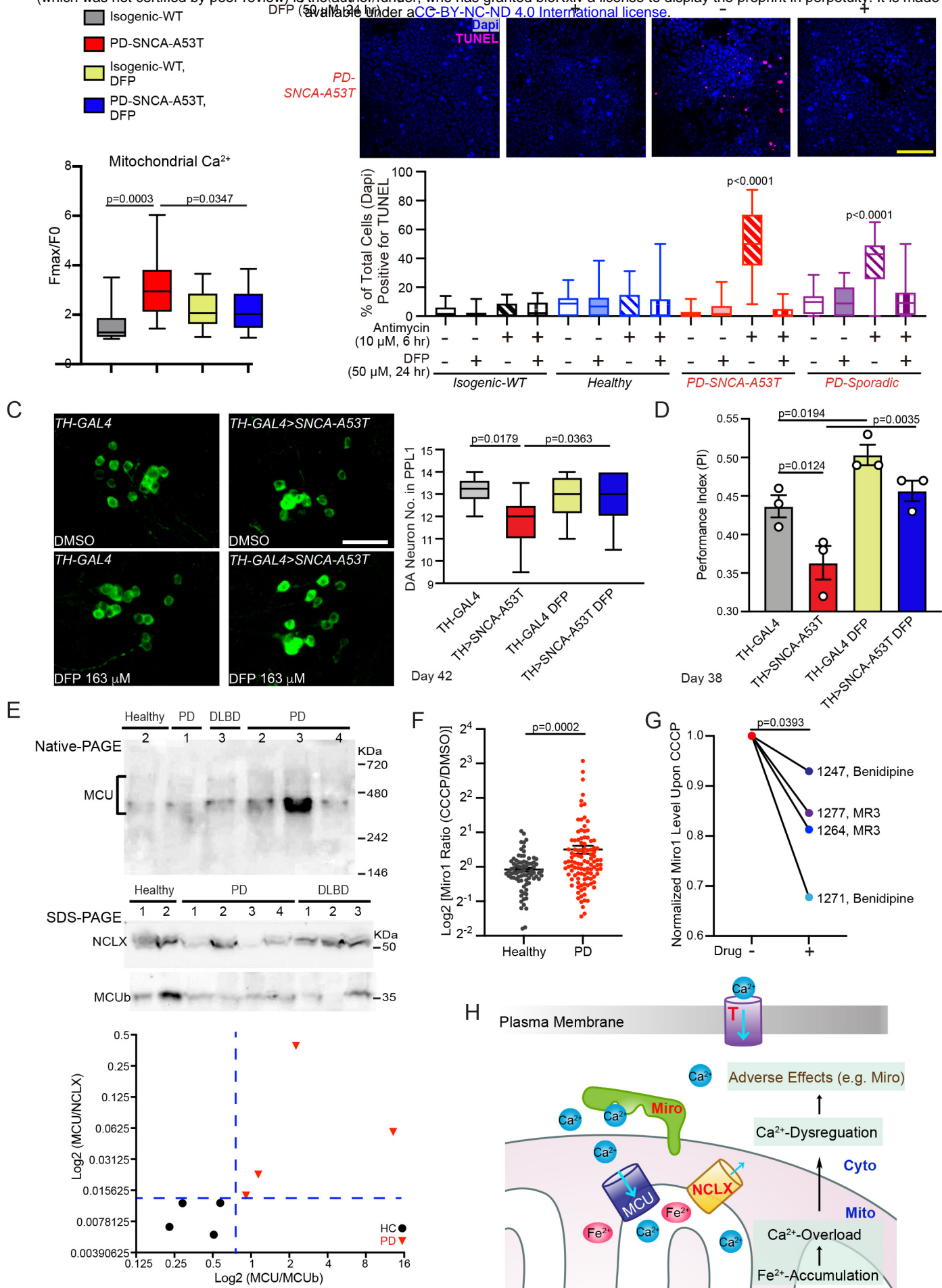342   healthy) of 81.2% ($p<0.000001$; area under the Receiver Operator Curve (ROC)–AUC=0.822), and using

15

Figure 7. Chelating Iron Restores Ca2+ and neuronal homeostasis in PD neurons. (A) Similar to Figure 4, iPSC-derived neurons from a PD patient with SNCA-A53T and the isogenic control, with or without treatment of 100 uM DFP for 24 hours, were stimulated with thrombin, and mitochondrial Ca2+ (Rhod-2) was measured. Quantifications of the peak fluorescent intensity normalized to baseline. n=15 cell bodies from 3 independent coverslips. Control data without DFP treatment are the same as in Figure 4. One-Way Anova Post Hoc Tukey Test. (B) iPSC-derived neurons treated as indicated, were immunostained with TUNEL and Dapi, and imaged under a confocal microscope. Scale bar: 50 um. Below: Quantification of the percentage of TUNEL-positive neurons. n=20 images from 3 independent coverslips. P values are compared within each genotype (significant compared to every other condition) with One-Way Anova Post Hoc Tukey Test. (C) The DA neuron number was counted in the PPL1 cluster of flies with indicated genotypes and conditions. Drug treatment was started from adulthood (day 1). Scale bar: 20 um. n=6, 9, 8, 7 (from left to right). (D) The Performance Index was measured in flies. Drug treatment was started from embryogenesis. n=35, 33, 40, 34 flies (from left to right), 3 independent experiments. (C-D) One-Way Anova Post Hoc Tukey Test. (E) Postmortem brains were run in Native- or SDS-PAGE and blotted. The band intensity normalized to the total protein level measured by BCA is divided by that of the universal control on the same blot: CVD (cardiovascular disease), which was included on every blot. The MCU oligomer bands in Native-PAGE and the NCLX and MCUb bands in SDS-PAGE (average of 3 replicates) are used in the plot. HC: healthy control. (F) Miro1 protein levels were measured using ELISA in PBMCs treated with DMSO or 40 uM CCCP for 6 hours. Miro1 Ratio is calculated by dividing the Miro1 value treated with CCCP by that with DMSO from the same subject. Dot plot with Mean±S.E.M. n=80 healthy controls and 107 PD. Two-tailed Welch's T Test. (G) PBMCs from 4 PD patients were treated with 40 uM CCCP for 6 hours, or pretreated with 10 uM Benidipine or MR3 for 18 hours and then with 40 uM CCCP for another 6 hours, and Miro1 protein was detected using ELISA. Patient IDs are the same as in Table S4. Two-tailed paired T Test. (H) Schematic representation of the iron-calcium-Miro axis discovered in this study. Red texts show genes containing variants associated with PD status.

343   Miro1 Ratio, the accuracy was 67.6% (p=0.03; AUC=0.677). Notably, if both Miro1 Ratio and UPDRS

344   were considered, our model generated an improved accuracy of 87.8% (p=0.02; AUC=0.878), without the

345   interference of age or sex (Method, Figure S7E-F). Therefore, our results suggest that the molecular

346   (Miro1 Ratio) and symptomatic (UPDRS) evaluations may reveal independent information, and that

347   combining both tests may more accurately categorize individuals with PD and measure their responses to

348   experimental therapies.

349   To probe the potential utilization of this Miro1 assay in future clinical trials for stratifying patients or

350   monitoring drug efficacy, we treated PBMCs from 4 PD patients (Table S4) with either of the two

351   compounds known to reduce Miro1, Benidipine (Figures 1-2, S2) and Miro1 Reducer 3 (MR3) (Hsieh et

352   al., 2019; Li et al., 2021). Miro1 protein levels upon CCCP treatment were lowered by each compound in

353   all 4 patients (Figure 7G), showing that the Miro1 marker in PBMCs can respond to drug treatment.

354   Collectively, our results suggest that Miro1 protein in blood cells may be used to aid in diagnosis and drug

355   development.

356

357   **Rare variants in the iron-calcium-Miro pathway are associated with PD status**

358   After dissecting the functional impairment of this iron-calcium-Miro axis in PD, we explored its genetic

359   contribution to PD. Earlier, we showed that chelating iron, blocking Miro's binding to Ca^{2+}, or preventing

360   Ca^{2+}-entry into the cell all alleviated parkinsonian neurodegeneration (Figures 2, 3, 7). We evaluated the

361   genes encoding the protein targets of these approaches, which are spatially distinct and localized to three

362   subcellular locations: (1) IMM Ca^{2+}-channels and transporters (targeted by Fe^{2+}), (2) the Ca^{2+}-binding

363   protein Miro on the OMM, and (3) plasma membrane Ca^{2+}-channels (targeted by Benidipine and Tranilast)

364   (Figure 7H, Table S5). By analyzing common variants within or near any of the investigated genes in

365   GWAS reported in (Nalls et al., 2019), we did not observe significant association with PD clinical status.

16

366   We next employed the whole-genome sequencing (WGS) data from the Accelerating Medicines

367   Partnership–Parkinson's Disease (AMP–PD) (1,168 control; 2,241 PD), and assessed rare non-

368   synonymous and damaging variants using burden based and SKATO methods. We discovered polygenetic

369   vulnerability to PD at all three spatial hotspots: significant association with PD status of rare variants in

370   selective T-type Ca^{2+}-channel subtypes (*Cav3.2*, *3.3*) (cell surface), *Miro2* (OMM), and *NCLX* (IMM)

371   (Table S5). A SKATO Test on all variants of T-type or L-type Ca^{2+}-channel subtypes consistently showed

372   significant association with PD status of T-type channels, which survived multiple comparison correction,

373   but not of L-type channels (Table S5). Together, our analysis unravels genetic predisposition of this Ca^{2+}-

374   pathway to PD.

375   To functionally validate the selection of T-type Ca^{2+}-channels from our human genetic study, we

376   employed the same screening ICC assay described earlier (Figures S1-2) by which we discovered the non-

377   selective pan-Ca^{2+}-channel blocker, Benidipine, but now using 2 different specific L-type and 3 different

378   T-type Ca^{2+}-channel blockers. Intriguingly, we again discovered a striking selection of T-type versus L-

379   type channels, in the connection with Miro1 in PD fibroblasts (Figure S7G): only T-type blockers

380   promoted Miro1 degradation following depolarization, just like Benidipine, supporting the human genetic

381   finding. Similar to Miro1, Miro2 was also resistant to depolarization-triggered degradation in PD

382   fibroblasts (Hsieh et al., 2019) and responded to Benidipine treatment (Figure S3C). NCLX functionally

383   interacts with MCU (Lee et al., 2016), and was coregulated with MCUb and MCU by Fe^{2+} (Figure 5B,

384   6B, S5) and PD (Figure 7E). Although it remains elusive why rare variants in these genes are selectively

385   associated with PD status, our combined, unbiased analyses of human genetics, tissues, cell, and in vivo

386   models corroborate the complexity and multifactorial nature of PD etiology, and indicate that the

387   polygenetic architecture built around this iron-calcium-Miro axis might influence an individual's risk to

388   develop PD.

17

389

390   **Discussion**

391   In this work, we have established a pathway of iron-calcium-Miro dysregulation in our PD models (Figure

392   7H). Elevation of iron concentrations may cause mitochondrial Ca^{2+}-overload by promoting the MCU

393   activity and reducing NCLX levels (Figures 4-7, S5). It is possible that the initial mitochondrial Ca^{2+}-

394   accumulation subsequently disrupts Ca^{2+}-homeostasis at the cellular level, eliciting responses of

395   additional Ca^{2+}-binding proteins, such as Miro (Figure 7H). We have shown multiple ways to target this

396   axis. Chelating iron, reducing Ca^{2+}-entry into the cell, or blocking Miro's binding to Ca^{2+} is each

397   neuroprotective (Figures 2, 3, 7). Of note, the impairment of this axis can be reflected in blood cells using

398   a Miro1 assay with high content capacity (Figure 7F-G) and can be detected in the genome of PD patients

399   (Table S5). Hence, this ionic axis may be important for PD pathogenesis and can be leveraged for better

400   detecting and treating the disease.

401

402   Although the precise course of PD pathogenesis remains unclear, emerging evidence has demonstrated its

403   complex and polygenetic nature. Interactions among multiple organs and cell types, systemic immune

404   activation, and environmental triggers act uniquely in different individuals. Although GWAS and

405   segregation studies have unveiled many PD risk or causal loci, rare genetic variants may be particularly

406   important for conferring individual heterogeneity in disease onset and etiology. However, rare variants

407   associated with PD risk are difficult to discover given the limitations in sample collection and the unmet

408   need for exome-sequencing as opposed to sparse genotyping on microarrays. In our work, we have located

409   several potential risk genes by first connecting them in the same biological pathway. In an individual

410   prone to PD, there might be already mitochondrial malfunction causing mitochondrial proteins to release

411   labile iron, or impairments in systemic iron uptake and circulation causing iron accumulation at the cellular

18

412 level. Any predisposed genetic perturbations in this iron-calcium-Miro axis, which could be due to

413 different rare variants in each person, would further exacerbate its dysfunction. With or without another

414 trigger such as a virus infection or environmental insult, the tipping point for disease onset might be

415 reached. Thus, combining complementary genetic and functional studies may help us better understand

416 the destructive paths leading to the disease and identify network hubs for therapeutic targeting.

417

418 Screening people for genetic variants in this iron-calcium-Miro axis and detecting Miro in blood cells may

419 help stratify a unique population of patients and at-risk individuals, who will particularly benefit from

420 therapeutic interventions targeting this axis. For example, treating people bearing genetic variants in Ca^{2+}-

421 channels with Ca^{2+}-channel blockers, iron-chelators, or both, may yield the best efficacy. Similarly, people

422 who test positive for the Miro1 phenotype in PBMCs may respond best to drugs reducing Miro1.

423 Integrating genetic screening of this axis with Miro1 detection in peripheral tissues may enhance the

424 accuracy of risk evaluation and help design personalized treatment, such as a cocktail of different Ca^{2+}-

425 channel blockers, iron chelators, and Miro reducers, to improve prevention and treatment efficacy.

426

427 Calcium mishandling and iron accumulation have been widely observed in PD neurons (Angelova et al.,

428 2020; Apicco et al., 2021; Belaidi and Bush, 2016; Buttner et al., 2013; Kim et al., 2020; Lee et al., 2018;

429 Surmeier et al., 2017; Tabata et al., 2018; Verma et al., 2017; Vuuren et al., 2020). Now we have provided

430 a mechanistic link. Fe^{2+} elicits mitochondrial Ca^{2+}-overload through acting on IMM Ca^{2+}-channels and

431 transporters. Further investigations are needed to dissect how Fe^{2+} regulates MCUb and NCLX levels.

432 One hypothesis is that these proteins are targeted by Ca^{2+}-activated mitochondrial proteases. Our results

433 (Figure 6J) have suggested that Fe^{2+}-triggered Ca^{2+}-efflux delay depends on mitochondrial Ca^{2+}-overload.

434 A recent study has shown that NCLX protein levels are lowered in another mitochondrial iron-dependent

435   disease, Friedreich's Ataxia (FA), through a possible mechanism dependent on calpain cleavage (Britti et

436   al., 2021). Intriguingly, iron chelators have displayed promising therapeutic benefits in preclinical models

437   of both PD (Clark et al., 2020) and FA (Llorens et al., 2019), and is currently in multiple clinical trials for

438   treating symptoms of PD patients (Clark et al., 2020). Thus, the underlying molecular mechanisms may

439   be shared by multiple diseases with mitochondrial iron accumulation.

440

441   The consequences to intracellular Ca^{2+}-dysfunction could be profound and detrimental. Dissecting these

442   downstream details in PD will help us find more powerful targets and biomarkers. Mitochondrial Ca^{2+}-

443   overload could overwhelm the oxidative phosphorylation system (Ashrafi et al., 2020), damaging the

444   electron transport chain. Malfunctions of MCU and NCLX may disrupt spatially discrete Ca^{2+} transients

445   and oscillations adjacent to ER membranes, leading to defects in autophagy (Zheng et al., 2022), which

446   may consequently cause protein aggregation and lysosomal dysfunction. Intriguingly, chelating iron can

447   induce selective autophagy pathways (Wilhelm et al., 2022), raising the question whether MCU and local

448   Ca^{2+} dynamics are involved in iron-mediated autophagy. Ca^{2+} may be also important for safeguarding

449   mitochondrial quality. Miro appears to be a molecular switch between distinct routes of mitochondrial

450   quality control. Proteasomal degradation of Miro dissociates mitochondria from microtubules and allows

451   the entire damaged mitochondria to enter the mitophagy pathway (Chan et al., 2011; Hsieh et al., 2016;

452   Wang et al., 2011), whereas sustaining Miro on the microtubule motors is required for the biogenesis of

453   mitochondrial derived vesicles (MDVs) that deliver a subset of mitochondrial proteins (including Miro)

454   to lysosomes (Konig et al., 2021). Notably, MDVs occur under mild oxidative stress or at steady state,

455   and before mitophagy which is triggered by extended damage (Konig et al., 2021; Lin et al., 2017;

456   McLelland et al., 2014). Miro may also ride with those damaged mitochondria expelled from neurons

457   through nanotunnels and vesicles (Ahmad et al., 2014; Davis et al., 2014; Melentijevic et al., 2017; Rosina

20

458   et al., 2022; Saha et al., 2022). Perhaps it starts with only one of these biological processes impaired by

459   Ca^{2+} signals, leading to Miro retention on damaged mitochondria, which further affects more Miro-

460   dependent quality control pathways. More studies are needed to unravel the precise roles of Miro and

461   other Ca^{2+}-binding proteins in PD pathogenesis and how Ca^{2+} regulates these roles. The physical and

462   functional interactions of these Ca^{2+}-binding proteins with specific Ca^{2+}-channels in plasma,

463   mitochondrial, or additional organellar membranes, may constitute the core underlying mechanisms.

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638

Author contributions

640   R.V. did drug screens. S.E.R. and R.B.A. performed pathway analysis. V.B., A.S.D., C.-H.H., L.L., and

641   S.C. performed human cell experiments. L.L. conducted fly work. C.M.M. and L.F. did in vitro work.

642   Y.LG. and M.D.G. analyzed human genetic data. P.N. analyzed PBMC data. X.W. conceived and

643   supervised the project. All authors designed the experiments and wrote the paper.

644

Competing interests

646   The authors declare the following competing interests: X.W. is a co-founder, adviser, and shareholder of

647   AcureX Therapeutics, and a shareholder of Mitokinin Inc. V.B., L.L., C.-H.H., and R.V. are shareholders

648   of AcureX Therapeutics. P.N. is employed by Vroom Inc. Patents based on this study were filed by

649 Stanford University with X.W., R.V., V.B., L.L., C.-H.H. as inventors. The remaining authors declare no

650 competing interests.

651

Data availability

653 Further information and reagents are available from the corresponding author.

654

655 **FIGURE LEGENDS**

656 **Figure 1. HTP Screens Identify Ca^{2+}-Related Drug Hits for PD.** (A) Schematic representation of a

657 custom-designed drug screen for Miro1 in PD fibroblasts. (B) Pathway analysis identified calcium as a

658 shared factor in the primary hit-Miro1 network. Each individual pathway is generated using a primary hit

659 and Miro1 as search query and the resulting subnetwork is visualized and curated using docs2graph–a

660 knowledge-graph browser. The visualization shows a subgraph generated by docs2graph from the

661 collection of curated supporting documents for each pathway.

662

663 **Figure 2. Benidipine Rescues PD Relevant Phenotypes.** (A-B) iPSC-derived neurons from a PD patient

664 with *SNCA-A53T* and the isogenic control, treated as indicated, were immunostained with anti-TH (A) or

665 TUNEL and Dapi (B), and imaged under a confocal microscope. Scale bars: 100 μm. Below:

666 Quantifications of the density of TH-positive neurons (A) or the percentage of TUNEL-positive neurons

667 (B). n=20 images from 3 independent coverslips. P values are compared with the far-left bar, except

668 indicated otherwise. (C) 40-day-old fly brains were immunostained with anti-TH and the DA neuron

669 number was counted in the PPL1 cluster. Scale bar: 20 μm. n=4, 7, 7, 4 (from left to right). (D) The

670 Performance Index was measured in 30-day-old flies, fed as indicated. n=59, 57, 54, 57 flies (from left to

26

671   right), 3 independent experiments. (C-D) Drug treatment was started from adulthood (day 1). One-Way

672   Anova Post Hoc Tukey Test for all panels.

673

674   **Figure 3. The EF-hands of Miro Play a Role in Causing PD Relevant Phenotypes.** (A) Representative

675   still images from live Mito-dsRed and GFP-Miro1 imaging movies of axons of indicated genotypes,

676   following 100 μM Antimycin A treatment. Scale bar: 10 μm. (B) Left: Quantification of the GFP-Miro1

677   intensity at 25 minutes divided by that at 0 minute following 100 μM Antimycin A treatment from the

678   same axonal region. Right: Quantification of the reduction speed of the GFP-Miro1 intensity within 25

679   minutes following 100 μM Antimycin A treatment. (C) Left: Quantification of the Mito-dsRed intensity

680   at 58 minutes divided by that at 0 minute following 100 μM Antimycin A treatment from the same axonal

681   region. Right: Quantification of the Mito-dsRed intensity reduction speed within 58 minutes following

682   100 μM Antimycin A treatment. (B-C) n=5, 4, 5 (from left to right) axons (one axon per coverslip). (D)

683   The DA neuron number was counted in the PPL1 cluster of flies with indicated genotypes. Scale bar: 20

684   μm. n=7, 4, 6, 5 (from left to right). (E) The Performance Index was measured in flies with indicated

685   genotypes and age. n (from left to right)=49, 47, 40, 47 flies (day 12); 49, 47, 39, 47 (day 14); 48, 45, 37,

686   44 (day 20); 3 independent experiments. One-Way Anova Post Hoc Tukey Test for all panels.

687

688   **Figure 4. PD Mitochondria Import More Ca^{2+}.** iPSC-derived neurons from a PD patient with *SNCA-*

689   *A53T* and the isogenic control were stimulated with thrombin and mitochondrial (Rhod-2) and cytosolic

690   Ca^{2+} levels (Calcium Green) were measured. Left: Representative traces of Ca^{2+} ions in neurons. Right:

691   Quantifications of the peak fluorescent intensity normalized to baseline. n=15 cell bodies from 3

692   independent coverslips. Two-tailed Welch's T Test.

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27

694   **Figure 5. Iron Promotes MCU Oligomerization.** (A) Elution profiles of MCU from SEC samples. (B)

695   HEK cells were treated similarly as above, run in Native-PAGE, and blotted. Right: Qualifications of the

696   band intensities normalized to the total protein amount measured by BCA. n=5 independent experiments.

697   One-Way Anova Post Hoc Tukey Test. (C) Elution profiles of additional proteins from SEC samples.

698

699   **Figure 6. MCU Binds to Fe^{2+}.** (A) HEK cells were treated with or without 5 mM Fe^{2+} for 21 hours, then

700   IPed with rabbit IgG or anti-MCU, and Fe^{2+} concentrations in the IP samples were detected. n=4

701   independent experiments. Top western blots demonstrated the success of IP. Anti-MCU was validated

702   using *MCU-/-* HEK cell lysate (bottom panel). Arrow indicates the MCU band. (B) HEK cells were treated

703   with 5 mM Fe^{2+} or Co^{2+} for 22 hours and lysed in buffer containing the same metal. Lysates were run in

704   Native- or SDS-PAGE and blotted with anti-MCU. Similar results were seen for at least 3 times. (C) HEK

705   cells treated with 5 mM Fe^{2+} or 500 μM Co^{2+} for 22 hours were stimulated with thrombin and

706   mitochondrial Ca^{2+} levels (Rhod-2) were measured. The peak fluorescent intensity normalized to baseline

707   is quantified. n=20 cells from 4 independent coverslips. (D-E) Fluorescence-detection SEC profiles of

708   purified human MCU. The annotation of the peaks is based on (Fan et al., 2018; Fan et al., 2020). (F)

709   *MCU-/-* HEK cells transfected as indicated were treated with 5 mM Fe^{2+} for 20 hours, then IPed with anti-

710   Flag, and Fe^{2+} concentrations in the IP samples were detected. Two-tailed paired T Test. (G) Left:

711   Representative blots of IP with anti-Flag using cell lysates as indicated, run in Native- or SDS-PAGE.

712   Right: Quantification of the band intensity of MCU-Flag in Native-PAGE normalized to that in SDS-

713   PAGE from the same experiment. n=4 independent experiments. (H) HEK cells treated as indicated were

714   stimulated with thrombin and mitochondrial Ca^{2+} levels (Rhod-2) were measured. Representative traces

715   of Ca^{2+} ions. (I-J) Based on traces like in (H), the peak fluorescent intensity normalized to baseline (I) or

28

716   efflux rate (J) is quantified. n=17 cells from 4 independent coverslips. Two-tailed Welch's T Test for all

717   panels except (F).

718

719   **Figure 7. Chelating Iron Restores Ca^{2+} and neuronal homeostasis in PD neurons.** (A) Similar to

720   Figure 4, iPSC-derived neurons from a PD patient with *SNCA-A53T* and the isogenic control, with or

721   without treatment of 100 μM DFP for 24 hours, were stimulated with thrombin, and mitochondrial Ca^{2+}

722   (Rhod-2) was measured. Quantifications of the peak fluorescent intensity normalized to baseline. n=15

723   cell bodies from 3 independent coverslips. Control data without DFP treatment are the same as in Figure

724   4. One-Way Anova Post Hoc Tukey Test. (B) iPSC-derived neurons treated as indicated, were

725   immunostained with TUNEL and Dapi, and imaged under a confocal microscope. Scale bar: 50 μm.

726   Below: Quantification of the percentage of TUNEL-positive neurons. n=20 images from 3 independent

727   coverslips. P values are compared within each genotype (significant compared to every other condition)

728   with One-Way Anova Post Hoc Tukey Test. (C) The DA neuron number was counted in the PPL1 cluster

729   of flies with indicated genotypes and conditions. Drug treatment was started from adulthood (day 1). Scale

730   bar: 20 μm. n=6, 9, 8, 7 (from left to right). (D) The Performance Index was measured in flies. Drug

731   treatment was started from embryogenesis. n=35, 33, 40, 34 flies (from left to right), 3 independent

732   experiments. (C-D) One-Way Anova Post Hoc Tukey Test. (E) Postmortem brains were run in Native- or

733   SDS-PAGE and blotted. The band intensity normalized to the total protein level measured by BCA is

734   divided by that of the universal control on the same blot: CVD (cardiovascular disease), which was

735   included on every blot. The MCU oligomer bands in Native-PAGE and the NCLX and MCUb bands in

736   SDS-PAGE (average of 3 replicates) are used in the plot. HC: healthy control. (F) Miro1 protein levels

737   were measured using ELISA in PBMCs treated with DMSO or 40 μM CCCP for 6 hours. Miro1 Ratio is

738   calculated by dividing the Miro1 value treated with CCCP by that with DMSO from the same subject. Dot

739   plot with Mean±S.E.M. n=80 healthy controls and 107 PD. Two-tailed Welch's T Test. (G) PBMCs from

740   4 PD patients were treated with 40 μM CCCP for 6 hours, or pretreated with 10 μM Benidipine or MR3

741   for 18 hours and then with 40 μM CCCP for another 6 hours, and Miro1 protein was detected using ELISA.

742   Patient IDs are the same as in Table S4. Two-tailed paired T Test. (H) Schematic representation of the

743   iron-calcium-Miro axis discovered in this study. Red texts show genes containing variants associated with

744   PD status.

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