

1 **PPTC7 limits mitophagy through proximal and dynamic interactions with BNIP3 and NIX**
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11 **Abstract**

12
13 PPTC7 is a mitochondrial-localized PP2C phosphatase that maintains mitochondrial protein
14 content and metabolic homeostasis. We previously demonstrated that knockout of *Pptc7* elevates
15 mitophagy in a BNIP3- and NIX-dependent manner, but the mechanisms by which PPTC7
16 influences receptor-mediated mitophagy remain ill-defined. Here, we demonstrate that loss of
17 PPTC7 upregulates BNIP3 and NIX post-transcriptionally and independent of HIF-1 α stabilization.
18 On a molecular level, loss of *PPTC7* prolongs the half-life of BNIP3 and NIX while blunting their
19 accumulation in response to proteasomal inhibition, suggesting that PPTC7 promotes the
20 ubiquitin-mediated turnover of BNIP3 and NIX. Consistently, overexpression of PPTC7 limits the
21 accumulation of BNIP3 and NIX protein levels in response to pseudohypoxia, a well-known
22 inducer of mitophagy. This PPTC7-mediated suppression of BNIP3 and NIX protein expression
23 requires an intact PP2C catalytic motif but is surprisingly independent of its mitochondrial
24 targeting, indicating that PPTC7 influences mitophagy outside of the mitochondrial matrix. We
25 find that PPTC7 exists in at least two distinct states in cells: a longer isoform, which likely
26 represents full length protein, and a shorter isoform, which likely represents an imported, matrix-
27 localized phosphatase pool. Importantly, anchoring PPTC7 to the outer mitochondrial membrane
28 is sufficient to blunt BNIP3 and NIX accumulation, and proximity labeling and fluorescence co-
29 localization experiments suggest that PPTC7 associates with BNIP3 and NIX within the native
30 cellular environment. Importantly, these associations are enhanced in cellular conditions that
31 promote BNIP3 and NIX turnover, demonstrating that PPTC7 is dynamically recruited to BNIP3
32 and NIX to facilitate their degradation. Collectively, these data reveal that a fraction of PPTC7
33 dynamically localizes to the outer mitochondrial membrane to promote the proteasomal turnover
34 of BNIP3 and NIX.

35 **Introduction**

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37
38 Mitophagy, or mitochondrial-specific autophagy, is a conserved organellar quality control
39 process that promotes the selective turnover of damaged or superfluous mitochondria across
40 cellular conditions (Pickles *et al*, 2018; Uoselis *et al*, 2023). During mitophagy, mitochondria are
41 selectively tagged for degradation by the activation of either ubiquitin-associated pathways or
42 specific mitophagy receptors. Multiple human diseases result from mutations within
43 mitophagy-associated genes, such as Parkinson Disease (Valente *et al*, 2004; Kitada *et al*, 1998;
44 Zimprich *et al*, 2011) and Amyotrophic Lateral Sclerosis (Maruyama *et al*, 2010). Most of these
45 disease-associated mutations lessen the activation or efficiency of mitophagy, suggesting that
46 enhancing mitophagy may promote mitochondrial function and alleviate various human
47 pathologies (Mishra & Thakur, 2023; Lee & Kim, 2014; Wang *et al*, 2023). Interestingly, however,
48 recent studies show that unrestrained mitophagy may also trigger pathophysiology, particularly
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52 through disrupting the regulation of the mitophagy receptors BNIP3 and NIX (Bonnen *et al*, 2013;
53 Gai *et al*, 2013; Cao *et al*, 2023; Elcocks *et al*, 2023; Nguyen-Dien *et al*, 2023).

54 BNIP3 and NIX have long been associated with mitochondrial turnover. Well-
55 characterized as transcriptional targets of Hypoxia Inducible Factor 1 α (HIF-1 α), BNIP3 and NIX
56 are upregulated during hypoxia to decrease mitochondrial content due to limited oxygen
57 availability (Ney, 2015). NIX promotes mitochondrial clearance during erythropoiesis to prevent
58 mature red blood cells from consuming the oxygen they carry before it is delivered to distal tissues
59 (Schweers *et al*, 2007). Similarly, BNIP3 and NIX induce mitochondrial turnover and subsequent
60 metabolic reprogramming in various models of cellular differentiation, including neurons
61 (Ordureau *et al*, 2021), myoblasts (Sin *et al*, 2016), and cardiomyocytes (Esteban-Martínez &
62 Boya, 2018; Zhao *et al*, 2020). These studies demonstrate that BNIP3 and NIX can potently
63 decrease mitochondrial content across cell types, indicating the levels of these mitophagy
64 receptors must be tightly regulated to prevent excessive mitochondrial clearance. Consistently,
65 recent studies have shown that loss of the mitochondrial E3 ubiquitin ligase FBXL4 unleashes
66 BNIP3- and NIX-mediated mitophagy, leading to decreased mitochondrial protein levels, mtDNA
67 depletion, and perinatal lethality in mice (Alsina *et al*, 2020; Cao *et al*, 2023; Elcocks *et al*, 2023;
68 Nguyen-Dien *et al*, 2023). Mutations in human *FBXL4* cause Mitochondrial DNA Depletion
69 Syndrome 13 (MTDPS13) a severe pathology characterized by encephalopathy, stunted growth,
70 and metabolic deficiencies (Bonnen *et al*, 2013; Gai *et al*, 2013; Dai *et al*, 2017; Ballout *et al*,
71 2019). Importantly, these human mutations disrupt the ability of FBXL4 to promote BNIP3 and
72 NIX turnover (Cao *et al*, 2023; Elcocks *et al*, 2023; Nguyen-Dien *et al*, 2023), suggesting that
73 excessive BNIP3 and NIX accumulation constitutes a substantial organismal liability. Despite this,
74 the mechanisms restraining these mitophagy receptors under basal conditions have not been fully
75 defined.

76 We previously identified the mitochondrial-resident protein phosphatase PPTC7 as a
77 regulator of BNIP3- and NIX-mediated mitophagy (Niemi *et al*, 2023). Knockout of *Pptc7* in mice
78 led to fully penetrant perinatal lethality concomitant to metabolic defects, including hypoketotic
79 hypoglycemia (Niemi *et al*, 2019). Strikingly, tissues and cells isolated from *Pptc7* knockout
80 animals showed robustly decreased mitochondrial protein levels as well as consistently elevated
81 BNIP3 and NIX protein expression (Niemi *et al*, 2019), indicating that unchecked BNIP3 and NIX
82 expression may drive mitochondrial loss through excessive mitophagy. Indeed, knockout of *Bnip3*
83 and *Nix* within the *Pptc7* knockout background largely rescues mitochondrial protein levels and
84 elevated mitophagy (Niemi *et al*, 2023). Additionally, we found that BNIP3 and NIX are
85 hyperphosphorylated in *Pptc7* knockout systems, and that PPTC7 can directly interact with BNIP3
86 and NIX to facilitate their dephosphorylation in vitro (Niemi *et al*, 2023). These data demonstrate
87 that PPTC7 acts as a critical negative regulator of BNIP3- and NIX-mediated mitophagy. However,
88 the precise molecular mechanism(s) by which PPTC7 influences BNIP3 and NIX protein levels
89 and mitophagic flux remain unclear, particularly given that these proteins reside in separate
90 mitochondrial compartments (Rhee *et al*, 2013; Hung *et al*, 2017). Here, we use a combination of
91 biochemical and cellular assays to demonstrate that PPTC7 proximally and dynamically interacts
92 with BNIP3 and NIX to promote their turnover and limit basal receptor mediated mitophagy.

93 Results

94 PPTC7 regulates BNIP3 and NIX post-transcriptionally and independent of HIF-1 α

95 We previously reported that BNIP3 and NIX were significantly upregulated in tissues and cells
96 derived from *Pptc7*^{-/-} mice (Niemi *et al*, 2019, 2023). Consistently, *Pptc7* knockout (KO) mouse
97 embryonic fibroblasts (MEFs, Figure 1A) and PPTC7 KO 293T cells (Figure 1B) showed elevated
98 expression of BNIP3 and NIX relative to wild-type cells. To understand the mechanisms
99 underlying BNIP3 and NIX upregulation upon PPTC7 loss, we investigated the involvement of

103 Hypoxia Inducible Factor-1 α (HIF-1 α) in mediating this response. *BNIP3* and *NIX* are well-
104 established transcriptional targets of HIF-1 α in conditions of hypoxia, as well as pseudohypoxia
105 through various pharmacological stimuli (e.g., the iron chelators deferoxamine (DFO) and
106 deferiprone (DFP) as well as cobalt chloride) (Allen *et al*, 2013; Wang & Semenza, 1993a, 1993b).
107 We thus hypothesized that the increase in BNIP3 and NIX protein levels in *PPTC7* KO cells may
108 be due to elevated HIF-1 α activity. To test this, we immunoblotted for HIF-1 α expression in wild-
109 type and *PPTC7* KO cells in both basal conditions as well as in the presence of baflomycin A1
110 (Baf-A1), a compound previously shown to stabilize HIF-1 α (Hubbi *et al*, 2013). These
111 experiments revealed no differences in HIF-1 α protein expression between wild-type and *PPTC7*
112 KO cells across tested conditions (Figure 1C). Additionally, we found no significant changes in
113 the abundance of select HIF-regulated proteins relative to other proteins across our previously
114 collected proteomics datasets in *Pptc7* KO mouse tissues (Niemi *et al*, 2019) (Supplemental
115 Figure 1A). We next tested whether BNIP3 and NIX were transcriptionally upregulated but found
116 no significant differences in *BNIP3* or *NIX* mRNA levels between wild-type and *PPTC7* KO 293T
117 cells (Figure 1D). Consistently, we found that the transient transfection of plasmids encoding myc-
118 BNIP3 or FLAG-NIX led to increased protein expression in *PPTC7* KO 293T cells relative to wild-
119 type 293T cells (Supplemental Figures 1B, C). As these plasmids are driven by the same CMV
120 promoter in both wild-type and *PPTC7* KO cells, the elevated BNIP3 and NIX protein expression
121 seen in *PPTC7* KO likely occurs independent of transcriptional changes. Together, these data
122 indicate that *PPTC7* influences BNIP3 and NIX protein expression post-transcriptionally and
123 independent of HIF-1 α .

124 The HIF-1 α -independent upregulation of BNIP3 and NIX in *PPTC7* KO cells suggests that
125 HIF-1 α and *PPTC7* regulate BNIP3 and NIX through parallel pathways. If true, we hypothesized
126 that loss of *PPTC7* would not alter BNIP3 and NIX transcriptional induction upon HIF activation,
127 but would promote an additive increase in BNIP3 and NIX protein levels. Indeed, treatment of
128 both wild-type and *PPTC7* KO cells with the iron chelator DFO induced similar fold changes in
129 *BNIP3* (Figure 1E) and *NIX* (Figure 1G) mRNA transcripts across genotypes. At the protein level,
130 expression of BNIP3 (Figure 1F) and NIX (Figure 1H) were substantially higher in DFO-treated
131 *PPTC7* KO cells relative to other tested conditions. Notably, basal protein expression of BNIP3
132 and NIX in untreated *PPTC7* KO cells exceeded BNIP3 and NIX induction in DFO-treated wild-
133 type cells (Figures 1F and 1H), indicating the magnitude of BNIP3 and NIX upregulation in the
134 absence of *PPTC7*. These experiments further indicate that neither BNIP3 nor NIX is maximally
135 upregulated in *PPTC7* knockout cells in basal conditions.

136 The additive increase in BNIP3 and NIX expression in *PPTC7* KO cells upon DFO
137 treatment led us to hypothesize that mitophagic flux would be elevated in DFO-treated *PPTC7*
138 knockout cells relative to either condition independently. To test this, we assayed wild-type and
139 *Pptc7* KO MEFs expressing mt-Keima, a pH-sensitive fluorescent mitophagy sensor (Sun *et al*,
140 2017) using flow cytometry. We first exposed wild-type or *Pptc7* KO MEFs to varying
141 concentrations of DFO and found that only the highest tested dose of DFO, 100 μ M, induced
142 mitophagy in wild-type cells (Supplemental Figures 1D-F). Notably, the percentage of cells
143 undergoing high mitophagic flux in wild-type cells treated with 100 μ M DFO remained below the
144 rates of mitophagic induction in untreated *Pptc7* KO cells (Supplemental Figures 1D-F). We
145 repeated these experiments in wild-type, *Pptc7* KO, and *Pptc7/Bnip3/Nix* triple knockout (TKO)
146 MEFs and found that 100 μ M DFO induced an approximate three-fold increase in mitophagic flux
147 in both wild-type and *Pptc7* KO cells (Figures 1I-K). However, the absolute levels of mitophagy in
148 DFO-treated *Pptc7* KO cells significantly exceeded those of DFO-treated wild-type cells as well
149 as untreated *Pptc7* KO cells (Figures 1I-K). Importantly, mt-Keima-positive *Pptc7/Bnip3/Nix* TKO
150 cells fail to undergo appreciable mitophagy in the presence of 100 μ M DFO, demonstrating their
151 necessity in increasing mitophagic flux in *Pptc7* knockout cells (Figures 1I-K). Collectively, these
152 data demonstrate that BNIP3 and NIX are post-transcriptionally upregulated to induce mitophagy

153 in *PPTC7* KO cells, and that transcriptional activation of HIF-1 α can substantially enhance
154 BNIP3/NIX protein expression and mitophagy in the absence of *PPTC7*.
155

156 **PPTC7 enables BNIP3 and NIX turnover through proteasomal degradation**

157
158 The elevated protein expression of BNIP3 and NIX in *PPTC7* knockout cells implies that *PPTC7*
159 alters the synthesis or turnover rates of these mitophagy receptors. BNIP3 and NIX turnover has
160 emerged as a critical regulatory step in limiting basal mitophagy, as evidenced by recent studies
161 on the E3 ubiquitin ligase *FBXL4* (Cao *et al*, 2023; Elcocks *et al*, 2023; Nguyen-Dien *et al*, 2023).
162 Loss of *FBXL4* phenotypically mirrors *PPTC7* KO in mice, and *PPTC7* and *FBXL4* have significant
163 and positively correlated essentiality profiles across over one-thousand cancer cell lines
164 (Supplemental Figure 2A, (Dempster *et al*, 2019)). These data suggest that *PPTC7* and *FBXL4*
165 influence BNIP3 and NIX via similar mechanisms, leading us to hypothesize that BNIP3 and NIX
166 have decreased turnover rates in *PPTC7* knockout cells relative to wild-type cells.
167

168 To test this, we first sought to identify an experimental condition in which we could quantify
169 endogenous BNIP3 and NIX turnover. We noted that DFO-mediated iron chelation was previously
170 shown to decrease the protein level of select mitochondrial proteins in a manner that was
171 reversible upon compound washout (Rensvold *et al*, 2013). As BNIP3 and NIX accumulate in
172 response to DFO (Figure 1), we hypothesized that washout of DFO would induce BNIP3 turnover
173 due to its short half-life (Schäfer *et al*, 2022) (Figure 2A). Indeed, treatment of wild-type 293T cells
174 with DFO increased BNIP3 levels in a time-dependent manner, which returned to at or near
175 baseline (i.e., untreated) levels 24 hours after DFO washout (Figure 2B). Thus, DFO washout
176 constitutes an experimental system in which we could test the effects of *PPTC7* on the turnover
177 of endogenous BNIP3. We repeated these experiments in wild-type and *PPTC7* knockout 293T
178 cells and found that BNIP3 and NIX exhibited blunted turnover in *PPTC7* KO cells (Figures 2C-F,
179 Supplemental Figure 2B-E). Modeling of BNIP3 and NIX decay rates showed that loss of *PPTC7*
180 extends the half-life of monomeric and dimeric populations of BNIP3 and NIX (Figures 2C-F).
181 While the dimer populations of BNIP3 and NIX have at least a doubling of protein half-life in
182 *PPTC7* KO cells relative to wild-type cells, the half-lives of the monomeric populations of each
183 mitophagy receptor could not be effectively modeled in *PPTC7* KO cells, consistent with
184 substantial suppression of the turnover of these species of BNIP3 and NIX in the absence of
185 *PPTC7* (Figures 2C-F).

186 Given the slowed rates of BNIP3 and NIX turnover in *PPTC7* KO cells, we sought to
187 understand the pathway(s) contributing to their degradation. The phenotypic similarities between
188 loss-of-function models of *PPTC7* and *FBXL4*, as described above, suggest that these two
189 proteins function in a similar pathway. Additionally, previous work has shown that BNIP3
190 accumulates in response to the proteasomal inhibitor MG-132 (Park *et al*, 2013; Poole *et al*, 2021).
191 If knockout of *PPTC7* suppresses the proteasomal degradation of BNIP3 and NIX, we
192 hypothesized that *PPTC7* KO cells would be less responsive to the proteasomal inhibitor MG-132
193 than matched wild-type cells. We found that BNIP3 and NIX levels increased in wild-type cells
194 upon MG-132 treatment (Figures 2G, H), but that the level of each receptor was significantly less
195 responsive to MG-132 in *PPTC7* KO cells (Figures 2G, H). To further test this model, we exploited
196 the DFO washout assay, predicting that if BNIP3 and NIX were turned over by proteasomal
197 degradation upon DFO washout, MG-132 treatment would slow their turnover rates in wild-type
198 cells but have a diminished effect in *PPTC7* KO cells. We found that, upon DFO washout, MG132
199 treatment causes BNIP3 and NIX accumulation in wild-type cells, while the levels of these proteins
200 remain largely unchanged in *PPTC7* KO cells under identical conditions (Figure 2I). Overall, these
201 data demonstrate that *PPTC7* enables the efficient turnover of BNIP3 and NIX in a manner that
202 largely depends upon proteasomal degradation.

203 **PPTC7 requires an intact active site but not a mitochondrial targeting sequence to limit**
204 **BNIP3 and NIX accumulation**

205
206 As loss of *PPTC7* increases BNIP3 expression, we hypothesized that PPTC7 overexpression
207 may diminish BNIP3 upregulation induced by pseudohypoxia. To test this, we overexpressed
208 either cytosolic GFP or PPTC7-GFP in HeLa cells that were treated with the pseudohypoxia
209 inducer cobalt chloride (CoCl₂). We fixed and immunolabeled these cells to examine endogenous
210 BNIP3 levels relative to the general mitochondrial marker TOMM20. CoCl₂ robustly upregulated
211 BNIP3 protein expression, and mitochondrial BNIP3 staining could be detected in nearly all cells
212 transfected with cytosolic GFP (Figure 3A). In contrast to cytosolic GFP, PPTC7-GFP co-localized
213 with the mitochondrial marker TOMM20 as expected (Figure 3A). Notably, mitochondrial BNIP3
214 signal was rarely detected in the subset of cells expressing PPTC7-GFP (Figures 3A, B). On a
215 functional level, overexpression of PPTC7 additionally suppressed CoCl₂-induced mitophagic flux
216 relative to identically treated wild-type mt-Keima cells (Figure 3C, Supplemental Figure 3A).
217 Finally, overexpression of PPTC7 in *Pptc7* KO MEFs rescued basal BNIP3 protein expression to
218 levels seen in wild-type MEFs (Supplemental Figure 3B). Collectively, these data show that
219 overexpressed PPTC7 limits BNIP3 protein expression and mitophagy induction in both wild-type
220 and *Pptc7* KO cells.

221 As these experiments indicated that overexpressed PPTC7 was properly localized and
222 functional, we generated a series of mutants to understand the mechanism by which PPTC7 limits
223 BNIP3 and NIX accumulation. PPTC7 consists of a PP2C phosphatase domain that is preceded
224 by a predicted 38-residue disordered N-terminus (Figure 3C), which includes a mitochondrial
225 targeting sequence (i.e., MTS) that is processed at amino acid 14 (Calvo *et al*, 2017). Interestingly,
226 when western blotting for overexpressed PPTC7, we found that the protein ran as a doublet, while
227 a ΔMTS-PPTC7 mutant ran at the same molecular weight as the bottom band (Figure 3D),
228 suggesting that wild-type PPTC7 expressed as both a full-length and processed form. Given these
229 molecular insights, we used this overexpression system to test the necessity of PPTC7 catalytic
230 activity and mitochondrial targeting in suppressing BNIP3 and NIX induction under pseudohypoxia.

231 We first mutated the PP2C phosphatase domain of PPTC7 at a key catalytic residue, D78.
232 We previously demonstrated that recombinant PPTC7 D78A was unable to dephosphorylate
233 BNIP3 and NIX on mitochondria isolated from *Pptc7* KO MEFs (Niemi *et al*, 2023). Consistently,
234 recombinant wild-type PPTC7, but not D78A PPTC7, caused a collapse in the laddering pattern
235 of monomeric BNIP3 in mitochondria isolated from wild-type and *PPTC7* knockout 293T cells
236 (Supplemental Figure 3C). These data show that not only that PPTC7 D78A lacks catalytic activity,
237 but also that the upper monomeric bands seen on BNIP3 western blots represent phosphorylated
238 intermediates that can be directly dephosphorylated by PPTC7. While the D78A mutant lacks
239 phosphatase activity, mutation of PPTC7 D78 may also disrupt its physical interaction with BNIP3
240 or NIX, as AlphaFold2 multimer models indicate that BNIP3 and NIX dock to PPTC7 proximal to
241 D78 (Supplemental Figures 3D,E). Via either mechanism, we predicted that a D78A mutant would
242 be unable to suppress BNIP3 and NIX accumulation during pseudohypoxia. We overexpressed
243 wild-type and D78A PPTC7 in HeLa FLP-IN T-REx cells and found that both constructs were
244 doxycycline-induced to similar extents and, interestingly, also expressed at higher levels in the
245 presence of CoCl₂, similar to BNIP3 and NIX (Figure 3F). While overexpression of wild-type
246 PPTC7 decreased BNIP3 and NIX accumulation in response to CoCl₂ treatment, the D78A mutant
247 failed to suppress the induction of these mitophagy receptors (Figure 3F), indicating that PPTC7
248 requires an intact catalytic motif to influence BNIP3 and NIX.

249 We next examined whether disrupting the mitochondrial localization of PPTC7 would
250 affect its ability to suppress CoCl₂-induced BNIP3 and NIX levels. We overexpressed the ΔMTS-
251 PPTC7 construct HeLa FLP-IN T-REx cells, hypothesizing that it would fail to influence CoCl₂-
252 induced BNIP3 and NIX expression due to its inability to target to mitochondria. Surprisingly, we
253 found that ΔMTS-PPTC7 fully suppressed CoCl₂-mediated NIX accumulation, and partially

254 suppressed BNIP3 accumulation (Figure 3G). This rescue of BNIP3 and NIX expression is
255 consistent with a model in which a non-targeted PPTC7 can influence mitophagy at sufficient (i.e.,
256 overexpressed) levels. This, in combination with the fact that wild-type PPTC7 runs as a doublet,
257 suggested that PPTC7 may exist in two populations: one outside of mitochondria (e.g., a full
258 length, unprocessed form) and one that localizes to the mitochondrial matrix (e.g., Δ MTS-PPTC7,
259 which lacks its mitochondrial targeting sequence upon processing after import). If true, full length
260 PPTC7 may reside outside of mitochondria prior to its import, placing it proximal to BNIP3 and
261 NIX. We thus hypothesized that artificially anchoring PPTC7 to the outer mitochondrial membrane
262 (OMM) would block BNIP3 and NIX accumulation in response to pseudohypoxia. We engineered
263 a Δ MTS-PPTC7-OMP25 construct that both lacks an MTS and is fused to OMP25, a C-terminal
264 tail anchored protein that targets to the OMM (Horie *et al*, 2002). Consistent with our hypothesis,
265 Δ MTS-PPTC7-OMP25 blunts the accumulation of BNIP3 and NIX under both basal and CoCl_2 -
266 treated conditions (Figure 3H), suggesting an OMM-localized pool of PPTC7 influences BNIP3
267 and NIX protein expression. Collectively, these data show that PPTC7 requires an intact active
268 site but not its mitochondrial targeting sequence to suppress BNIP3 and NIX expression in
269 pseudohypoxic conditions, consistent with a role for PPTC7 in regulating these mitophagy
270 receptors outside of mitochondria.

271

272 PPTC7 proximally and dynamically interacts with BNIP3 and NIX in cells

273

274 Our data demonstrating that anchoring PPTC7 to the OMM blunts BNIP3 and NIX accumulation
275 (Figure 3H) indicates that a pool of PPTC7 may reside outside of mitochondria to directly interact
276 with BNIP3 and NIX to promote their proteasomal turnover. We sought to test this model by
277 determining whether BNIP3 and NIX interact with PPTC7 in cells through miniTurbo-based
278 proximity labeling experiments. We expressed PPTC7-V5-miniTurbo, as well as two control
279 constructs, in 293T cells with or without DFO treatment. We treated half of the samples with
280 exogenous biotin (which facilitates miniTurbo-based proximity labeling) and left the remaining
281 cells untreated to control for potential non-specific interactions. After biotinylation, we lysed cells,
282 performed a pulldown with streptavidin beads to enrich for biotinylated proximal interactors, and
283 probed for an interaction with BNIP3 and NIX via western blot. While V5-PPTC7-miniTurbo
284 expressed equally across all conditions, the streptavidin pulldown revealed interactions with
285 BNIP3 and NIX only in the biotin-treated samples, demonstrating specific proximal labeling
286 (Figure 4A). Importantly, these interactions were also specific to PPTC7-V5-miniTurbo and were
287 not seen in either vector only or V5-miniTurbo control samples (Figure 4A). These data, combined
288 with our data demonstrating that recombinant PPTC7 can directly dephosphorylate BNIP3 *in vitro*
289 (Supplemental Figure 2D), strongly suggest that PPTC7 directly interacts with BNIP3 and NIX
290 within the native cellular environment.

291 These data support a model in which PPTC7 is directly recruited to BNIP3 and NIX to
292 promote their turnover. Our DFO washout experiments offer insights into the kinetics of this
293 turnover, which allowed us to test whether the PPTC7-BNIP3/NIX interactions are dynamic
294 throughout the turnover process. Our experiments show that BNIP3 is present at near peak levels
295 4 hours post-DFO washout (Figures 2B, C), leading us to hypothesize that PPTC7 would have
296 enhanced recruitment to BNIP3 and NIX during acute DFO washout to facilitate the turnover of
297 BNIP3 and NIX. To test this, we repeated proximity labeling of PPTC7-V5-miniTurbo under
298 conditions of acute (i.e., 4 hour) DFO washout. Interestingly, while the PPTC7-BNIP3/NIX
299 interactions are enriched upon 24 hours of DFO treatment, similar to our previous experiment,
300 they are further enhanced upon acute 4-hour DFO washout (Figure 4B). These data show that
301 PPTC7 is dynamically recruited to BNIP3 and NIX to promote their turnover upon resolution of
302 DFO-mediated pseudohypoxia.

303 To further explore the dynamic nature of the PPTC7-BNIP3 interaction, we exploited the
304 recent observation that BNIP3 enriches at LC3-positive punctate structures which likely represent

305 nascent mitophagosomes (Gok *et al*, 2023). We hypothesized that PPTC7 would also enrich at
306 these punctate structures under conditions of pseudohypoxia, and that this localization would be
307 enriched upon resolution of pseudohypoxia. We overexpressed PPTC7-GFP in U2OS cells
308 treated with the iron chelator deferiprone (DFP) for 24 hours, and then fixed and immunolabeled
309 for BNIP3 and TOMM20. Examination of over 400 BNIP3-enriched foci in DFP-treated cells
310 revealed that ~14% of these structures were co-enriched for PPTC7-GFP (Figure 4C). Importantly,
311 these foci were co-localized with the mitochondrial marker TOMM20 (Figure 4C), demonstrating
312 these interactions occur at mitochondria and not other organelles, such the ER, where BNIP3 has
313 been reported to localize (Zhang *et al*, 2009; Hanna *et al*, 2012). Remarkably, we found that
314 PPTC7-GFP showed almost a three-fold increase co-enrichment (~46%) with BNIP3-enriched
315 foci 4 hours after DFP washout relative to DFP treatment alone (Figure 4D). These data, along
316 with our proximity labeling experiments, suggest that PPTC7 is actively recruited to BNIP3 and
317 NIX under conditions that promote their turnover. Collectively, our data are consistent with a
318 model in which a pool of PPTC7 dynamically localizes outside of mitochondria to associate with
319 BNIP3 and NIX—likely through a direct interaction—to promote their ubiquitin-mediated turnover.
320

321 Discussion

322 PPTC7 is one of twelve phosphatases that localize to mammalian mitochondria (Niemi &
323 Pagliarini, 2021). Conserved through budding yeast (where it is named Ptc7p), PPTC7 has been
324 linked to the maintenance of metabolism and mitochondrial homeostasis across organisms
325 (Martín-Montalvo *et al*, 2013; Guo *et al*, 2017a, 2017b; Gonzalez-Mariscal *et al*, 2017; González-
326 Mariscal *et al*, 2018; Niemi *et al*, 2019, 2023). While we previously have performed
327 phosphoproteomics to identify candidate substrates of Ptc7p (Guo *et al*, 2017a, 2017b) and
328 PPTC7 (Niemi *et al*, 2019, 2023), the precise roles of these phosphatases in regulating
329 mitochondrial homeostasis remain unclear. In this study, we begin to illuminate important aspects
330 underlying the regulation and function of PPTC7 and how this phosphatase influences BNIP3-
331 and NIX-mediated mitophagy.
332

333 The data presented in this study demonstrate that BNIP3 and NIX turnover is a tightly
334 regulated and highly dynamic process. Using a model of DFO-mediated iron chelation followed
335 by compound washout, we show that endogenous BNIP3 and NIX rapidly turn over in wild-type
336 cells in a manner that is slowed by proteasomal inhibition. Our data further show that BNIP3 and
337 NIX have longer half-lives and are less responsive to proteasomal inhibition in PPTC7 KO cells,
338 consistent with a model in which the phosphatase functions to promote the ubiquitin-mediated
339 turnover of these mitophagy receptors. Indeed, a study published during the preparation of this
340 manuscript showed that PPTC7 coordinates BNIP3 and NIX degradation by acting as a molecular
341 scaffold for the E3 ligase FBXL4 (Sun *et al*, 2023) at the outer mitochondrial membrane.
342 Consistently, we find that PPTC7 does not require its mitochondrial targeting sequence to
343 suppress BNIP3 and NIX accumulation, and anchoring PPTC7 on the outer mitochondrial
344 membrane decreases BNIP3 and NIX protein levels under pseudohypoxia. Furthermore, we find
345 that PPTC7 associates with BNIP3 and NIX via proximity labeling experiments, demonstrating a
346 likely direct interaction between the phosphatase and these mitophagy receptors in cells. These
347 data indicate that PPTC7 has meaningful functions at the outer mitochondrial membrane to
348 regulate BNIP3- and NIX-mediated mitophagy.

349 The apparent dual functionality of PPTC7 across mitochondrial compartments leads to
350 interesting questions regarding its regulation. The data in this study indicate that PPTC7 is actively
351 recruited to BNIP3 and NIX to promote their degradation, as the proximal interactions between
352 PPTC7 and BNIP3/NIX are enriched upon resolution of pseudohypoxia (i.e., the washout of iron
353 chelator). This conclusion is further supported by the enhanced colocalization of fluorescently
354 tagged PPTC7 with BNIP3-positive punctae in cells in conditions of iron chelator washout. These
355 data suggest not only a model of dynamic recruitment, but also a precisely coordinated spatial

356 organization of BNIP3 and PPTC7 at foci that likely represent mitophagosome formation sites
357 (Gok *et al*, 2023). Interestingly, TMEM11 was also found to co-localize with BNIP3 and NIX at
358 mitophagic punctae, and its knockout increases both BNIP3-positive foci as well as mitophagic
359 flux (Gok *et al*, 2023). These data suggest that PPTC7 and TMEM11 may function in similar
360 complexes to regulate BNIP3 and NIX mediated turnover and/or mitophagy. Whether these
361 complexes include FBXL4 and how they may actively remodel to promote or limit mitophagy are
362 key questions regarding the dynamic regulation of BNIP3- and NIX-mediated mitophagy.

363 Previous studies of *Fbxl4* and *Pptc7* knockout mouse models show that each display similar
364 pathophysiological profiles, including metabolic dysfunction, broad loss of mitochondrial protein
365 levels, and perinatal lethality. Importantly, knockout of *Bnip3* and *Nix* alleviates many of these
366 phenotypes in cells (Cao *et al*, 2023; Elcocks *et al*, 2023; Nguyen-Dien *et al*, 2023; Niemi *et al*,
367 2023) and mice (Cao *et al*, 2023; Sun *et al*, 2023), suggesting that loss of *Fbxl4* or *Pptc7* drives
368 excessive BNIP3- and NIX-mediated mitophagy. Despite these advances, the precise mechanism
369 by which PPTC7 influences BNIP3 and NIX protein levels remain unclear. We previously found
370 that BNIP3 and NIX are hyperphosphorylated in PPTC7 knockout cells and tissues, suggesting
371 PPTC7 may influence mitophagy via their dephosphorylation (Niemi *et al*, 2019, 2023). Indeed,
372 phosphorylation of BNIP3 and NIX can enhance their stability or their ability to induce mitophagy
373 (He *et al*, 2022; Poole *et al*, 2021; Zhu *et al*, 2013; Rogov *et al*, 2017), suggesting
374 dephosphorylation may be required to suppress their activity. However, Sun *et al*. suggest that
375 PPTC7 promotes BNIP3 and NIX degradation independent of its phosphatase activity, as retains
376 its ability to limit BNIP3 and NIX accumulation in the presence of cadmium, a PP2C phosphatase
377 inhibitor (Sun *et al*, 2023). Interestingly, however, their data demonstrate that an inactive PPTC7
378 mutant (D78A/G79A) cannot suppress BNIP3 and NIX accumulation or mitophagy induction,
379 which is consistent with our data demonstrating that D78A PPTC7 neither dephosphorylates
380 BNIP3 in vitro, nor blocks BNIP3 and NIX accumulation in conditions of pseudohypoxia (Figure
381 3). One possible explanation for this discrepancy is that PPTC7 may directly bind to BNIP3 and
382 NIX within its active site, consistent with Alphafold multimer models (Supplemental Figure 3). If
383 so, mutation of the PPTC7 active site could disallow BNIP3 and NIX binding independent of its
384 catalytic activity. Alternatively, it is possible that cadmium does not completely block PPTC7 in
385 cells, allowing low level phosphatase activity that can facilitate BNIP3 and NIX degradation.
386 Careful dissection of the mechanisms by which the D78A mutation PPTC7 limits BNIP3- and NIX-
387 mediated mitophagy, as well as how phosphorylation affects BNIP3 and NIX stability or activity
388 should be active areas of investigation in the future.

389 Collectively, our data reveal an unexpected role for the mitochondrial protein phosphatase
390 PPTC7 in the regulation of receptor-mediated mitophagy at the outer mitochondria membrane.
391 While knockout of BNIP3 and NIX largely rescue the decreases in mitochondrial protein levels
392 present in *Pptc7* KO tissues and cells, their collective knockout fails to fully rescue metabolic
393 defects seen in *Pptc7* KO cells (Niemi *et al*, 2023). This, together with our data indicating PPTC7
394 exists in two distinct pools in cells, suggests that PPTC7 regulates additional mitochondrial
395 functions within the matrix. It is possible that PPTC7 mediates such functions by influencing the
396 mitophagic selectivity of matrix-localized substrates, as occurs in yeast (Tal *et al*, 2007; Abeliovich
397 *et al*, 2013; Kolitsida *et al*, 2019, 2023). Alternatively, it is possible that PPTC7 maintains
398 mitochondrial metabolism completely independent of its role in mitophagic signaling, potentially
399 through the regulation of one or more candidate substrates previously identified via
400 phosphoproteomic analyses (Niemi *et al*, 2019, 2023). Disentangling the matrix-mediated
401 functions of PPTC7 versus those that regulate functions outside of mitochondria, and how these
402 ultimately relate to mitophagic flux, will be a key next step in understanding the role of this
403 phosphatase in modulating mitochondrial homeostasis.

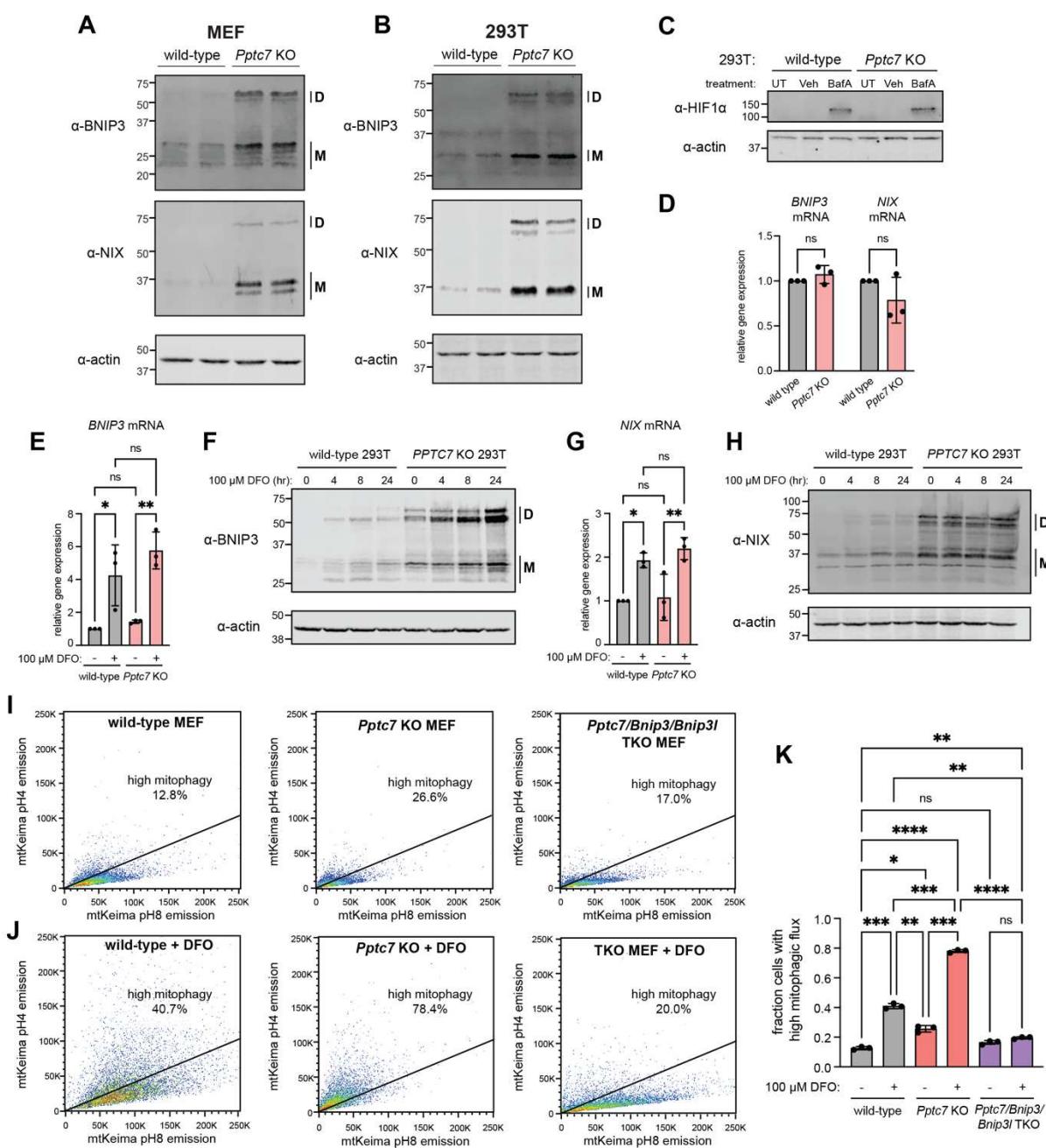
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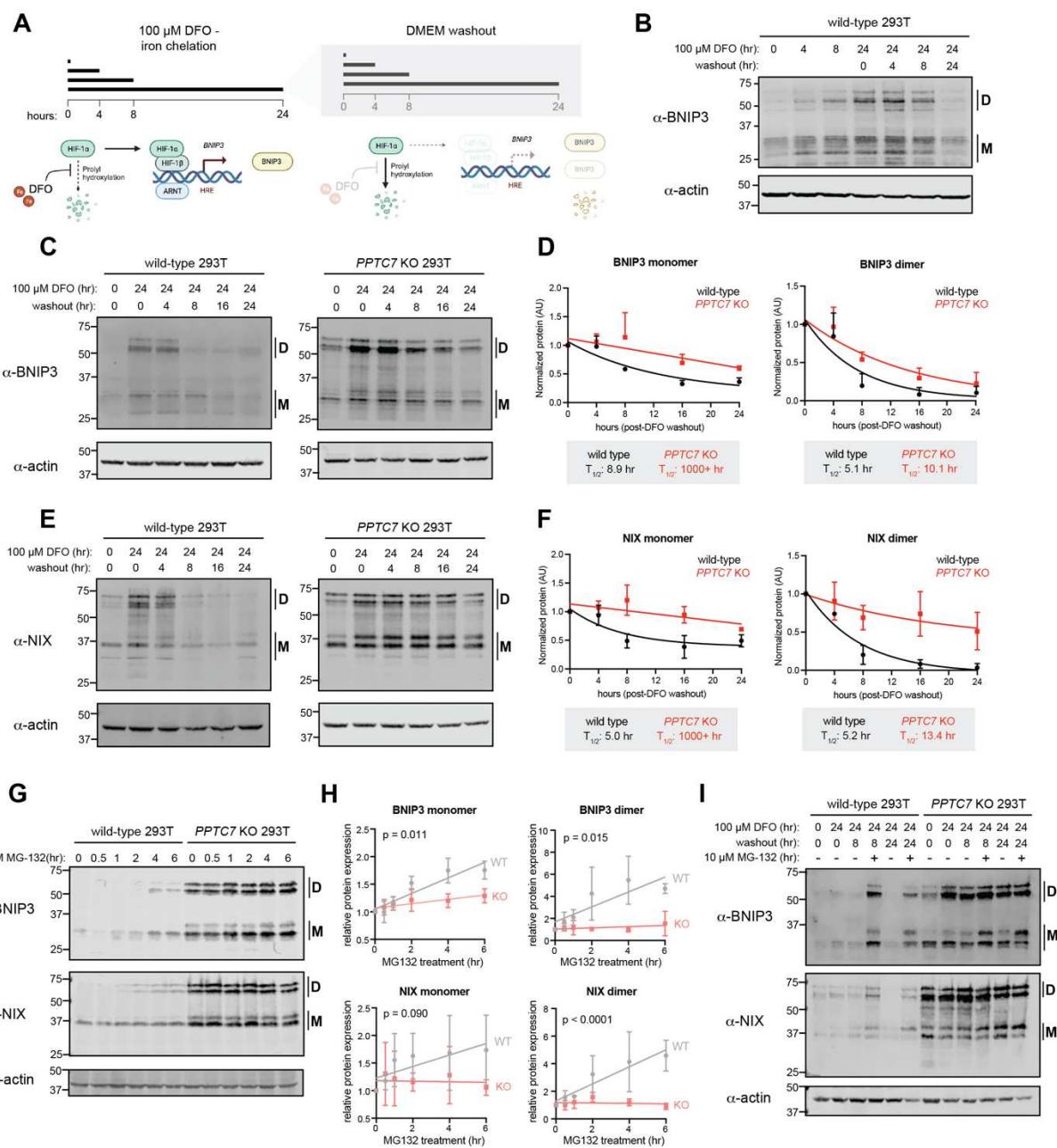
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407 **Acknowledgements**

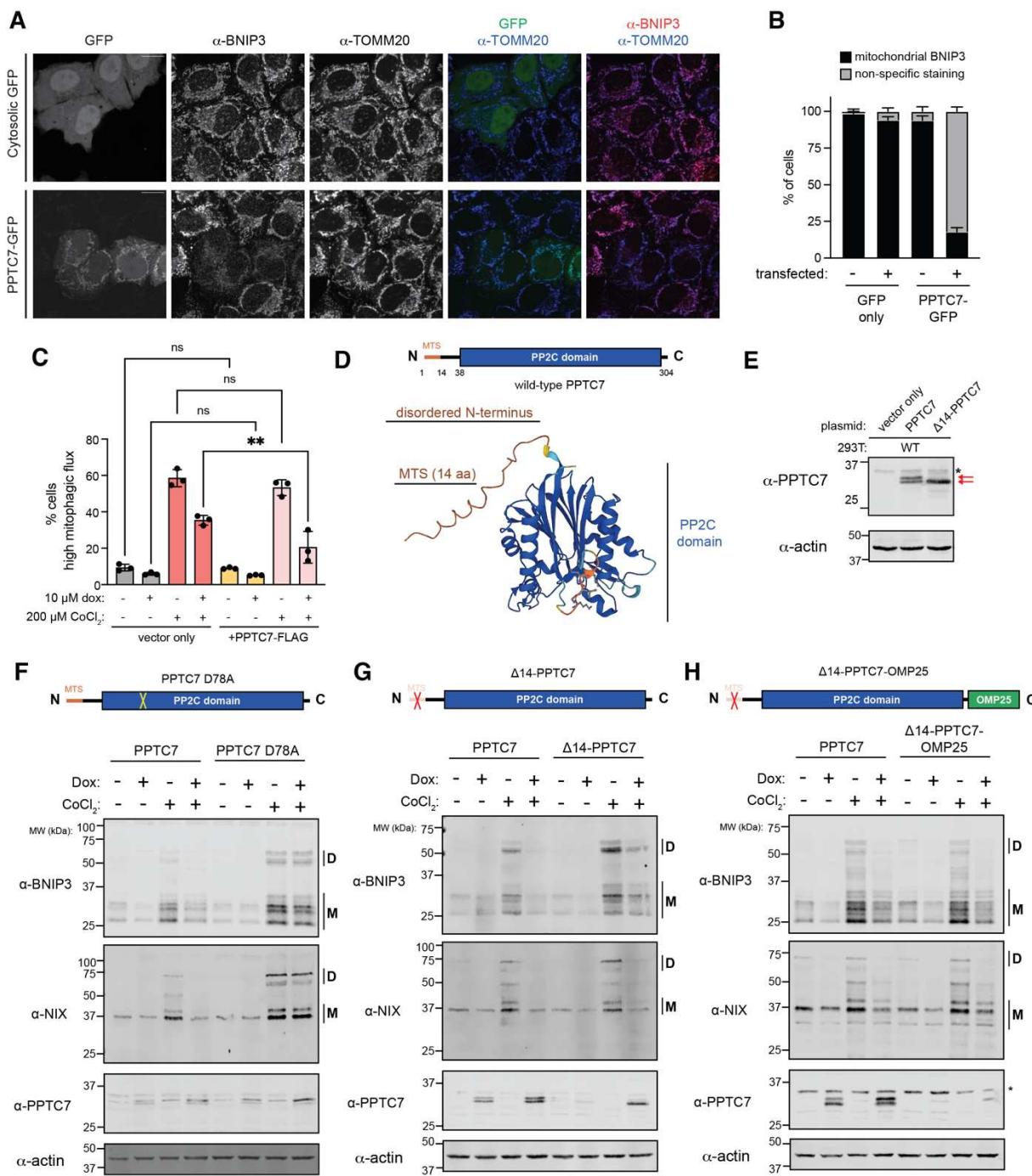
408
409 We thank Edrees Rashan, Keshav Kailash, Michael McKenna, and the Niemi Laboratory for
410 careful reading of the manuscript and for helpful discussions on this work. We thank Julia Pagan
411 and Keri-Lyn Kozul (University of Queensland, Australia) for key resources and for important
412 discussions related to this manuscript. We thank the Flow Cytometry and Fluorescence-Activated
413 Cell Sorting Core at Washington University School of Medicine for equipment and support for the
414 flow cytometry experiments. This work was supported by R35GM151130 (to N.M.N.) and
415 R35GM137894 (to J.R.F.). The UT Southwestern Quantitative Light Microscopy Facility, which is
416 supported in part by NIH P30CA142543, provided access to the Nikon SoRa microscope
417 (purchased with NIH 1S10OD028630-01). L.W. was supported by the MilliporeSigma Predoctoral
418 Fellowship in Honor of Dr. Gerty T. Cori at Washington University. J.S. was supported through
419 the Summer Undergraduate Research Program put on within the Department of Biochemistry and
420 Molecular Biophysics at Washington University School of Medicine in St. Louis.



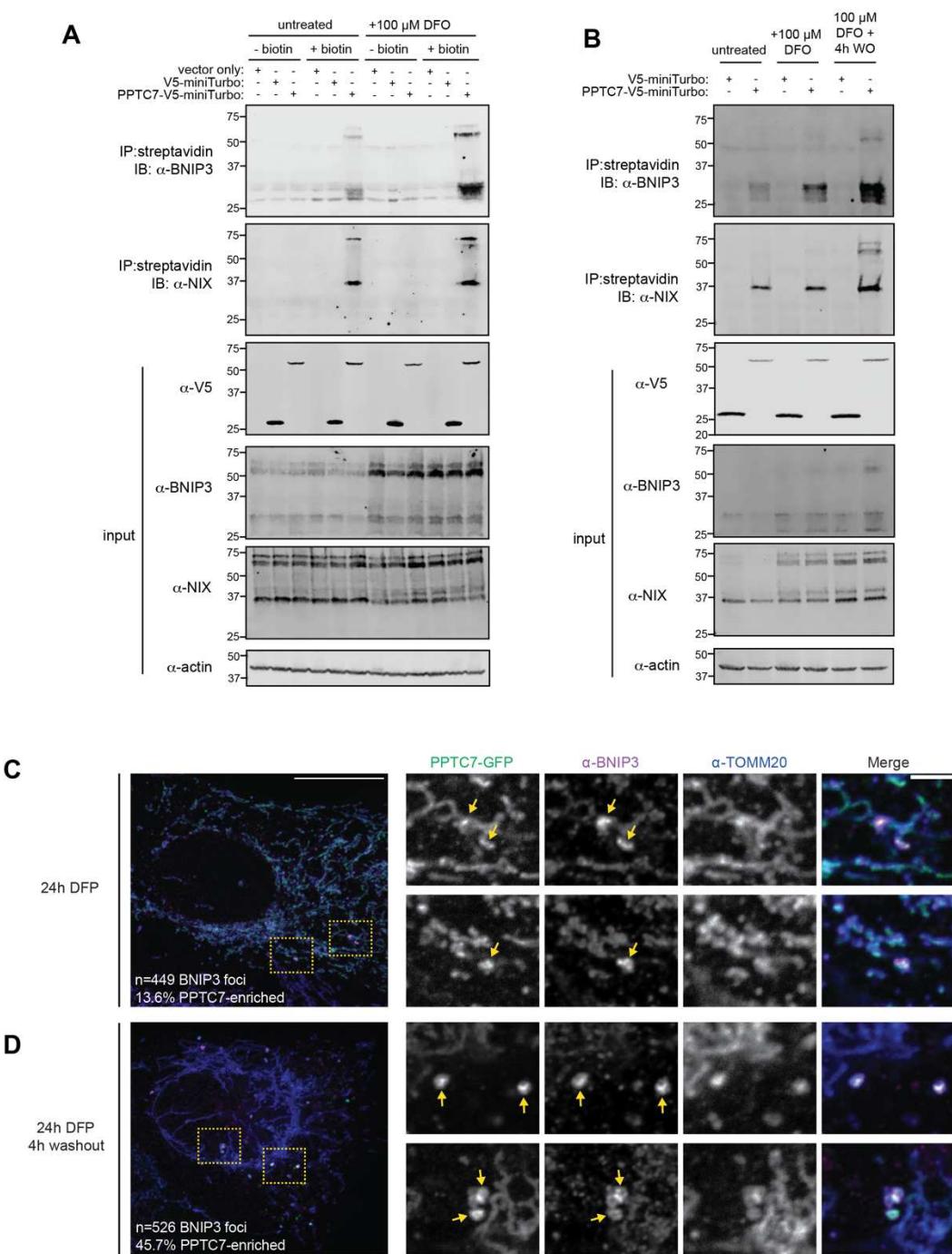
422 **Figure 1: BNIP3 and NIX are upregulated post-transcriptionally and independent of HIF-1 α in**
423 **PPTC7 KO cells. A., B.** Western blots of BNIP3 (top panels), NIX (middle panels) and actin
424 (serving as a load control, bottom panels) in wild-type or *Pptc7* knockout (KO) mouse embryonic
425 fibroblasts (MEF, A.) or in wild-type or *PPTC7* KO 293T cells (B.). “D” indicates dimer species;
426 “M” indicates monomer species. **C.** Western blot for HIF-1 α in untreated (UT), vehicle only (veh,
427 0.2% DMSO) or 100 nM baflomycin A (BafA) for 16 hours in wild-type and *PPTC7* KO 293T cells.
428 Actin shown as a loading control. **D.** qRT-PCR of BNIP3 and NIX endogenous mRNA levels in
429 wild-type (gray) and *PPTC7* KO (pink) 293T cells. **E.** qRT-PCR of *BNIP3* RNA levels in untreated
430 and DFO-treated (100 μ M, 24 hours) wild-type (gray) and *PPTC7* KO (pink) 293T cells. **p<0.01,
431 *p<0.05, ns = not significant, Ordinary one-way ANOVA. Error bars represent standard deviation;
432 data points represent independent experiments. **F.** Western blotting for endogenous BNIP3 levels
433 in wild-type or *PPTC7* KO 293T cells treated with 100 μ M DFO for indicated times. Actin shown
434 as a loading control. **G.** qRT-PCR of *NIX* RNA levels in untreated and DFO-treated (100 μ M, 24
435 hours) wild-type (gray) and *PPTC7* KO (pink) 293T cells. **p<0.01, *p<0.05, ns = not significant,
436 Ordinary one-way ANOVA. Error bars represent standard deviation; data points represent
437 independent experiments. **H.** Western blotting for endogenous NIX levels in wild-type or *PPTC7*
438 KO 293T cells treated with 100 μ M DFO for indicated times. Actin shown as a loading control. **I.**
439 FACS histograms of basal mitophagy in wild-type (left), *Pptc7* KO (middle) and *Pptc7/Bnip3/Nix*
440 TKO MEFs using the mt-Keima fluorescence assay. Cells undergoing high mitophagy are above
441 the diagonal line; percentages indicated in figure. **J.** FACS histograms of mitophagy rates upon
442 24 hours of 100 μ M DFO treatment in wild-type (left), *Pptc7* KO (middle) and *Pptc7/Bnip3/Nix*
443 TKO MEFs using the mt-Keima fluorescence assay. **K.** Quantification of mt-Keima data shown in
444 I., J. ****p<0.0001, ***p<0.001, **p<0.01, *p<0.05, ns = not significant, Ordinary one-way ANOVA.
445 Error bars represent standard deviation; data points represent individual biological replicates.
446



448 **Figure 2: BNIP3 and NIX have decreased turnover rates and are less responsive to proteasomal**
449 *inhibition in PPTC7 KO cells. A.* Schematic of DFO treatment and washout timeline and
450 mechanism. **B.** Western blot of endogenous BNIP3 protein after indicated times of DFO treatment
451 and washout, when applicable. “D” indicates dimer species; “M” indicates monomer species. Actin
452 shown as a loading control. **C.** Western blot of endogenous BNIP3 protein after indicated times
453 of DFO treatment and washout in wild-type (left panel) and *PPTC7* KO (right panel) 293T cells.
454 “D” indicates dimer species; “M” indicates monomer species. Actin shown as loading control. **D.**
455 Quantification of data shown in C. BNIP3 monomer (left graph) or dimer (right graph) bands were
456 quantified using densitometry, averaged, and plotted over time. Data were fit with a one phase
457 decay model to calculate protein half-lives ($T_{1/2}$), which are shown below each graph. Error bars
458 represent standard deviations of normalized densitometry across three independent experiments.
459 **E.** Western blot of endogenous NIX protein after indicated times of DFO treatment and washout
460 in wild-type (left panel) and *PPTC7* KO (right panel) 293T cells. “D” indicates dimer species; “M”
461 indicates monomer species. Actin shown as loading control. **F.** Quantification of data shown in
462 E. NIX monomer (left graph) or dimer (right graph) bands were quantified using densitometry,
463 averaged, and plotted over time. Data were fit with a one phase decay model to calculate protein
464 half-lives ($T_{1/2}$), which are shown below each graph. Error bars represent standard deviations of
465 normalized densitometry across three independent experiments. **G.** Western blots of endogenous
466 BNIP3 (top panel) and NIX (bottom panel) in wild-type and *PPTC7* KO 293T cells upon treatment
467 with 10 μ M MG-132 for the indicated timeframes. “D” indicates dimer species; “M” indicates
468 monomer species. Actin shown as a loading control. **H.** Quantification of BNIP3 and NIX monomer
469 (left graphs) and dimer (right graphs) populations in wild-type (gray) and *PPTC7* KO (pink) cells
470 shown in G. Bands were quantified using densitometry, averaged, and plotted over time. Data
471 were analyzed via linear regression, and significance between slopes was calculated using
472 Analysis of Covariance (ANCOVA). **I.** Western blot of endogenous BNIP3 and NIX proteins in
473 wild-type and *PPTC7* KO cells after DFO treatment and subsequent washout in the presence or
474 absence of 10 μ M MG-132. “D” indicates dimer species; “M” indicates monomer species. Actin
475 shown as a loading control.
476

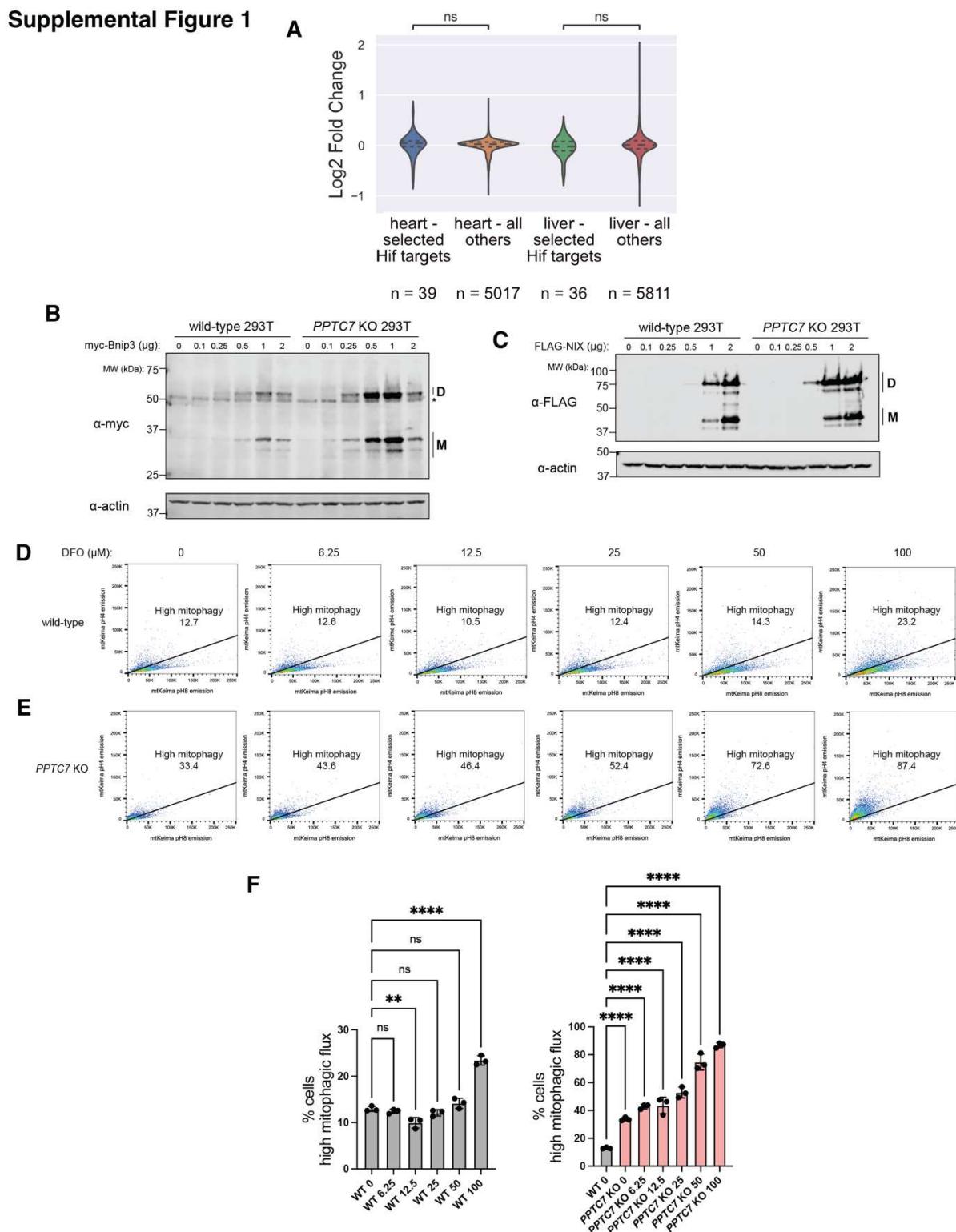


478 **Figure 3: PPTC7 requires an intact catalytic motif but not mitochondrial targeting to suppress**
479 **BNIP3 and NIX accumulation. A.** Representative single plane confocal images of GFP only (top
480 panels) or PPTC7-GFP (bottom panels) expressed in HeLa cells treated for 12 hours with 500
481 μ M CoCl₂. Cells were stained for BNIP3 (second column) or TOMM20 (third column). Overlays
482 are shown for GFP and TOMM20 (fourth column) and GFP and BNIP3 (fifth column). Scale bar
483 = 20 μ m. **B.** Quantification of data shown in A, mitochondrial BNIP3 staining versus non-specific
484 staining in cells overexpressing GFP only or PPTC7-GFP versus matched untransfected controls
485 for each experiment. Error bars represent standard error of the mean of three independent
486 experiments. **C.** Quantification of mt-Keima positive mitophagic flux in HeLa FLP-IN TRex cells
487 expressing vector only (left) or PPTC7-FLAG in the presence of 10 μ M doxycycline (dox, to
488 promote PPTC7 expression), 200 μ M cobalt chloride (CoCl₂), or both. **p<0.01, ns = not
489 significant, ordinary one-way ANOVA. Error bars represent standard deviation. Each dot
490 represents an independent biological replicate. **D.** Schematic of PPTC7 features, including a
491 mitochondrial targeting sequence (MTS) and PP2C phosphatase domain, top. Bottom,
492 AlphaFold2 representation of PPTC7 structure; predicted disordered N-terminus and PP2C
493 phosphatase domains indicated. **E.** Western blot of 293T cells overexpressing PPTC7 or a Δ MTS-
494 PPTC7 mutant. Red arrows indicate dual species in wild-type PPTC7 expression. * represents a
495 non-specific band. Actin shown as a loading control. **F.-H.** Western blots of BNIP3, NIX, and
496 PPTC7 depicting the ability of various mutants of PPTC7 to suppress BNIP3 and NIX
497 accumulation in response to CoCl₂ treatment. “D” indicates dimer species, “M” indicates
498 monomer species. In F, a catalytic mutant of PPTC7, D78A, cannot effectively suppress BNIP3
499 and NIX accumulation relative to wild-type PPTC7. In G, the Δ MTS-PPTC7 mutant partially or
500 fully suppresses BNIP3 and NIX accumulation, respectively. In H, a mutant that artificially anchors
501 PPTC7 to the outer mitochondrial membrane, Δ MTS-PPTC7-OMP25, suppresses BNIP3 and NIX
502 accumulation. Actin shown as a loading control.
503



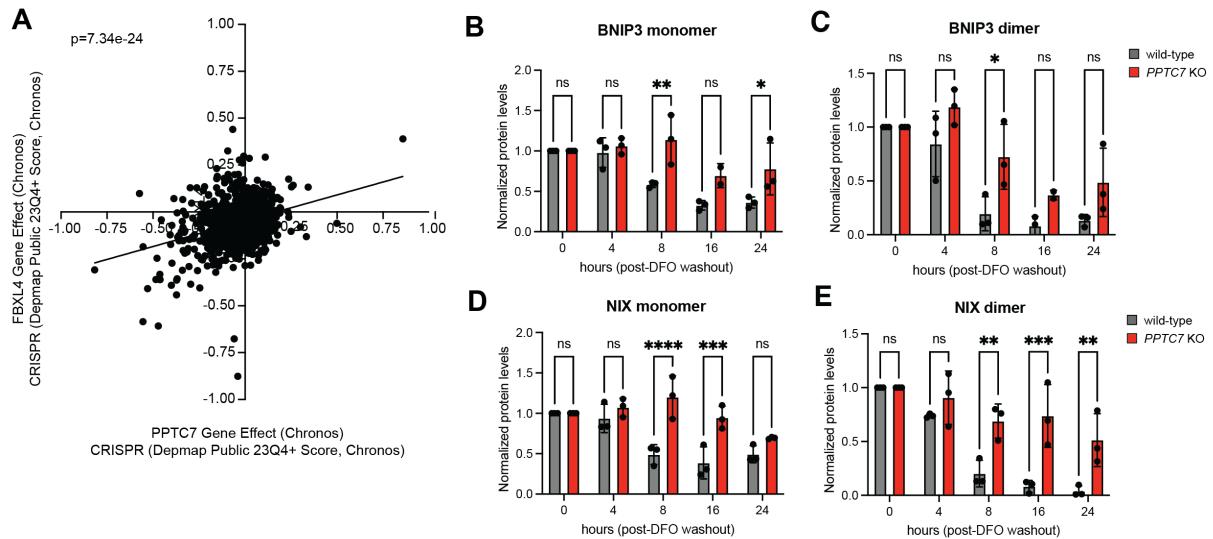
505 **Figure 4: PPTC7 proximally and dynamically interacts with BNIP3 and NIX in cells. A.** Proximity
506 labeling of PPTC7-V5-miniTurbo in 293T cells with or without 24-hour deferoxamine (DFO)
507 treatment. PPTC7-V5-miniTurbo, as well as vector only or V5-miniTurbo only controls, were
508 transfected into 293T cells. Streptavidin pulldowns were used to enrich for PPTC7-V5-miniTurbo
509 interactors, which were run on SDS-PAGE gels and western blotted for BNIP3 (top blot) or NIX
510 (2nd blot). Only PPTC7-V5-miniTurbo + biotin samples pulled down BNIP3 and NIX (lanes 6 and
511 12, streptavidin pull down gels), indicating specific binding. Western blots shown for reaction input
512 for pulldowns for V5 (showing miniTurbo constructs), BNIP3, NIX, and actin (serving as a load
513 control). **B.** Proximity labeling of PPTC7-V5-miniTurbo in 293T cells with after 24-hour DFO
514 treatment with or without 4 hour DFO washout. Streptavidin pulldowns were used to enrich for
515 PPTC7-V5-miniTurbo interactors as described in A. Western blots shown for reaction input for
516 pulldowns as described in A. **C.** A representative maximum z-projection confocal image (left) and
517 corresponding single plane insets (right) are shown of a U2OS cell overexpressing PPTC7-GFP
518 and treated with deferiprone (DFP) for 24 hours. Cells were fixed and stained for BNIP3 and
519 TOMM20 to visualize co-enrichment of PPTC7 with BNIP3-enriched foci (n=449). **D.** As in C for
520 a cells treated for 24h with DFP and washed for an additional 4h prior to fixation. Cells were
521 stained to visualize co-enrichment of PPTC7 with BNIP3-enriched foci (n=526).
522

Supplemental Figure 1



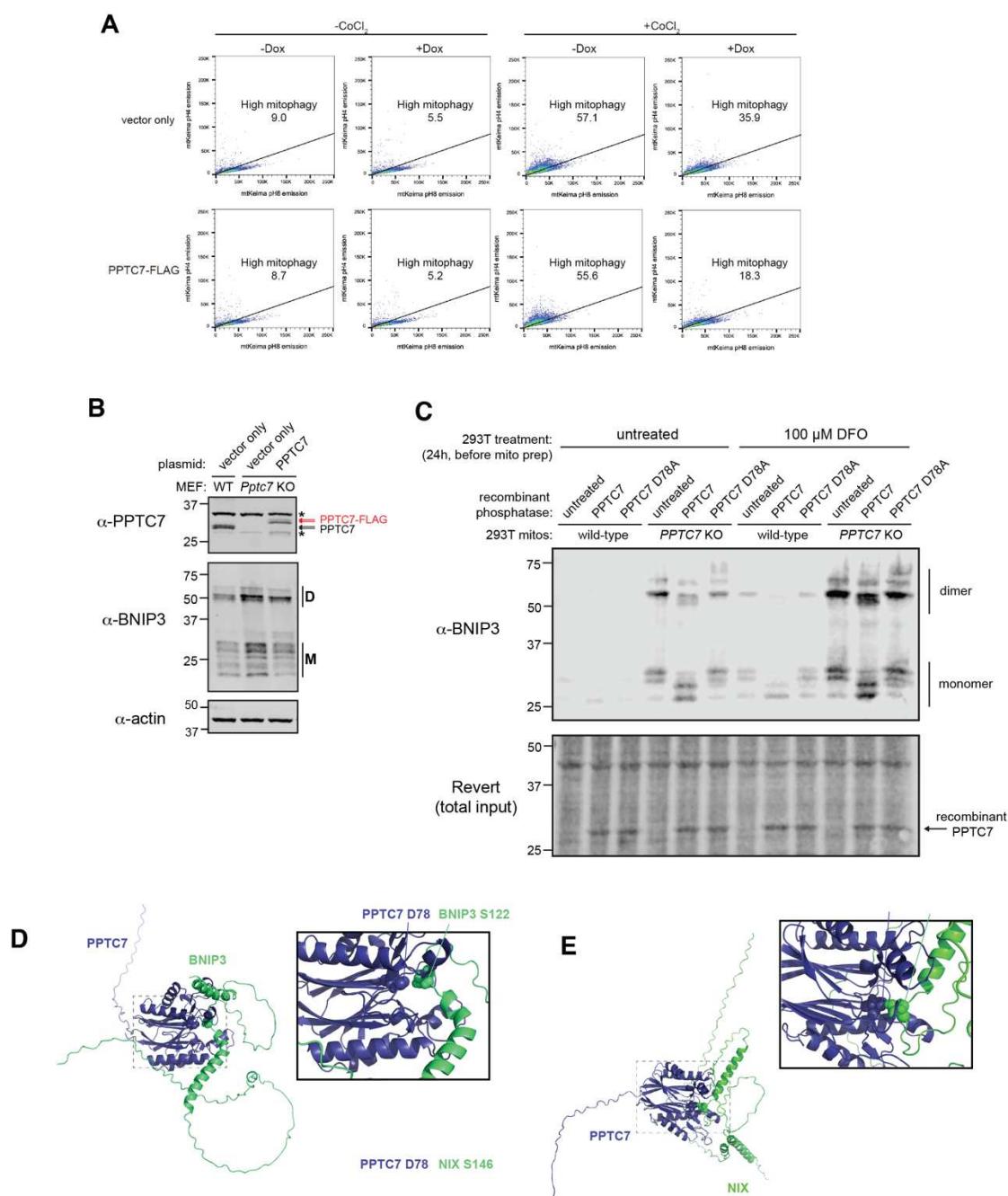
524 **Supplemental Figure 1. A.** Analysis of select HIF responsive targets in *Pptc7* KO heart and liver
525 proteomics datasets collected in Niemi et al., Nat Commun, 2019. **B.,C.** Western blots of
526 exogenous BNIP3 (B) or NIX (C) expressed in wild-type of *PPTC7* KO 293T cells at various
527 plasmid concentrations. “D” indicates dimer species, “M” indicates monomer species. Actin shown
528 as a loading control. **D., E.** Histograms of FACS analysis of mt-Keima positive wild-type (D) and
529 *Pptc7* KO (E) MEFs showing high mitophagy rates in response to variable DFO concentrations.
530 **F.** Statistical analysis of data shown in D., E., grey bars = wild-type samples, pink bars = *Pptc7*
531 KO samples. ****p<0.0001, ***p<0.001, **p<0.01, ns = not significant, ordinary one-way ANOVA.
532 Error bars represent standard deviation, data points represent individual biological replicates.

Supplemental Figure 2



534 **Supplemental Figure 2: A.** DepMap essentiality profiles of *PPTC7* gene effect (x-axis) and
535 *FBXL4* gene effect (y-axis). Linear regression analysis and associated p-value shown. **B.-E.**
536 Statistical analysis of BNIP3 and NIX monomer and dimer turnover rate data in wild-type (gray
537 bars) and *PPTC7* KO (red bars) 293T cells shown in Figure 2. ****p<0.0001, ***p<0.001,
538 **p<0.01, *p<0.05, ns = not significant, ordinary one-way ANOVA. Error bars represent standard
539 deviation, data points represent individual biological replicates.

Supplemental Figure 3



541 **Supplemental Figure 3: A.** FACS data shown for data represented in Figure 2D. Cells
542 undergoing high mitophagy are above the diagonal line; percentages indicated in figure. **B.**
543 Western blot of PPTC7 expressed in wild-type MEFs (black arrows), *Pptc7* KO MEFs, or *Pptc7*
544 KO MEFs rescued with human PPTC7 (red arrows). * represents a non-specific band. Basal
545 BNIP3 levels across samples shown below; actin shown as a loading control. **C.** Western blot of
546 BNIP3 in crude mitochondria isolated from wild-type or *PPTC7* KO 293T cells. Mitochondria were
547 left untreated, treated with recombinant PPTC7, or treated with recombinant PPTC7 D78A. Revert
548 stain is shown for loading; equal loading of recombinant proteins can be seen as depicted by
549 arrows. **D., E.** AlphaFold2 multimer predictions of the BNIP3-PPTC7 (**D.**) and the NIX-PPTC (**E.**)
550 interaction. Residues of BNIP3 and NIX proximal to PPTC7 D78 highlighted in zoom-in image.
551
552

553

554 Methods

555

556 Supplies and reagents

557

558 Deferoxamine (DFO) and cobalt chloride were purchased from MilliporeSigma (Burlington, MA).
559 MG132 and polyethylenimine (PEI) was purchased from Fisher Scientific (Hampton, NH).
560 Lipofectamine 3000 was purchased from ThermoFisher (Waltham, MA). Fugene 6 was purchased
561 from Promega (Madison, WI). Multiple plasmids were ordered or generated for this study,
562 including: myc-*MmBNIP3-FL* in pcDNA3.1, which was a kind gift from Joseph Gordon (Addgene
563 #100796) and V5-miniTurbo-NES in pcDNA3.1, which was a kind gift from Alice Ting (Addgene
564 #107170). pCMV-SPORT6-*MmNIX* was purchased from Horizon Discoveries. pcDNA3.1-PPTC7-
565 FLAG, pcDNA3.1-FLAG-*MmNIX*, pcDNA3.1-PPTC7-GFP, pcDNA3.1-PPTC7-OMP25-FLAG,
566 pcDNA3.1-PPTC7-V5-miniTurbo, and pcDNA3.1-ΔMTS-PPTC7-FLAG were generated for this
567 study through standard PCR and restriction-enzyme based cloning techniques. Plasmids for the
568 HeLa FLP-IN TREx system were generated using Gateway cloning. Briefly, constructs were PCR
569 amplified with primers containing attB1 or attB2 sequences. These fragments were incubated with
570 pDONR221 (a kind gift from Julia Pagan) and BP clonase for recombination. Positive constructs
571 were incubated with LR clonase and the pcDNA5/FRT/TO-Venus-Flag-Gateway destination
572 vector, which was a kind gift from Jonathon Pines (Addgene #40999). All cloned plasmids were
573 validated via Sanger sequencing.

574

575 Cell culture and Transfection

576

577 293T were acquired from the American Type Culture Collection (Manassas, VA). *PPTC7* KO 293T
578 cells were generated as previously described (Meyer *et al*, 2020) and MEF cells were generated
579 from wild-type and *Pptc7*^{−/−} mice embryos as previously described (Niemi *et al*, 2023). Wild-type,
580 *Pptc7* KO, and *Pptc7/Bnip3/Nix* TKO MEFs were transduced with mt-Keima as previously
581 described (Niemi *et al*, 2023). HeLa FLP-IN TREx cells stably expressing mt-Keima were a kind
582 gift from Dr. Julia Pagan. Cells were cultured in growth media (Delbecco's Modified Eagle Media
583 supplemented with 10% heat inactivated Fetal Bovine Serum and 1× penicillin/streptomycin).
584 Cells were grown in a temperature-controlled incubator at 37°C and 5% CO₂. Transient plasmid
585 transfection into 293T wild-type and *PPTC7* KO cells was performed with polyethylenimine (PEI)
586 for 24 to 48 hours. Transient plasmid transfection into U2OS and HeLa cells was performed with
587 Lipofectamine 3000 for 5 hours. Stable plasmid transfection into HeLa FLP-IN cells were
588 performed in the presence of pOG44 in a ratio of 0.5 µg pcDNA5 to 2 µg pOGG44. Transfections
589 were performed with Fugene 6 (Promega) per manufacturer's directions for 24-48 hours before
590 selection with 400 µg/mL hygromycin B.

591

592 SDS-PAGE and immunoblotting

593

594 Cells were lysed with radioimmunoprecipitation buffer (RIPA; 0.5% w/v sodium deoxycholate,
595 150mM sodium chloride, 1.0% v/v IGEPAL CA-630, 1.0% sodium dodecyl sulfate (SDS), 50mM
596 Tris pH8.0. 1mM EDTA pH8.0 in water) unless otherwise specified. After generating cell lysates,
597 samples were clarified by centrifugation (21,100×g) at 4°C for 10 minutes, snap frozen in liquid
598 nitrogen, and stored at -80°C before use. All samples were quantified with the bicinchoninic acid
599 (BCA) assay kit (Thermo Scientific). Lysates were mixed with 5x sample buffer (312 mM Tris-
600 Base, 25% w/v sucrose, 5% w/v SDS, 0.05% w/v bromophenol blue, 5% v/v β-mercaptoethanol,
601 pH6.8) and boiled at 95°C for 10 minutes. Lysates (20-40 µg) were run on SDS-PAGE gels with
602 Precision All-Blue Protein Standards (BioRad) before being transferred onto nitrocellulose
603 membranes. Membranes were incubated with primary antibodies with 2% BSA or 3% nonfat diary
604 milk in TBS-T for periods as indicated above. Primary antibodies used in immunoblotting include:
605 anti-human BNIP3 (Cell Signaling Technology (CST) catalog #44060, dilution 1:1000, 48 hour
606 incubation at 4°C), anti-rodent BNIP3 (CST catalog #3769, dilution 1:1000, 48 hour incubation at

604 4°C), anti-NIX (CST catalog #12396, dilution 1:1000, 48 hour incubation at 4°C), anti-PPTC7
605 (Novus catalog #NBP190654, dilution 1:1000, 48 hour incubation at 4°C), anti-HIF1α (CST
606 catalog #36169, dilution 1:1000, overnight incubation at 4°C), anti-β-actin (CST catalog #3700,
607 dilution 1:1000; CST catalog #4970, dilution 1:1000; and Abcam catalog #ab170325, dilution
608 1:1000; overnight incubation at 4°C), anti-FLAG (Sigma catalog #F1804, dilution 1:2000,
609 overnight incubation at 4°C), anti-V5 (Fisher catalog #PIMA515253, dilution 1:1000, overnight
610 incubation at 4°C), and anti-myc (Fisher catalog #MA121316, dilution 1:1000, overnight
611 incubation at 4°C). Membranes were washed 2-3x with TBS-T for 5 minutes per wash and
612 incubated with corresponding fluorophore-conjugated antibodies for 30 minutes at room
613 temperature. Anti-680 or anti-800 conjugated mouse or rabbit antibodies (LiCOR) were used for
614 detection. Membranes were then washed 2-3x with TBS-T for 5 minutes per wash and were
615 imaged with a LiCOR OdysseyFC instrument using Image Studio software (LiCOR; version 5.2).
616

617 *RNA extraction and qRT-PCR*

618 For RNA extraction, the collected cell pellets were processed using Monarch RNA extraction kit
619 (New England BioLabs). The RNA samples were then quantified with nanodrop and normalized
620 before cDNA synthesis (Lambda Biotechnologies). Quantitative PCR analyses with SYBR Green
621 PCR Master Mix (Applied Biosystems) were done on a BioRad CFX-96 Touch Real-Time PCR
622 Detection System controlled by CFX Maestro (ver2.2) on a computer. Primers used were: GAPDH
623 forward 5'-TTCGCTCTGCTCCTGTT-3', GAPDH reverse 5'-
624 GCCCAATACGACCAATCCGTTGA-3', BNIP3 forward 5'-GCCCACCTCGCTCGCAGACAC-3',
625 BNIP3 reverse 5'-CAATCCGATGCCAGCAAATGAGA-3', NIX forward 5'-
626 CTACCCATGAACAGCAGCAA-3', and NIX reverse 5'-ATCTGCCATCTTCTGTGG-3'.
627

628 *Flow cytometry*

629 For all experiments, wild-type MEFs or HeLa cells (i.e., with no mt-Keima expressed) were used
630 as a negative control and were plated at the same density as other cells in the corresponding
631 experiment. Cells were grown in standard DMEM media (25 mM glucose, 2 mM glutamine, 10%
632 heat-inactivated FBS, 1x penicillin/streptomycin) for 48 hours. Cells were harvested by
633 trypsinization and were resuspended in FluoroBrite media with 0.8% heat-inactivated FBS in 5mL
634 polystyrene round-bottom tubes (Falcon) immediately prior to the flow cytometry experiments.
635 The LSR-Fortessa (BD Biosciences) flow cytometer was used for the experiment, and the flow
636 cytometer was controlled by BD FACSDiva (version 9.0). FSC (488 nm), SSC (excitation 488nm,
637 emission 488 nm), QDot 605 (excitation 405nm, emission 610 nm), and PE-TexasRed (excitation
638 585 nm, emission 610 nm). The laser intensities for Qdot 605 and PE-TexasRed were changed
639 based on the emission profile of MEF wild-type cells for each experiment and were kept constant
640 throughout the experiment. Cells were gated to select for live cells, single cells, and mt-Keima
641 positive cells sequentially. Once gates were established in a given experiment, they were used
642 for the duration of that experiment. Flow cytometry data were processed with FlowJo (version
643 10.2.2), and the high mitophagy gate was drawn during analysis.
644

645 *Immunofluorescence assay and confocal microscopy*

646 For immunofluorescence assay, cells grown on glass-bottom cover dishes (CellVis) were fixed in
647 4% paraformaldehyde solution in PBS (15 minutes, room temperature). Fixed cells were
648 permeabilized (0.1% Triton X-100 in PBS), blocked (10% FBS and 0.1% Triton X-100 in PBS),
649 and then incubated overnight at 4°C with the indicated primary antibodies in blocking buffer.
650 Primary antibodies used in immunofluorescence assays include anti-TOMM20 (Abcam catalog
651 #56783, dilution 1:400) and anti-human BNIP3 (CST catalog #44060, dilution 1:100). After three
652 washes (5 minutes each) in PBS, cells were incubated with secondary antibodies in blocking
653 buffer for 30 min. The secondary antibodies used in immunofluorescence assays include donkey

654 anti-mouse Alexa Fluor 647 (Fisher catalog #A-31571, dilution 1:400) and donkey anti-rabbit
655 Alexa Fluor 555 (Fisher catalog #A-31572, dilution 1:400). Cells were subsequently washed three
656 times in PBS prior to imaging. Images were acquired on a Nikon Ti2 microscope equipped with
657 Yokogawa CSU-W1 spinning disk confocal and SoRa modules, a Hamamatsu Orca-Fusion
658 sCMOS camera and a Nikon 60x objective (for Figure 3A-B) or Nikon 100x 1.45 NA (for Figure
659 4A-B) objective. All images were acquired using a 0.2- μ m step size with the spinning disk module,
660 and image adjustments were made with ImageJ/Fiji.

661

662 *DFO-induced iron chelation and washout*

663 For immunoblotting experiments to visualize changes of BNIP3 and NIX protein levels upon iron
664 chelation, 293T wild-type and *PPTC7* KO cells were treated with 100 μ M DFO for 0-24 hours as
665 indicated in the results section. After the indicated incubation time, the cells were harvested by
666 cell scraping in phosphate-buffered saline (PBS) before cell lysis and immunoblotting analysis.

667

668 For qRT-PCR experiments to quantify changes in *BNIP3* and *NIX* transcript level, 293T WT and
669 *PPTC7* KO cells were treated with 100 μ M DFO for 24 hours, and the cells were harvested by cell
670 scraping in PBS before RNA extracton and qRT-PCR analysis.

671

672 For DFO washout experiments, after treating 293T WT and *PPTC7* KO cells with 100 μ M DFO for
673 24 hours, cells were washed with PBS and was switched to fresh DMEM media (for Figure 2B-F)
674 or with DMEM containing 0.1% EtOH (as vehicle control) or 10 μ M MG132 (for Figure 2I) for 0-
675 24 hours as indicated in the results section. After the indicated incubation time, the cells were
676 harvested by cell scraping in phosphate-buffered saline (PBS) before cell lysis and
677 immunoblotting analysis.

678

679 For flow cytometry experiments to determine the change of mitophagic flux with DFO dosage,
680 MEF mt-Keima wild-type and *Pptc7* KO cells were treated with 0-100 μ M DFO as indicated in the
681 results section for 24 hours before downstream processing for flow cytometry. To understand
682 whether the DFO-induced mitophagy is BNIP3- and NIX-mediated, MEF mt-Keima wild-type,
683 *Pptc7* KO, and *Pptc7/Bnip3/Nix* TKO cells were treated with 100 μ M DFO for 24 hours before
684 downstream processing for flow cytometry.

685

686 *MG132 proteasomal inhibition time course*

687 For MG132 proteasomal inhibition time course experiment done in Figure 2G-H, 293T WT and
688 *PPTC7* KO cells were treated with 0.1% EtOH (as vehicle control) or 10 μ M MG132 for 0-6 hours
689 as indicated in the results section. After the indicated incubation time, the cells were harvested
690 by cell scraping in PBS before cell lysis and immunoblotting analysis.

691

692 *Analysis of PPTC7 localization relative to BNIP3*

693 U2OS cells were transiently transfected with PPTC7-GFP plasmid. Cells were then passaged into
694 glass-bottom dishes. Cells were allowed to adhere for 12 hours and subsequently treated with
695 freshly prepared DFP (1 mM; Sigma-Aldrich) for 24 hours prior to fixation. Where indicated, cells
696 were then washed with fresh media, and fixed after an additional 4 hours of incubation.
697 Immunofluorescence staining and confocal microscopy were then performed on cells. To
698 determine the co-enrichment of PPTC7 with BNIP3, enlarged foci enriched for BNIP3 signal
699 relative to TOMM20 were manually counted in Fiji by examining single plane images throughout
700 z-series of individual cells blinded to the corresponding PPTC7 image, followed by assessment
701 of whether PPTC7 was co-enriched.

702

703 *Analysis of the effect of PPTC7 overexpression on BNIP3 immunofluorescence*

704 For analysis of BNIP3 staining by immunofluorescence, HeLa cells were transiently transfected
705 with cytosolic GFP or PPTC7-GFP plasmids. Cells were allowed to adhere to glass-bottom dishes
706 for 12 hours and treated with freshly prepared CoCl₂ (500 μM; Sigma-Aldrich) 12 hours prior to
707 fixation. Cells were imaged with identical imaging conditions between experimental replicates.
708 Cells that had GFP signal above an arbitrary threshold that was consistently maintained between
709 experiments were blindly identified and manually categorized as having mitochondrial or diffuse
710 non-mitochondrial BNIP3 signal in Fiji by examining maximum z-projections.
711

712 *Analysis of the effect of PPTC7 mutant expression on BNIP3 and NIX total protein levels*
713 HeLa FLP-IN T-Rex cells expressing wild-type PPTC7 or various mutants (e.g., D78A) were
714 generated as described above. To induce expression of these constructs, cells were treated with
715 10 μM doxycycline for 24-32 hours, after which BNIP3 and NIX expression was induced by
716 treating cells with 200 μM CoCl₂ for 16 hours. Cells were collected, lysed in RIPA buffer, run via
717 SDS-PAGE and western blotted for each protein as described above (see “SDS-PAGE and
718 immunoblotting”).
719

720 *Proximity labeling*
721 Wild-type 293T cells were transiently transfected with pcDNA3.1-MCS (empty vector), pcDNA3.1-
722 V5-miniTurbo-NES, or pcDNA3.1-PPTC7-V5-miniTurbo before cells were treated with either
723 water or 100 μM DFO (supplemented in growth media) for another 24 hours. For labeling, DMSO
724 or 250 μM biotin were added to cells, and cells were incubated at 37°C for 30 minutes. Labeling
725 reactions were quenched by washing the cells three times with ice-cold PBS and incubating the
726 cells at 4°C. Cells were then harvested in ice cold PBS before lysed with RIPA, clarified, and
727 quantified via BCA. The lysates were then incubated with 80 μL streptavidin magnetic beads (New
728 England Biolabs) at 4°C overnight. The beads were then washed twice with RIPA buffer, once
729 with Wash Buffer 2 (500 mM sodium chloride, 0.1% w/v deoxycholate, 1% Triton X-100, 1 mM
730 EDTA, 50 mM HEPES, pH 7.5), once with Wash Buffer 3 (250 mM sodium chloride, 0.5% Triton
731 X-100, 0.5% w/v deoxycholate, 1 mM EDTA, 50 mM HEPES, pH 8.1), once with Wash Buffer 4
732 (150 mM sodium chloride, 50 mM HEPES, pH 7.4), and once with Wash buffer 5 (50 mM
733 ammonium bicarbonate in MS-grade water). The bound species were eluted by adding 20 μL 5x
734 sample buffer, boiling at 95°C for 10 minutes, vortexing for 10 minutes, and boiling at 95°C for 10
735 minutes. The samples were then run on SDS-PAGE gels, and immunoblotting was performed to
736 probe for BNIP3 and NIX.
737

738 *Data/statistical analysis and figure generation*
739

740 Statistical analysis was performed using Microsoft Excel and/or Prism software (GraphPad,
741 version 10). Supplemental Figure 1A was generated using Seaborn library on Python. A selected
742 list of HIF target genes were curated (Dengler *et al*, 2014) as mined from datasets in mouse *Pptc7*
743 KO heart and liver proteomics (Niemi *et al*, 2019). Figure 2C was generated using AlphaFold2.
744 Supplemental Figures 2C-D were generated using AlphaFold multimer mode; images were
745 generated using PyMOL (Version 2.5.2). Figure 3A was generated using BioRender with an
746 appropriate license. All figures were generated using Adobe Illustrator.

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