

1 Post-Transcriptional Control of Coenzyme Q 2 Biosynthesis Revealed by Transomic Analysis 3 of the RNA-Binding Protein Puf3p

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15 SUMMARY

16 Coenzyme Q (CoQ) is a redox active lipid required for mitochondrial oxidative phosphorylation (OxPhos).
17 How CoQ biosynthesis is coordinated with the biogenesis of OxPhos protein complexes is unclear. Here,
18 we show that the *Saccharomyces cerevisiae* RNA-binding protein (RBP) Puf3p directly regulates CoQ
19 biosynthesis. To establish the mechanism for this regulation, we employed a transomic strategy to identify
20 mRNAs that not only bind Puf3p, but also are regulated by Puf3p *in vivo*. The CoQ biosynthesis enzyme
21 Coq5p is a critical Puf3p target: Puf3p regulates the level of Coq5p and prevents its toxicity, thereby
22 enabling efficient CoQ production. In parallel, Puf3p represses a specific set of proteins involved in
23 mitochondrial protein import, translation, and OxPhos complex assembly — pathways essential to prime
24 mitochondrial biogenesis. Our data reveal a mechanism for post-transcriptionally coordinating CoQ
25 production with OxPhos biogenesis and, more broadly, demonstrate the power of transomics for defining
26 genuine targets of RBPs.

27 HIGHLIGHTS

- 28 • The RNA binding protein (RBP) Puf3p regulates coenzyme Q (CoQ) biosynthesis
- 29 • Transomic analysis of RNAs, proteins, lipids, and metabolites defines RBP targets
- 30 • Puf3p regulates the potentially toxic CoQ biosynthesis enzyme Coq5p
- 31 • Puf3p couples regulation of CoQ with a broader program for controlling mitochondria

33 INTRODUCTION

34 Mitochondria are complex organelles central to cellular metabolism, and their dysfunction is implicated in
35 over 150 human diseases (Vafai and Mootha, 2012). Alterations in metabolic demands induce adaptive
36 changes in mitochondrial mass and composition (Labbe et al., 2014), but precisely how these changes are
37 regulated is unclear. Transcriptional regulators of mitochondria have been identified (Kelly and Scarpulla,
38 2004), yet the roles of post-transcriptional regulators remain poorly defined.

39 Remodeling and biogenesis of mitochondria is uniquely complicated. It requires synchronized
40 expression of proteins encoded by both nuclear DNA and mitochondrial DNA (mtDNA) (Couvillion et al.,
41 2016), and coordinated assembly of protein complexes, such as those of oxidative phosphorylation
42 (OxPhos), with specific metabolites and lipids, such as coenzyme Q (CoQ). CoQ is a redox active lipid in
43 the electron transport chain whose deficiency causes numerous human diseases (Laredj et al., 2014). Its
44 biosynthesis likewise requires assembly of a multi-protein complex of enzymes (“complex Q”) and lipids in
45 the mitochondrial matrix (Floyd et al., 2016; He et al., 2014; Stefely et al., 2016b), and production of the
46 water-soluble CoQ head group precursor 4-hydroxybenzoate (4-HB) (Payet et al., 2016; Stefely et al.,
47 2016a). Thus, mitochondrial biogenesis demands integrated remodeling of the proteome, the metabolome,
48 and the lipidome. The mechanisms for this “transomic” regulation are largely uncharacterized.

49 As described in this report, analysis of our publicly available “Y3K” data set (Stefely et al., 2016a)
50 reveals an unexpected link between CoQ abundance and the *Saccharomyces cerevisiae* gene *puf3*. The
51 encoded protein, Puf3p, belongs to the conserved PUF family of RNA-binding proteins (RBPs), which play
52 key roles throughout Eukarya (Quenault et al., 2011; Spassov and Jurecic, 2003; Wickens et al., 2002).
53 Puf3p binds to mRNAs containing “Puf3p-binding elements” (PBEs), conforming to the consensus
54 UGUANAUUA (Gerber et al., 2004; Olivas and Parker, 2000; Zhu et al., 2009), and regulates their translation
55 through a variety of mechanisms (Houshmandi and Olivas, 2005; Jackson et al., 2004; Lee and Tu, 2015;
56 Rowe et al., 2014). mRNAs that bind Puf3p have been identified, but which of those mRNAs are genuine
57 “targets” – mRNAs whose activity *in vivo* is controlled by Puf3p – is an open question. The few established
58 targets have connections to OxPhos complexes (e.g., *cox17*) and the mitochondrial ribosome (Chatenay-
59 Lapointe and Shadel, 2011; Garcia-Rodriguez et al., 2007; Olivas and Parker, 2000). Accordingly, yeast
60 that lack *puf3* (“ $\Delta puf3$ yeast”) exhibit mitochondria-related phenotypes, including reduced respiratory
61 growth (Eliyahu et al., 2010; Gerber et al., 2004; Lee and Tu, 2015) and increased respiratory activity
62 during fermentation (Chatenay-Lapointe and Shadel, 2011). However, it is unclear whether Puf3p affects
63 CoQ directly, via control of a key CoQ biosynthesis pathway enzyme, or indirectly, by controlling a protein
64 that subsequently affects the pathway.

65 Here, we employ a transomic strategy that analyzes mRNAs, proteins, lipids, and metabolites to
66 define high-confidence Puf3p targets, and we demonstrate that Puf3p directly regulates CoQ biosynthesis.
67 In addition to defining a specific Puf3p target responsible for CoQ control—Coq5p—we reveal 90 additional

68 high-confidence direct targets of Puf3p, which provides insight into how CoQ production is coordinated
69 with additional OxPhos biogenesis pathways. The results also demonstrate the power of transomic
70 strategies for dissecting the molecular functions of RBPs, which have widespread roles in human health
71 and disease (Gerstberger et al., 2014).

72 RESULTS

73 Puf3p Regulates CoQ Biosynthesis

74 The Y3K data set (Stefely et al., 2016a) reveals $\Delta puf3$ yeast to be significantly ($p < 0.05$) deficient for CoQ
75 when cultured under fermentation conditions, but not under respiration conditions (**Figures 1A, 1B, S1A**,
76 and **S1B**). Notably, the early CoQ biosynthesis intermediate polyprenylhydroxybenzoate (PPHB) was
77 elevated, while the later intermediate demethoxy-CoQ (DMQ) was decreased (**Figure 1A**), suggesting a
78 defect in one of the steps in the CoQ pathway that depends on the CoQ biosynthetic complex (“complex
79 Q” or “CoQ-Synthome”; comprised of Coq3p–Coq9p) (**Figure 1B**). The accumulation of PPHB in $\Delta puf3$
80 yeast was the largest such change across all yeast strains in the Y3K study under fermentation growth
81 conditions (**Figure S1A**), further suggesting a functionally important link between Puf3p and the CoQ
82 pathway.

83 To test these observations, we overexpressed Puf3p in wild type (WT) or $\Delta puf3$ yeast and
84 examined CoQ pathway lipids. Overexpression of Puf3p in WT yeast suppressed production of PPHB and
85 PPAB (the aminated analog of PPHB)—striking effects because they are the inverse of those observed in
86 $\Delta puf3$ yeast (**Figures 1C** and **S1C–S1E**). Furthermore, plasmid expression of Puf3p recovered CoQ
87 biosynthesis in fermenting $\Delta puf3$ yeast (**Figure 1C**). Thus, Puf3p regulates CoQ biosynthesis. We sought
88 to understand how it does so.

89 We hypothesized that Puf3p directly controls an enzyme in the CoQ biosynthesis pathway by
90 binding to and regulating the mRNA encoding it. To explore candidate enzymes, we examined existing
91 data sets from recent studies that used genome-wide technologies to identify mRNAs bound by Puf3p
92 (Freeberg et al., 2013; Kershaw et al., 2015; Lapointe et al., 2015; Wilinski et al., 2017). In aggregate, they
93 reported an astounding 2,018 mRNA putative “targets” of Puf3p—nearly a third of the yeast transcriptome
94 (**Table S1**). Putative Puf3p targets include seven enzymes directly involved in CoQ biosynthesis: *coq1*,
95 *coq2*, *coq3*, *coq5*, *coq6*, *coq7*, and *coq8* (**Figure 1D**). However, given the extensive length of the Puf3p-
96 bound mRNA list, we suspected that Puf3p regulates only a fraction of its 2,018 putative targets *in vivo*—
97 both for this specific case of Puf3p-mediated regulation of CoQ (**Figure 1E**) and for Puf3p functions in
98 general.

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102 **Transomic Analyses Define Puf3p Targets**

103 To identify high-confidence Puf3p targets, we integrated data from five experimental methods across four
104 omic planes (**Figure 2A**). We first identified Puf3p target mRNAs using data from two independent
105 approaches: HITS-CLIP (Licatalosi et al., 2008), which uses UV-crosslinking and immunopurification of
106 RBP-RNA complexes, and RNA Tagging (Lapointe et al., 2015), which employs an RBP-poly(U)
107 polymerase fusion protein to covalently tag mRNAs bound *in vivo* with 3' uridines ("U-tags") (**Figure S2A**).
108 We focused on these datasets because they were generated in-house and rely on orthogonal strategies
109 to identify mRNAs bound by a protein. HITS-CLIP (Wilinski et al., 2017) and RNA Tagging (Lapointe et al.,
110 2015) co-identified 269 Puf3p-bound mRNAs, out of the 467 and 476 mRNAs identified via each technique
111 on its own, respectively (**Figure 2A**) (hypergeometric test, $p < 10^{-211}$). Separating Puf3p-bound mRNAs
112 into classes based on detection (I–IV, with class I representing the strongest detection) showed that co-
113 identified mRNAs were the most robustly detected mRNAs in each method alone (**Figures 2B, S2B**, and
114 **S2C**), and 42% of mRNAs identified via both methods were class I or II in each individual approach (**Figure**
115 **S2D**). Moreover, the 3' untranslated regions (UTRs) of mRNAs identified via both methods were strongly
116 enriched for sequences that conform to high-affinity PBEs (UGUAHUA), and a remarkable 81% also
117 contained an upstream (−1 or −2) cytosine, which is required for particularly high-affinity interactions and
118 regulation *in vivo* (Lapointe et al., 2015; Zhu et al., 2009) (**Figures 2C** and **S2E**). In contrast, RNAs unique
119 to one method had more degenerate PBEs, with much less frequent upstream cytosines.

120 Integration of HITS-CLIP and RNA Tagging data thus yielded a high-confidence list of co-identified
121 Puf3p-bound mRNAs, referred to here as "Puf3p target mRNAs" (**Figure 2A** and **Table S1**). Importantly,
122 our analyses identified the CoQ-related mRNAs *coq2*, *coq5*, and *coq6* as high-confidence Puf3p targets,
123 but not *coq1*, *coq3*, *coq7*, or *coq8*. In searching for the target responsible for the $\Delta puf3$ CoQ deficiency
124 (**Figure 1A**), the observation of elevated PPHB and decreased CoQ suggested a defect in a complex Q
125 dependent step (**Figure 1B**) and allowed us to narrow our focus to *coq5* and *coq6*. However, which of
126 these Puf3p-mRNA interactions ultimately leads to regulation *in vivo* is unclear from the transcriptomic
127 data alone.

128 To determine how Puf3p impacts the abundance of proteins encoded by its target mRNAs, we
129 integrated these mRNA data with the proteomics feature of the Y3K data set (Stefely et al., 2016a). We
130 identified protein abundance changes due to loss of Puf3p in two metabolic conditions. In fermentation
131 culture conditions, 160 significant proteome changes were observed in $\Delta puf3$ yeast ($p < 0.05$ and fold
132 change [FC] $> 25\%$) (**Figure S2F**). In contrast, only 24 such changes in protein abundance were observed
133 in respiration culture conditions. Thus, we primarily leveraged the fermentation proteomics data set to
134 reveal Puf3p functions. The more drastic protein changes observed in fermentation mirror those of CoQ
135 pathway lipids, which were likewise significantly ($p < 0.05$) altered only in fermentation (**Figures 1A** and
136 **S1B**).

137 Of the 165 proteins encoded by Puf3p target mRNAs detected in fermenting yeast, 91 (55%) were
138 significantly more abundant by at least 25% in $\Delta puf3$ yeast relative to WT yeast ($p < 0.05$) (**Figure 2D**).
139 We collectively refer to these proteins as “Puf3p cis target proteins” (“cis targets”) (**Figure 2A**), and they
140 include Coq5p but not Coq6p. The 91 cis targets account for a striking 57% (91/160) of significantly ($p <$
141 0.05) altered proteins in the $\Delta puf3$ proteome. In contrast, Puf3p-bound RNAs uniquely identified by a single
142 RNA method, especially those detected weakly, were much less likely to be upregulated (**Figures 2E** and
143 **S2G**). Thus, the combination of these distinct “omic” approaches identifies RNA-binding events likely to
144 have regulatory effects in the cell. Collectively, these analyses provide high-confidence Puf3p targets
145 across both the transcriptome and the proteome (**Figure 2A** and **Table S2**). They also demonstrate that
146 Puf3p directly regulates Coq5p, suggesting a mechanistic link between loss of Puf3p and dysregulation of
147 CoQ pathway lipids.

148

149 **Puf3p Binding to *coq5* mRNA Prevents Toxicity**

150 To test the idea that Puf3p-mediated regulation of Coq5p abundance is required for proper CoQ
151 production, we analyzed how Coq5p overexpression (i.e., overriding Puf3p regulation) impacted yeast
152 growth and the CoQ biosynthetic pathway. Noticeably reduced CoQ levels can be sufficient for supporting
153 respiratory yeast growth (Stefely et al., 2015), and alterations in CoQ biosynthesis can be difficult to track
154 with growth assays. Thus, to facilitate mechanistic studies, we overexpressed Coq5p from a plasmid with
155 a strong promoter to make associated phenotypes more readily observable. Overexpression of Coq5p in
156 WT yeast slowed fermentative growth and essentially eliminated respiratory growth (**Figures 3A** and **3B**).
157 However, overexpression of Coq8p or Coq9p, which are also complex Q members (Floyd et al., 2016; He
158 et al., 2014; Stefely et al., 2016b), did not inhibit yeast growth, supporting the hypothesis that regulation of
159 Coq5p is particularly important for complex Q function (**Figures 3A**, **3B**, and **S3A**). To examine whether
160 Coq5p overexpression inhibits respiratory yeast growth by disrupting CoQ production, we examined CoQ
161 pathway intermediates. Indeed, Coq5p overexpression in WT yeast recapitulated the CoQ-related
162 phenotypes of $\Delta puf3$ yeast — deficiency of DMQ and CoQ, and elevation of PPAB and PPHB (**Figures**
163 **3C**, **S3B**, and **S3C**). Similar to the observed growth effects, CoQ intermediates were most affected by
164 overexpression of Coq5p compared to other proteins.

165 Coq5p overexpression could be deleterious due to excessive Coq5p methyltransferase activity or
166 inappropriate Coq5p physical interactions. Overexpression of Coq5p with mutations to putative catalytic
167 residues (D148A or R201A) inhibited yeast growth (**Figure S3D**), which suggests inhibition occurs through
168 inappropriate physical interactions rather than excessive Coq5p enzyme activity. Furthermore,
169 overexpression of Coq5p lacking its mitochondrial targeting sequence (MTS) (Coq5p^{-MTS}) reduced its
170 inhibitory effect in respiration (**Figure S3E**), suggesting that overexpressed Coq5p has inappropriate
171 interactors in the mitochondrial matrix.

172 To further test the idea that Puf3p directly regulates Coq5p, we generated yeast strains with
173 genomic mutations to the 3' UTR of *coq5* (**Figure 3D**). Removal of the *coq5* 3' UTR or the site-specific
174 mutation of the Puf3p-binding element (PBE) in *coq5* at its endogenous genomic loci significantly reduced
175 yeast growth in both fermentation and respiration ($p < 0.05$) (**Figures 3E, 3F, and S3F**). These growth
176 phenotypes recapitulate those observed when Coq5p is overexpressed from a plasmid, but are notably
177 different from those of $\Delta coq5$ yeast, which grow normally in fermentation (**Figures 3E and 3F**). As a control,
178 disruption of two genes that flank *coq5* (*bul2* and *zds2*) had no discernible effect on yeast growth, therefore
179 ensuring that the growth defects we observed are specific to loss of the PBE in *coq5*.

180 Our collective findings demonstrate that Puf3p modulates CoQ biosynthesis by directly regulating
181 Coq5p, a potentially promiscuous protein with toxic effects when overexpressed (**Figures 3G and S3G**).
182 By validating Coq5p as a Puf3p target, our findings strongly suggest that the additional 90 cis target
183 proteins identified by our transomic analysis are also very likely to be *bona fide* Puf3p targets *in vivo*. They
184 thus provide a foundation for identifying additional pathways that Puf3p coordinates with CoQ biosynthesis.

185 **Puf3p Coordinates CoQ Production with Mitochondrial Biogenesis Functions**

186 Our transomic data set enabled us to map additional Puf3p functions across multiple omic planes (**Figure**
187 **2A**), similar to how we mapped Puf3p to its mRNA target *coq5*, its cis target protein Coq5p, and its
188 downstream (trans) effect on CoQ lipids. We used the cis Puf3p protein targets to identify downstream
189 “trans effects” in the proteome. By a protein “trans effect,” we refer to proteins whose abundance is
190 dependent on Puf3p, but whose mRNA does not bind Puf3p (see Methods for details). The $\Delta puf3$ proteome
191 changes include the 91 cis targets, which are enriched for mitochondrial organization and translation
192 functions, and 49 trans effects, enriched for mitochondrial OxPhos and electron transport chain (ETC)
193 functions (**Figures 4A and S4A and Tables S2 and S3**).

194 We quantitatively compared properties of Puf3p cis targets and trans effects to those of either all
195 proteins or mitochondrial proteins. The latter is an informative control set because Puf3p target mRNAs
196 are highly enriched for mitochondrial proteins (**Figure S4B**). Both cis targets and trans effect proteins have
197 relatively low abundances in fermentation (Stefely et al., 2016a), but not in respiration, reflecting Puf3p-
198 mediated repression in fermentation (**Figures 4B and S4C**). In parallel, cis targets are upregulated across
199 early time points in the diauxic shift (the metabolic shift from fermentation to respiration) (Stefely et al.,
200 2016b) (**Figure S4D**), and yeast with respiration deficient mitochondria, which cannot complete the diauxic
201 shift, exhibit downregulation of Puf3p cis targets as part of their “respiration deficiency response” (Stefely
202 et al., 2016a) (**Figure S4E**). Cis targets are enriched for proteins that are co-translationally targeted to
203 mitochondria (Williams et al., 2014) (**Figures 4C and S4F**). Furthermore, they are enriched for proteins
204 that are toxic when overexpressed (**Figure 4D**), suggesting that regulation of toxic protein expression—
205 such as that observed with Coq5p—is a general mechanism of Puf3p action. Together, our analyses
206 support a model where Puf3p mediates repression of a set of mitochondrial proteins in fermentation, and

207 this repression is released, or potentially reversed (i.e., cis targets are activated), early during the diauxic
208 shift to help initiate the requisite biological changes, such as mitochondrial biogenesis (**Figure 4E**).

209 To reveal the specific biochemical pathways through which Puf3p regulates mitochondrial function,
210 we mapped individual Puf3p cis targets and trans effects (**Figures 4F** and **S5A**). Strikingly, 86 of the 87
211 Puf3p cis targets with known functions fit into pathways that support mitochondrial biogenesis, and in
212 particular converge to generate the OxPhos machinery. The first pathway (i) includes proteins that catalyze
213 the import, folding, and processing of nuclear DNA-encoded mitochondrial proteins, which include many
214 OxPhos subunits. The second pathway (ii) includes proteins that support transcription and translation of
215 mtDNA-encoded genes, which also encode OxPhos subunits. For example, cis Puf3p targets include over
216 half of the mitochondrial ribosomal proteins and critical translational activators (e.g., Cbp3p, Cbp6p,
217 Mam33p, Mba1p, and Mdm38p) (**Figures 4F** and **S5A–S5C**). Puf3p trans effects included increased
218 abundance of two proteins encoded by mitochondrial DNA (Var1p and Cox2p, the only two such proteins
219 observed). The third pathway (iii) encompasses assembly factors for each OxPhos subunit, which provide
220 ancillary support for OxPhos complex biogenesis. Collectively, these three pathways of Puf3p cis targets
221 are poised to prime OxPhos biogenesis. Consistently, Puf3p trans effects observed in $\Delta puf3$ yeast include
222 increased abundance of OxPhos complex subunits (iv). Thus, Puf3p regulates production and assembly
223 of both proteins and lipids required for OxPhos.

224 Two additional pathways of Puf3p cis targets include mitochondrial membrane transporters for
225 nucleotides, which sustain mtDNA transcription, and proteins such as Acp1p that support the TCA cycle
226 (**Figure 4F**). Downstream, citrate synthase (Cit1p), the rate limiting gateway enzyme to the TCA cycle and
227 a putative client protein of the Puf3p cis targets Tom70p and Hsp60p (Martin et al., 1992; Yamamoto et
228 al., 2009), and Ptc7p, a phosphatase that reactivates phosphorylated Cit1p (Guo et al., 2017), were also
229 significantly increased trans effect proteins (**Figure S6A**). Accordingly, the abundance of citrate was
230 significantly elevated in $\Delta puf3$ yeast ($p < 0.05$) (**Figure S6B**).

231 The tight functional association of cis Puf3p targets in mitochondrial biogenesis pathways (**Figure**
232 **4F**) suggests that the four uncharacterized proteins that are cis Puf3p targets — Rdl2p, Ynr040w, Mpm1p,
233 and Fmp10p — might also function in mitochondrial biogenesis. Previous large-scale screens detected
234 these proteins in mitochondria (Inadome et al., 2001; Reinders et al., 2006; Sickmann et al., 2003; Vogtle
235 et al., 2012). Immunocytochemistry analyses of FLAG-tagged Rdl2p, Ynr040w, Mpm1p, and Fmp10p,
236 validated these observations (**Figures S7A** and **S7B**), thereby confirming their classification as
237 mitochondrial uncharacterized (x) proteins (MXPs). While the gene deletion strains for these MXPs are
238 respiration competent, their overexpression in WT yeast inhibited respiratory growth (**Figures S7C** and
239 **S7D**), suggesting that they may interact with proteins required for OxPhos and have the potential to be
240 toxic like Coq5p. Given these data, all but one (Ahp1p) of the 91 cis targets localize to mitochondria, further
241 solidifying the role of Puf3p in mitochondrial function. Together, this transomic analysis provides a high-

242 resolution map of the molecular targets of Puf3p that will serve as a powerful resource for investigations
243 of mitochondrial biogenesis and function (**Tables S1 and S2**).

244 **DISCUSSION**

245 **Defining Genuine Targets *via* Transomics**

246 Our findings show that transomic strategies are powerful in the analysis of RBP function. In our case,
247 despite considerable effort to identify the mRNAs to which Puf3p binds *in vivo*, it was unclear which of the
248 many binding events were biologically productive – a central, common challenge in the analysis of RBPs
249 (Konig et al., 2012; Licatalosi and Darnell, 2010; Riley and Steitz, 2013). To understand how Puf3p
250 controlled CoQ biosynthesis, including whether there is a direct effect on an mRNA in the CoQ pathway,
251 we generated a high-confidence Puf3p mRNA target list by combining two distinct approaches, and
252 integrated that data with proteomic, metabolomic, and lipidomic studies. This strategy pinpointed Coq5p
253 as the key Puf3p target from a large pool of candidates (> 2,000) that included six other CoQ biosynthesis
254 enzymes (**Figure S3G**). Our analyses yielded a comprehensive roadmap of specific biochemical
255 processes controlled by Puf3p *in vivo*. Together, our findings revealed a post-transcriptional mechanism
256 to coordinate CoQ biosynthesis with the broader mitochondrial biogenesis program.

257 A key first step toward defining biochemical functions of Puf3p was the distillation of large lists of
258 Puf3p-RNA interactions to a smaller set of those mRNAs it actually controls *in vivo* – its genuine targets *in*
259 *vivo*. The HITS-CLIP and RNA Tagging data sets overlapped by ≈50%, which was greatest among the
260 mRNAs detected most robustly in each separate approach. Weakly detected Puf3p-bound mRNAs were
261 unlikely to overlap and likely arise through intrinsic biases of each method. Thus, our analyses illustrate
262 that HITS-CLIP and RNA Tagging are complementary, and we demonstrate that integration of their data
263 yields high-confidence RNA targets. Indeed, these analyses rapidly narrowed our focus to *coq5* or *coq6*,
264 from the other complex Q candidates *coq3*, *coq8*, and *coq9*. Importantly, our findings also demonstrate
265 that RBP-mRNA interaction data sets are stratified: interactions that lead to biological regulation are
266 concentrated at the top of the rank order. In the absence of a transomic data set, a sharp focus on the top
267 tier of candidates has great potential to reveal genuine targets.

268 A second key step was the integration of the Puf3p mRNA target set with $\Delta puf3$ proteome
269 alterations. Our integration revealed 91 Puf3p-mRNA interactions that directly regulate the abundance of
270 the encoded protein *in vivo*. The regulated Puf3p targets included Coq5p but not Coq6p, which yet again
271 narrowed our focus and was critical to reveal how Puf3p controls CoQ biosynthesis. In principle, the
272 remaining 74 Puf3p target mRNAs with unaffected protein abundances could be false positives. However,
273 it is more likely that Puf3p regulates their localization (Gadir et al., 2011; Saint-Georges et al., 2008)
274 independent of their translation, or that the yeast culture systems we used lacked an environmental stress
275 encountered by yeast in nature. Notably, many of these mRNA targets trended toward increased protein

276 abundance (**Figure 2D**), but did not cross our significance threshold ($p < 0.05$). They provide an initial
277 foothold for analyzing alternative forms of Puf3p-mediated control (e.g., localization), or Puf3p function
278 under varying environmental or cellular conditions.

279 **Post-Transcriptional Control of CoQ Biosynthesis and Toxic Proteins**

280 Our results reveal a mechanism for post-transcriptional regulation of CoQ biosynthesis. By suppressing
281 CoQ biosynthesis under fermentation conditions, Puf3p may help direct early CoQ precursors such as
282 tyrosine and isoprene subunits to other biosynthetic pathways, such as protein synthesis and sterol
283 biosynthesis, respectively.

284 Our biochemical work further demonstrated that Puf3p directly regulates the abundance of Coq5p,
285 which when overexpressed dramatically reduced yeast growth and CoQ production. These findings
286 suggest that the CoQ biosynthetic complex can be disrupted when particular subunits are too abundant,
287 thereby highlighting the importance of stoichiometry for complex Q activity. Interestingly, however,
288 overexpression of complex Q members Coq8p or Coq9p lacked the same deleterious effects as those
289 observed with Coq5p, demonstrating that some complex Q subunits are particularly poisonous at
290 inappropriately high protein abundance. Together, our findings suggest that Puf3p plays an important role
291 in coordinating expression of complex Q components with the larger program of OxPhos biogenesis,
292 through precise control of potentially toxic proteins. Analysis of the full Puf3p cis target set further
293 suggested that regulation of poisonous proteins is a broader Puf3p function. This provides an evolutionary
294 rationale for why particular proteins within larger pathways are targeted for regulation by Puf3p.

295 **Puf3p Coordinates Proteins and Lipids in Mitochondrial Biogenesis**

296 By controlling the abundance of proteins that catalyze production of mitochondrial proteins, lipids, and
297 metabolites, Puf3p regulation reaches across three omic planes. Transomic coordination may be critical
298 for efficient production of functional mitochondria, for example by linking production of OxPhos protein
299 complexes and the OxPhos lipid CoQ.

300 A similar mechanism was recently proposed for nuclear- and mitochondrial DNA-encoded proteins
301 via synchronization of cytoplasmic and mitochondrial translation programs (Couvillion et al., 2016). That
302 study suggested a critical role for cytosolic translational control of mitochondrial translational activators in
303 coordinating OxPhos biogenesis and, in particular, identified the COB (complex III subunit) translational
304 activator Cbp6p as a component of the mechanism. Importantly, our work now demonstrates that Puf3p
305 directly regulates Cbp6p and its binding partner Cbp3p, both of which are cis Puf3p targets. Thus, we
306 propose that Puf3p is a key part of the molecular mechanism that coordinates mitochondrial and cytosolic
307 translation programs. Mammalian RBPs that impact mitochondrial function have been identified (Gao et
308 al., 2014; Schatton et al., 2017), but whether any of these provide Puf3p-like transomic regulation of
309 mitochondrial biogenesis is currently unknown.

310 **A Roadmap of Puf3p Functions**

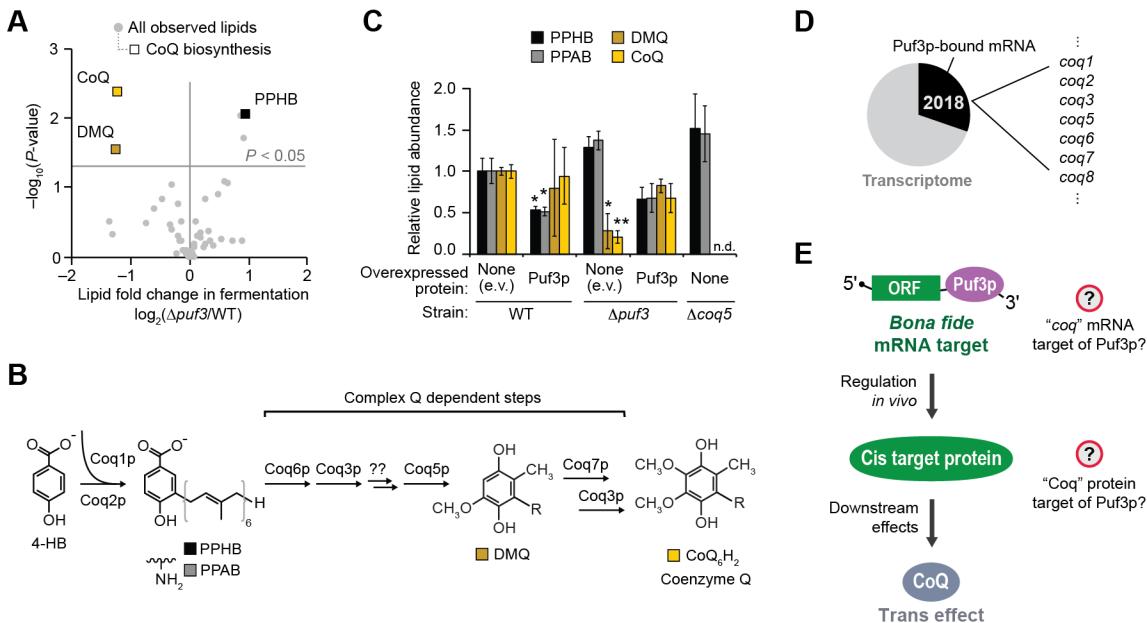
311 Our analyses provide substantial evidence that a primary function of Puf3p is to repress expression of
312 mitochondrial biogenesis factors in fermenting yeast, as opposed to Puf3p having many broad biological
313 functions (Kershaw et al., 2015). We identified 269 target mRNAs and 91 cis target proteins for Puf3p,
314 which can be separated into three pathways that converge to generate the OxPhos machinery. Our
315 strategy furthermore enabled us to map trans Puf3p effects across three omic planes, including protein
316 components of OxPhos complexes, TCA cycle metabolites, and mitochondrial lipids, such as CoQ. Trans
317 Puf3p effect proteins also include splicing factors and epigenetic regulators, and we speculate that $\Delta puf3$
318 yeast have “sensed” mitochondrial dysfunction through a mitochondria-to-nucleus retrograde signaling
319 pathway. Consistently, the Y3K proteomics data set (Stefely et al., 2016a) shows that $\Delta puf3$ yeast have
320 decreased levels of Ngg1p — a component of the SLIK complex, which regulates gene expression in
321 response to mitochondrial dysfunction (Pray-Grant et al., 2002).

322 We observed few effects in respiring $\Delta puf3$ yeast, which at first glance may seem to contradict a
323 recent study that reported Puf3p to activate translation of its mRNA targets as yeast enter the diauxic shift
324 (Lee and Tu, 2015). However, our cultures were harvested more than 5 hours after the diauxic shift, by
325 which time the activating function has disappeared (Lee and Tu, 2015). Indeed, our analyses of earlier
326 time points also suggest that Puf3p functions early in the diauxic shift (**Figure S4D**). Yeast that lack *puf3*
327 may only have mild phenotypes in respiration because transcriptional control compensates for the lack of
328 Puf3p-mediated regulation, or because other RBPs (e.g., Puf4p or Puf5p) compensate for its absence.
329 Regardless, Puf3p is a critical repressor of mitochondrial biogenesis in fermenting yeast — a function that
330 is conserved across more than 300 million years of evolution (Hogan et al., 2015; Wilinski et al., 2017).

331 The Puf3p mRNA targets that were not observed in our proteomics studies provide another
332 valuable resource (**Tables S1 and S2**). For example, they include previously uncharacterized genes (e.g.,
333 *aim11*, *aim18*, *ybr292c*, *ydr286c*, *ydr381c-a*, *ygr021w*, and *ygr161w-c*), which we can now potentially link
334 to roles in mitochondrial biogenesis given their identity as Puf3p target mRNAs. Thus, the pathways
335 mapped via transomics also provide a framework for retrospective analyses, which is particularly useful
336 since imperfect overlap between omic planes is a common feature of transomic studies (e.g., some
337 observed mRNAs are not detected at the protein level). Our high-confidence Puf3p cis target set provides
338 a snapshot of specific mitochondrial biogenesis pathways regulated by Puf3p (**Figure 4F**) that can guide
339 future analyses of the full set of Puf3p mRNA targets, which include additional OxPhos biogenesis factors.

340 Collectively, our work reveals post-transcriptional control of CoQ production linked into a larger
341 regulatory network that controls OxPhos biogenesis. Our findings also provide a foundation for further
342 exploring the molecular basis of mitochondrial biogenesis, including potential new roles for Puf3p-regulated
343 MXPs in OxPhos biogenesis. Moreover, our approach presents a generalizable transomic strategy to
344 identify biological roles for the many RNA-binding proteins that impact human health and disease.

345 **FIGURES**



346

347 **Figure 1. *puf3* Regulates Coenzyme Q Biosynthesis**

348 (A) Relative lipid abundances in $\Delta puf3$ yeast compared to WT (mean, $n = 3$) (fermentation condition) versus
349 statistical significance. Raw data from Y3K data set (Stefely et al., 2016a).

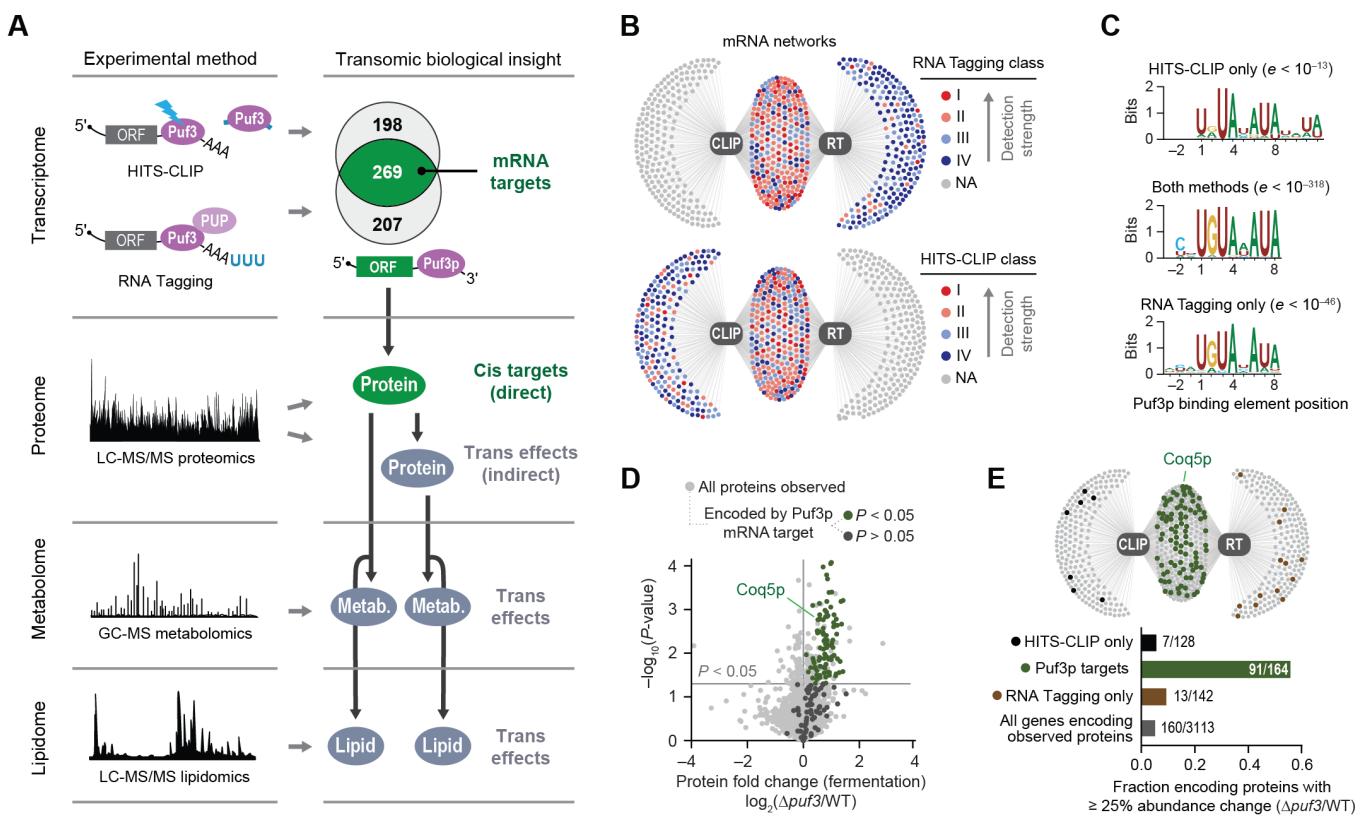
350 (B) Scheme of CoQ biosynthesis. 4-HB, 4-hydroxybenzoate; PPHB, polypropenylhydroxybenzoate; PPAB,
351 polypropenylaminobenzoate; DMQ, demethoxy-CoQ.

352 (C) Relative lipid abundances in yeast transformed with low copy plasmids overexpressing Puf3p (or empty
353 vector, e.v.) and cultured in fermentation media (mean \pm SD, $n = 3$). Bonferroni corrected * $p < 0.05$; ** $p <$
354 0.01. Two-sided Student's *t*-test for all panels.

355 (D) Pie chart illustrating the fraction of transcribed genes with mRNAs that are putative Puf3p targets,
356 which was derived by aggregating all Puf3p-bound mRNAs reported via HITS-CLIP (Wilinski et al., 2017),
357 RNA Tagging (Lapointe et al., 2015), PAR-CLIP (Freeberg et al., 2013), and RIP-seq (Kershaw et al.,
358 2015). Puf3p-bound mRNAs include 7 mRNAs encoding CoQ biosynthesis enzymes.

359 (E) Scheme of how Puf3p could impact CoQ production.

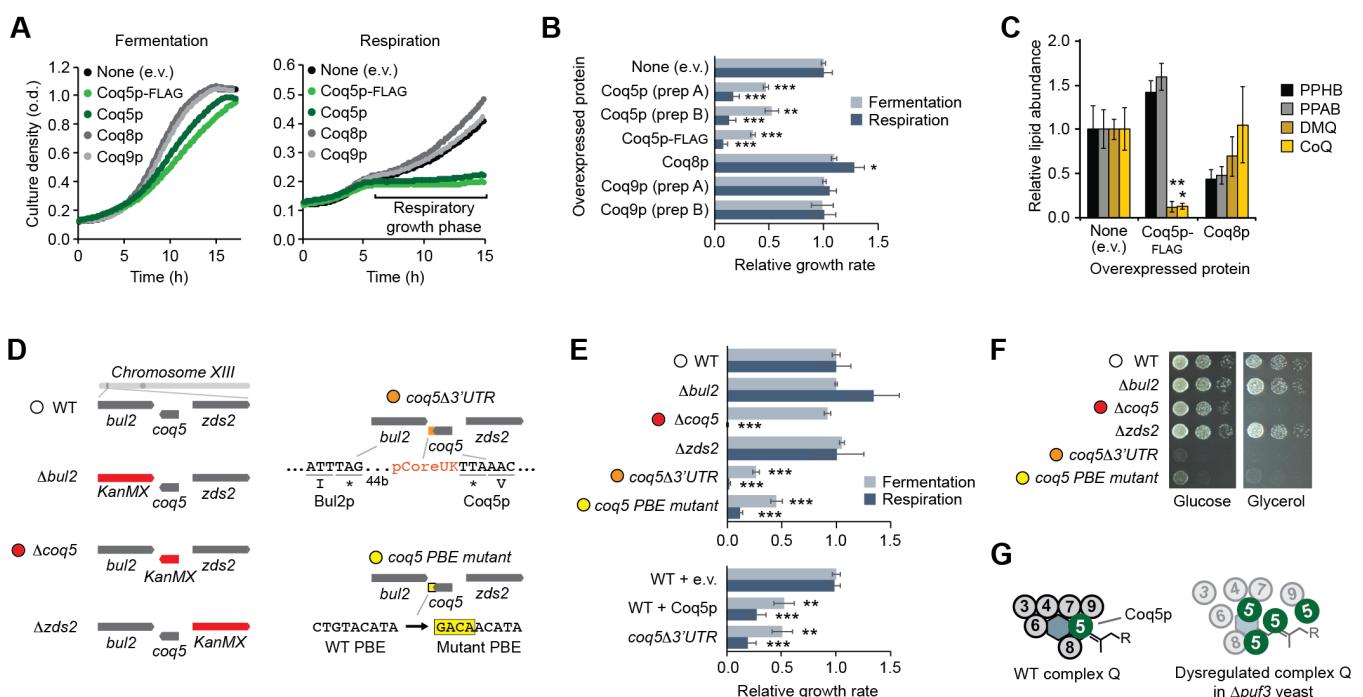
360



361

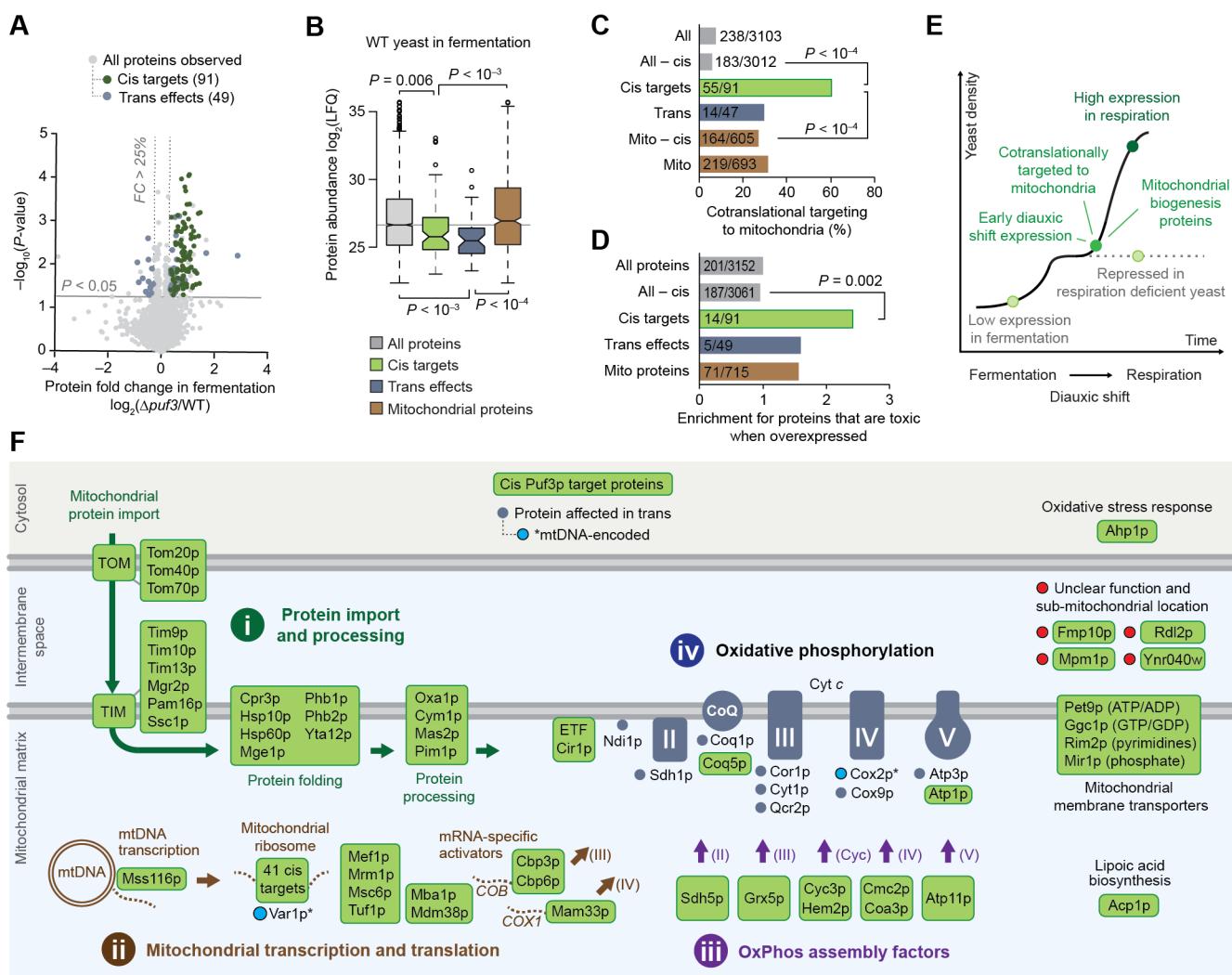
362 Figure 2. Transomics Maps High-Confidence Puf3p Targets

363 (A) Transomics approach overview. The Venn diagram indicates Puf3p-bound mRNAs co-identified by
364 RNA Tagging and HITS-CLIP.
365 (B) Network maps of Puf3p-bound mRNAs (dots) detected by RNA Tagging (RT) and/or HITS-CLIP (CLIP)
366 (edges).
367 (C) Enriched Puf3p-binding elements identified by MEME for the indicated groups of Puf3p-bound RNAs.
368 (D) Relative protein abundances in $\Delta puf3$ yeast compared to WT (mean, n = 3) versus statistical
369 significance (fermentation condition), highlighting proteins encoded by Puf3p mRNA targets. P-value cutoff
370 is for protein abundance changes.
371 (E) Bar graph and network map of Puf3p-bound mRNAs indicating mRNAs encoding proteins detected in
372 the Y3K proteomics data set with $\geq 25\%$ protein abundance change ($p < 0.05$, two-sided Student's *t*-test).
373 This figure includes new, integrated analyses of publicly available raw data from the RT (Lapointe et al.,
374 2015), HITS-CLIP (Wilinski et al., 2017), and Y3K multi-omic (Stefely et al., 2016a) data sets generated in
375 our labs.



377

378 **Figure 3. Puf3p Regulates the Potentially Poisonous CoQ Biosynthesis Enzyme Coq5p**
379 (A) Growth curves for WT yeast transformed with plasmids overexpressing the proteins shown and cultured
380 in either fermentation or respiration media.
381 (B) Relative growth rates of WT yeast transformed with plasmids overexpressing the proteins shown and
382 cultured in either fermentation or respiration media (mean \pm SD, n = 3). **p < 0.01; ***p < 0.001.
383 (C) Relative lipid abundances in WT yeast transformed with plasmids overexpressing the proteins shown
384 and cultured in respiration media (mean \pm SD, n = 3). Bonferroni corrected *p < 0.05; **p < 0.01.
385 (D) Scheme of genetic alterations in the yeast strains used in (E) and (F).
386 (E) Yeast strain growth rates in either fermentation or respiration media (mean \pm SD, n = 3). **p < 0.01;
387 ***p < 0.001.
388 (F) Serial dilutions of yeast strains cultured on solid media containing either glucose or glycerol.
389 (G) Model for how Coq5p overexpression dysregulates complex Q.
390 Two-sided Student's *t*-test for all panels.
391



392

393 Figure 4. Puf3p Targets Prime Biogenesis of Mitochondria and OxPhos

394 (A) Relative protein abundances in $\Delta puf3$ yeast compared to WT (mean, $n = 3$) versus statistical 395 significance, highlighting proteins with fold change (FC) $> 25\%$ and $p < 0.05$ (two-sided Student's t -test) 396 that were either identified as Puf3p targets by both RNA methods (cis targets, green) or neither RNA 397 method (trans effects, blue).

398 (B) Protein abundances (Stefely et al., 2016a) for each group of proteins shown. LFQ, label free 399 quantitation value. Center lines indicate medians, limits indicate 25th and 75th percentiles, whiskers extend 400 1.5 times the interquartile range, outliers are represented by dots, and P -values were determined with a 401 Student's t -test (two-tailed, homostatic). Protein set sizes: all proteins $n = 3152$, mitochondrial proteins $n = 715$, cis targets $n = 91$, trans effect proteins $n = 49$.

402 (C) Percent of each group of proteins shown that is cotranslationally targeted to mitochondria (Williams et 403 al., 2014). P -values determined by a Fisher's exact test.

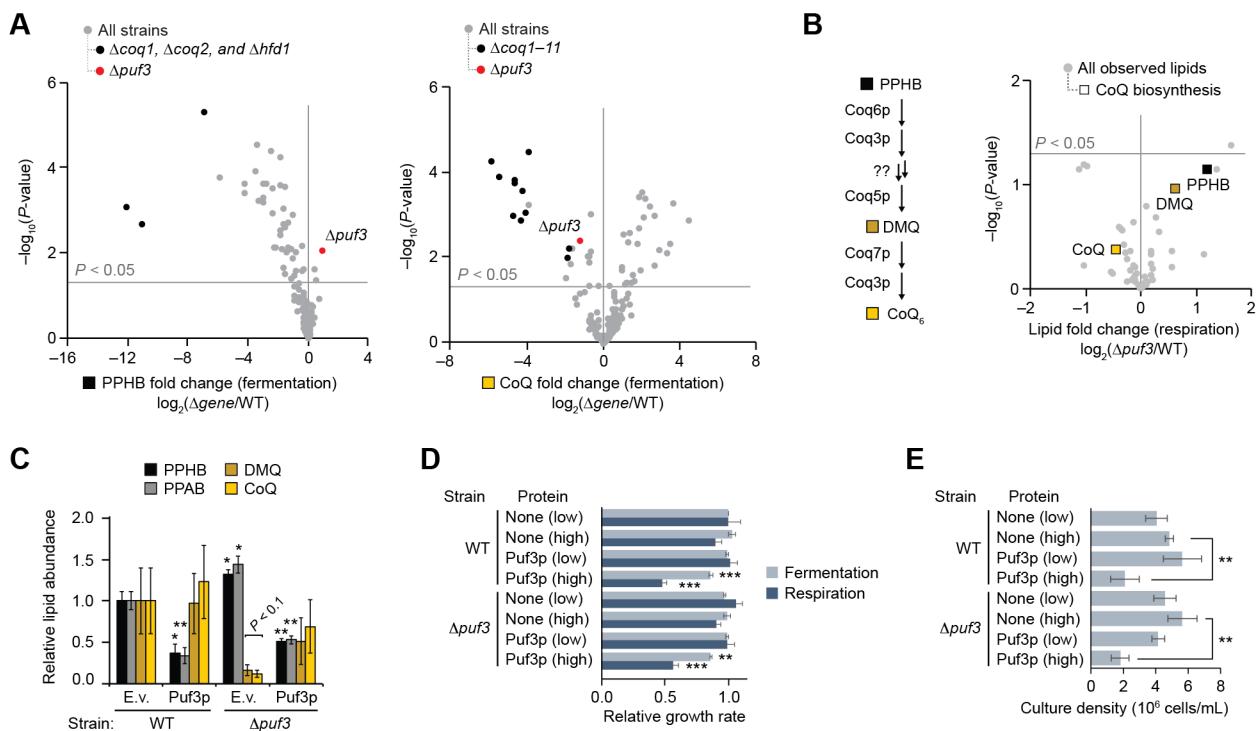
404 (D) Relative fraction of proteins that are toxic when overexpressed across the indicated protein groups. P - 405 values determined by a Fisher's exact test.

406 (E) Cartoon model of a yeast growth curve with key features and dynamics of Puf3p cis targets indicated.

407 (F) Cartoon map of all identified 91 Puf3p cis targets and select Puf3p trans effect proteins that are elevated 408 in $\Delta puf3$ yeast. Puf3p cis targets include numerous proteins that support (i) mitochondrial protein import 409 and processing, (ii) mitochondrial transcription and translation, and (iii) OxPhos assembly. Trans effects 410 include (iv) an increase in OxPhos proteins.

411

413 **SUPPLEMENTAL FIGURES**



414

415 **Figure S1, related to Figure 1. Puf3p Regulates CoQ Biosynthesis**

416 (A) Relative abundances of PPHB and CoQ (mean, n = 3) versus statistical significance across all yeast
417 strains in the Y3K data set (Stefely et al., 2016a) (fermentation culture condition).

418 (B) Lipid abundances in Δpuf3 yeast compared to WT (mean, n = 3) versus statistical significance
419 (respiration condition). Raw data from the Y3K data set (Stefely et al., 2016a).

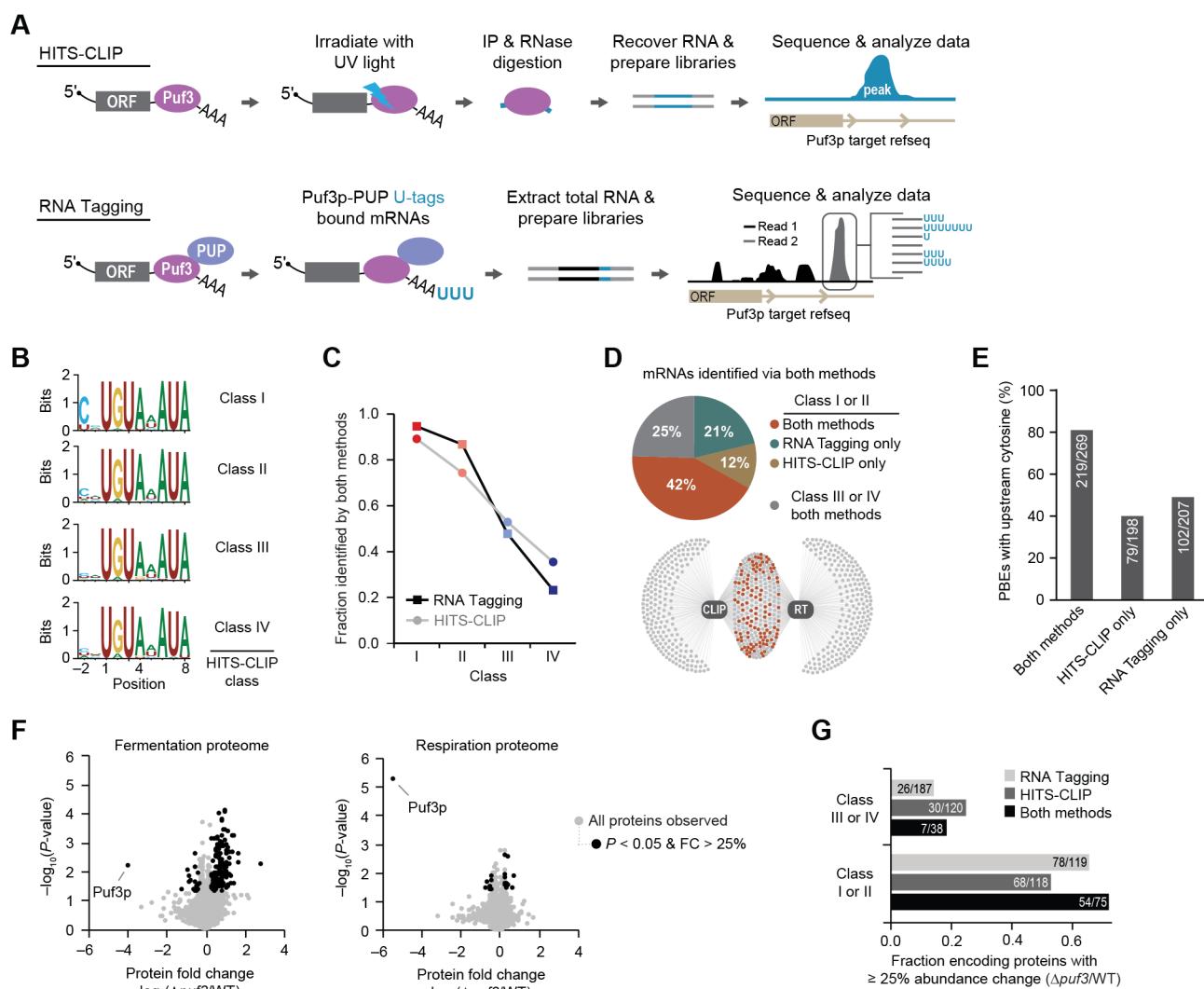
420 (C) Relative lipid abundances in yeast transformed with high copy plasmids overexpressing Puf3p (or
421 empty vector, e.v.) and cultured in fermentation media (mean \pm SD, n = 3). Bonferroni corrected *p < 0.05;
422 **p < 0.01.

423 (D) Growth rates of WT or Δpuf3 yeast transformed with plasmids overexpressing the proteins shown and
424 cultured in either fermentation or respiration media (mean \pm SD, n = 3).

425 (E) Culture densities of WT or Δpuf3 yeast transformed with plasmids overexpressing the proteins shown
426 and cultured in fermentation media (mean \pm SD, n = 3) at the time point of harvest for the lipid analyses
427 shown in Figure 1C and panel (C) of this figure.

428 Two-sided Student's *t*-test for all panels. *p < 0.05; **p < 0.01; ***p < 0.001

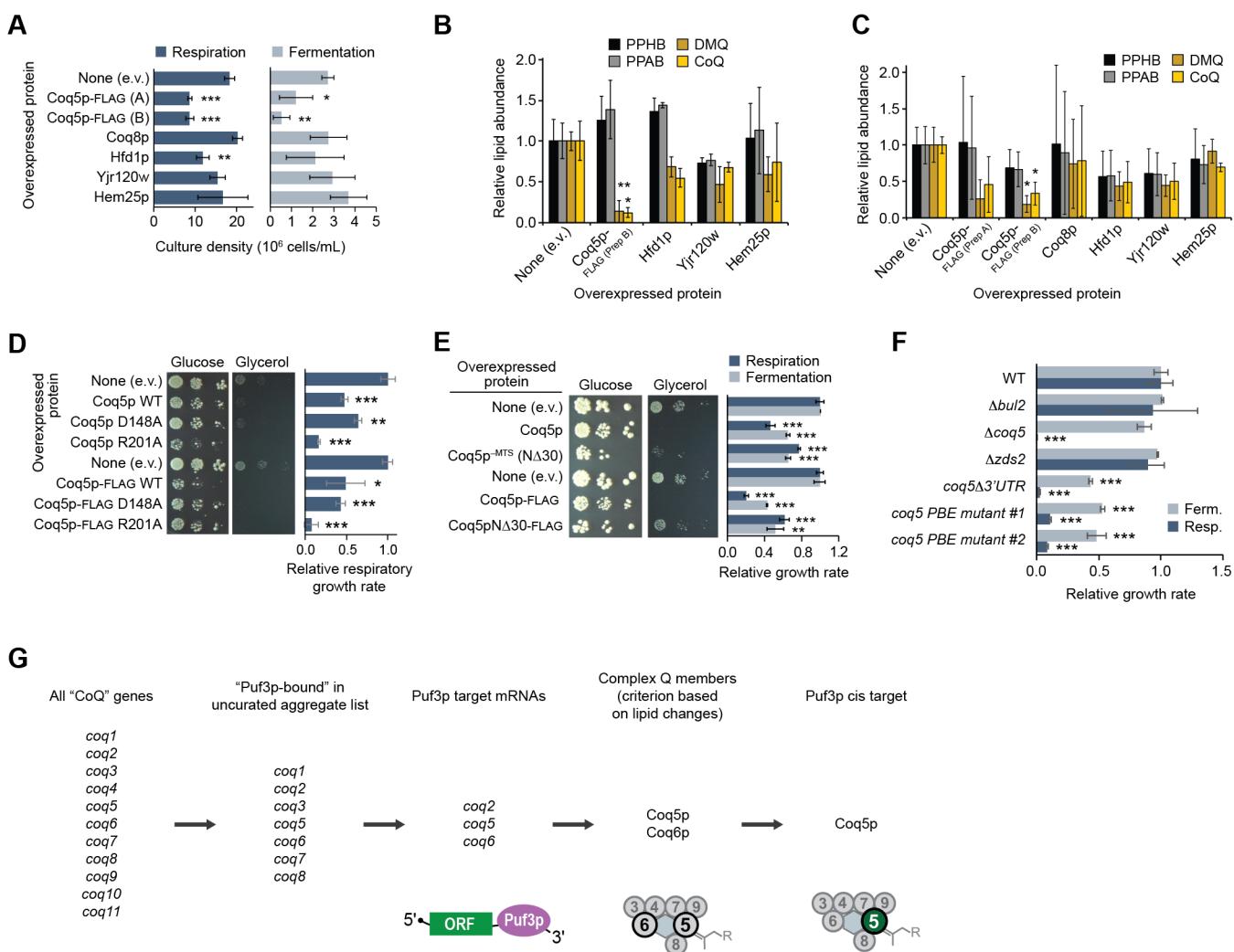
429



430

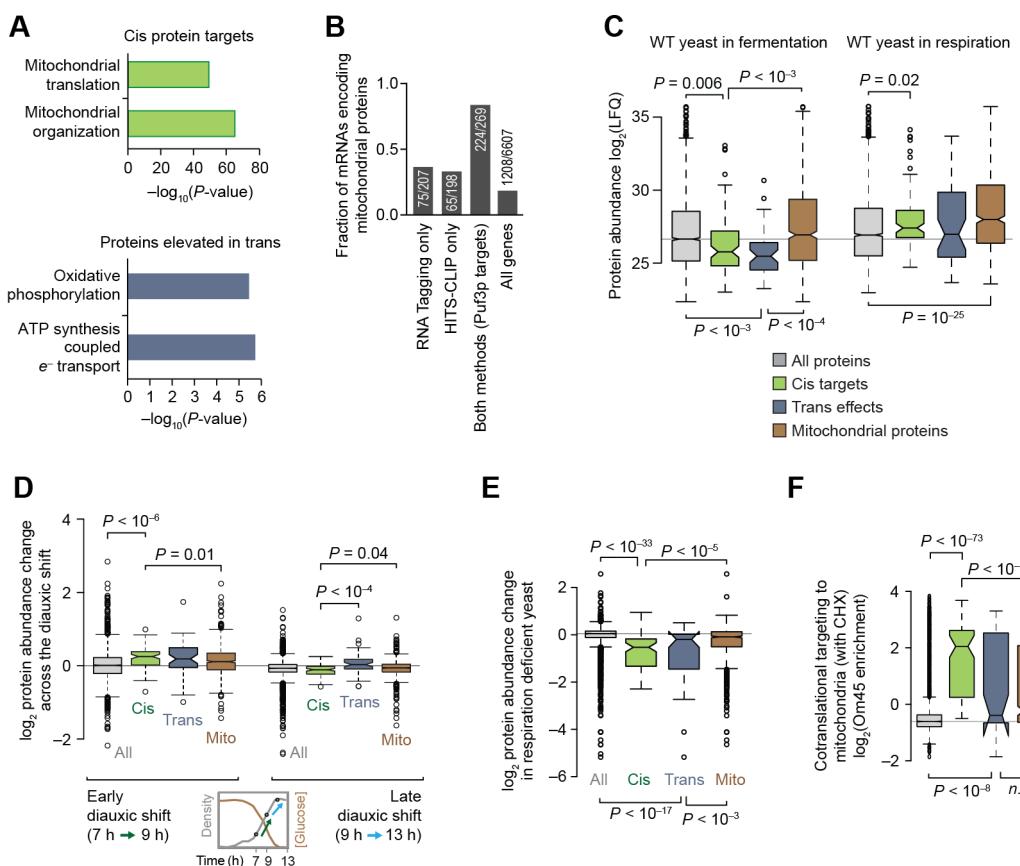
431 **Figure S2, related to Figure 2. Integration of HITS-CLIP, RT, & Proteomics Defines Puf3p Targets**

432 (A) Schematic of HITS-CLIP and RNA Tagging (RT) methods.
 433 (B) Position-weight matrices of PBEs identified under peaks for HITS-CLIP classes.
 434 (C) Overlap of RNAs identified as bound by Puf3p via both RNA Tagging and HITS-CLIP versus target
 435 class for the indicated method.
 436 (D) Class composition of Puf3p-bound mRNAs identified via both RNA Tagging (RT) and HITS-CLIP. The
 437 network map (bottom) shows Puf3p-bound mRNAs (dots) detected by RT and/or HITS-CLIP (CLIP)
 438 (edges). Puf3p-bound mRNAs present in class I or II of both methods are indicated in orange.
 439 (E) Percent of PBEs with upstream cytosine (-1 or -2 position).
 440 (F) Relative protein abundances in $\Delta puf3$ yeast compared to WT (mean, $n = 3$) versus statistical
 441 significance in fermentation and respiration conditions. Proteins with fold change (FC) $> 25\%$ and $p < 0.05$
 442 (160 and 24 proteins, respectively) are highlighted (two-sided Student's t -test).
 443 (G) Fraction of genes with at least a 25% change in protein abundance ($p < 0.05$, two-sided Student's t -
 444 test) for the indicated groups. Analyses were limited to genes with proteins detected in the Y3K proteomics
 445 study (Stefely et al., 2016a), which is the denominator of each ratio. This figure includes new, integrated
 446 analyses of publicly available raw data from the RT (Lapointe et al., 2015), HITS-CLIP (Wilinski et al.,
 447 2017), and Y3K multi-omic (Stefely et al., 2016a) data sets generated in our labs.
 448



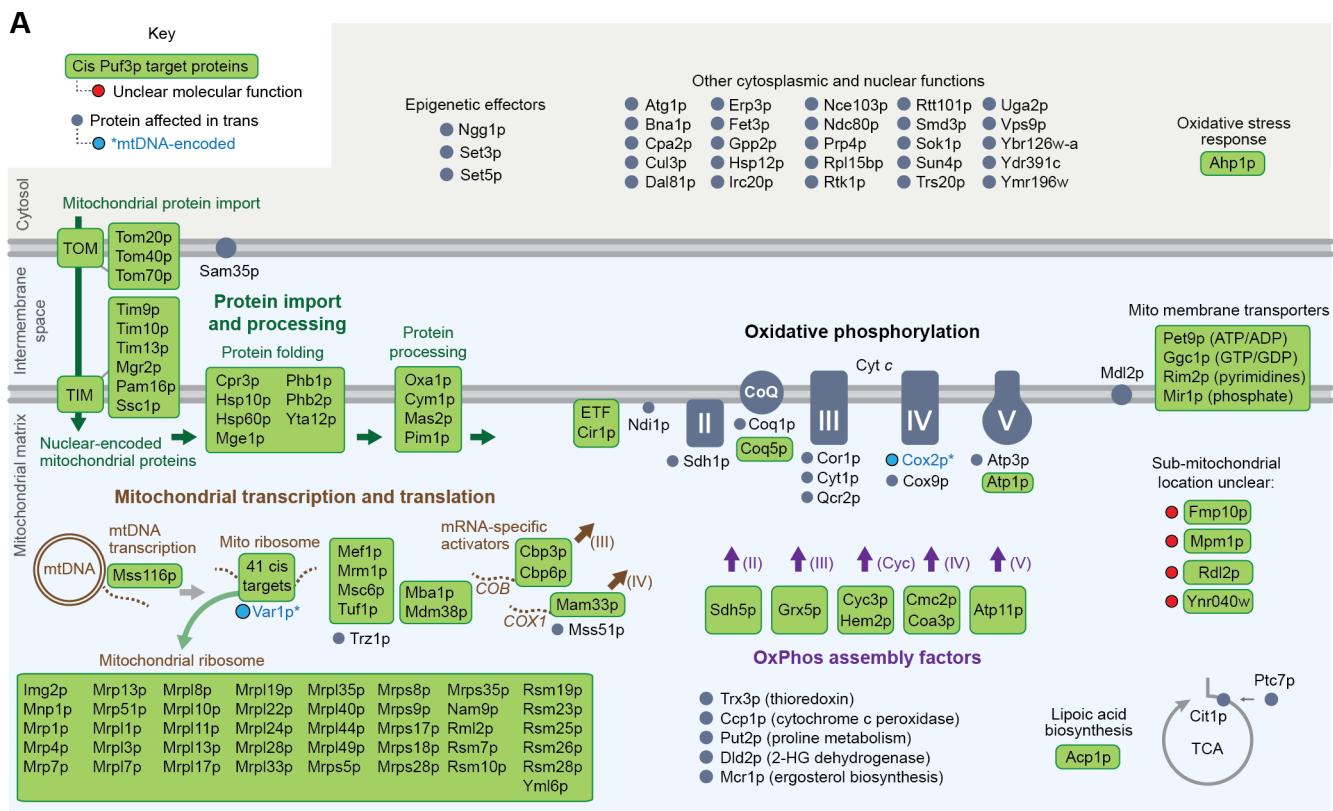
449

450 **Figure S3, related to Figure 3. Puf3p Regulates the CoQ Biosynthesis Enzyme Coq5p**
451 (A) Densities of yeast cultures at the time point of harvest for the lipid quantitation experiments depicted in
452 Figure 3C and panels (B) and (C) of this figure.
453 (B) and (C) Relative lipid abundances in WT yeast transformed with plasmids overexpressing the proteins
454 shown and cultured in respiration media (B) or fermentation media (C) (mean \pm SD, n = 3). Bonferroni
455 corrected *p < 0.05; **p < 0.01 compared to empty vector (e.v.) control.
456 (D) and (E) Left, serial dilutions of WT yeast transformed with plasmids overexpressing the proteins shown
457 and cultured on solid media containing either glucose or glycerol. Right, growth rates of WT yeast
458 transformed with plasmids overexpressing the proteins shown and cultured in liquid media (mean \pm SD, n
459 = 3).
460 (F) Growth rates of yeast strains cultured in either fermentation or respiration media (mean \pm SD, n = 3).
461 (G) Scheme of how Coq5p was identified as a key Puf3p target responsible for the Δ puf3 CoQ deficiency.
462 For all panels, P-values were determined by a two-sided Student's t-test. *p < 0.05; **p < 0.01; ***p < 0.001
463

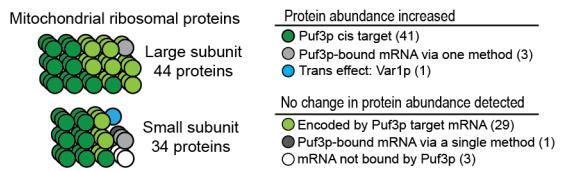


464

465 **Figure S4, related to Figure 4. Puf3p Targets Share Distinctive Properties and Dynamics**
466 (A) Gene Ontology (GO) terms enriched in cis Puf3p targets (top) or in elevated Puf3p trans effect proteins
467 (bottom).
468 (B) Fraction of genes annotated as encoding mitochondrial proteins for the indicated groups.
469 (C)–(F) Box plots comparing the distributions of various gene and protein properties for all proteins
470 observed in the Y3K $\Delta puf3$ proteomics data set (gray), cis Puf3p targets (green), trans Puf3p effects (blue),
471 and mitochondrial proteins (brown). Center lines indicate medians, limits indicate 25th and 75th percentiles,
472 whiskers extend 1.5 times the interquartile range, outliers are represented by dots, and P -values were
473 determined with a Student's *t*-test (two-tailed, homostatic). Protein set sizes: all proteins n = 3152,
474 mitochondrial proteins n = 715, cis targets n = 91, trans effect proteins n = 49.
475 (C) Protein abundances (Stefely et al., 2016a) for each group of proteins shown. LFQ, label free
476 quantitation value.
477 (D) Protein abundance changes (Stefely et al., 2016b) across the diauxic shift.
478 (E) Protein abundance changes (Stefely et al., 2016a) in respiration deficient yeast compared to respiration
479 competent yeast.
480 (F) Measure of cotranslational targeting to mitochondria in yeast treated with cycloheximide (CHX)
481 (Williams et al., 2014).
482



B



483

484 **Figure S5, related to Figure 4. Puf3p Targets Pathways of Mitochondrial Biogenesis Proteins**

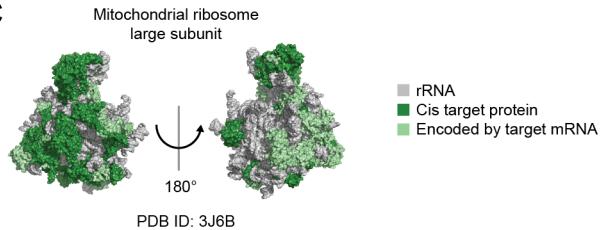
485 (A) Cartoon indicating all identified cis Puf3p targets and trans effects.

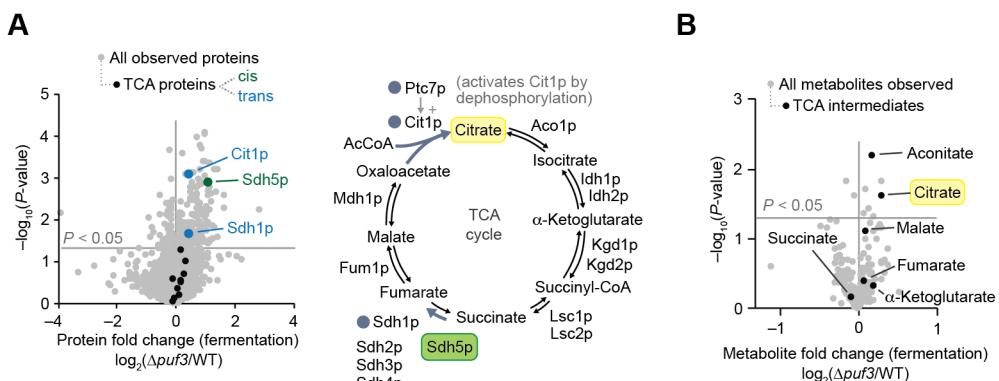
486 (B) Cartoon of mitochondrial ribosomal proteins with the effect by Puf3p indicated.

487 (C) Surface representation of the large subunit of the yeast mitochondrial ribosome (PDB: 3J6B) (Amunts
488 et al., 2014) indicating Puf3p targets.

489

C





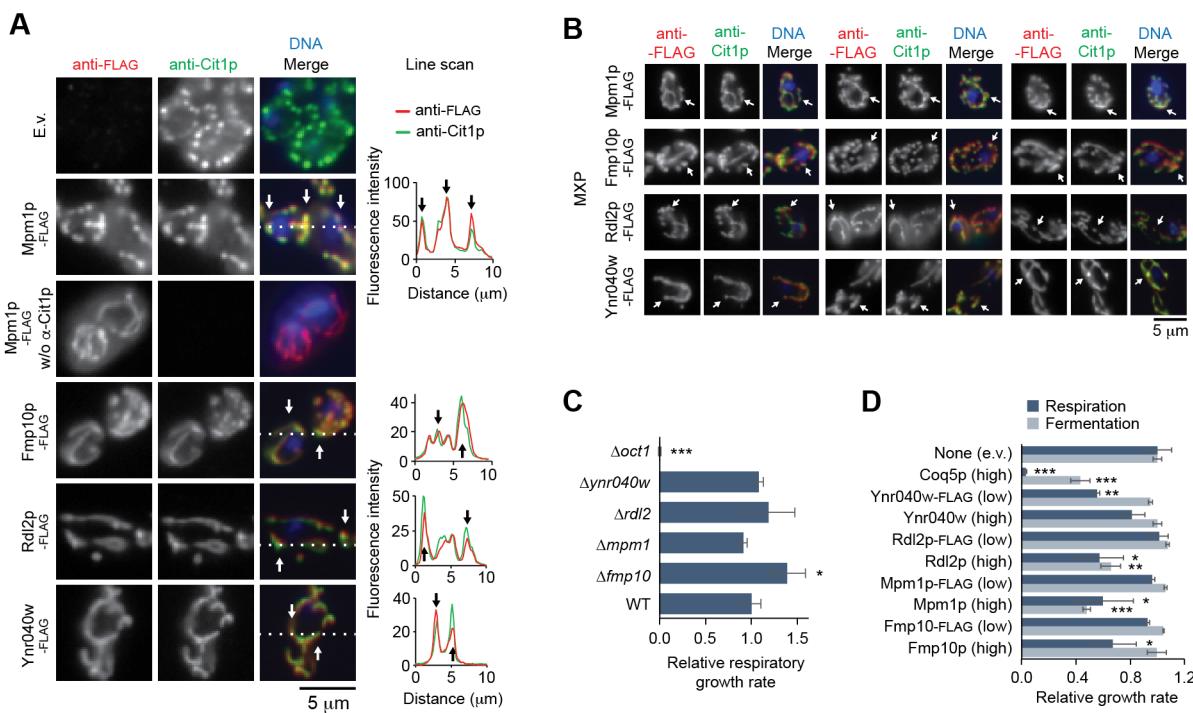
490

491 **Figure S6, related to Figure 4. Puf3p Regulates TCA Cycle Proteins**

492 (A) Relative protein abundances in $\Delta puf3$ yeast compared to WT highlighting TCA proteins (left), and
493 scheme of the TCA cycle highlighting proteins significantly ($p < 0.05$) elevated in $\Delta puf3$ yeast (right).

494 (B) Relative metabolite abundances in $\Delta puf3$ yeast compared to WT (mean, $n = 3$) versus statistical
495 significance (fermentation condition), highlighting TCA cycle metabolites. Raw data from the Y3K data set
496 (Stefely et al., 2016a).

497



498

499 **Figure S7, related to Figure 4. Uncharacterized Puf3p Targets Localize to Mitochondria**

500 (A) Fluorescent immunocytochemistry analysis of yeast transformed with the indicated FLAG tagged
501 proteins. An antibody against Cit1p was used to localize mitochondria. Arrows indicate landmarks on the
502 line scan (dotted line on image). Scale bar, 5 μm .

503 (B) Fluorescent immunocytochemistry analysis of WT yeast transformed with the indicated FLAG tagged
504 mitochondrial uncharacterized proteins (MXP). Arrows indicate spatial landmarks across each channel.
505 Scale bar, 5 μm .

506 (C) Relative growth rates of yeast strains cultured in respiration media (mean \pm SD, n = 3).

507 (D) Relative growth rates of WT yeast transformed with plasmids overexpressing the proteins shown and
508 cultured in either fermentation or respiration media (mean \pm SD, n = 3). High or low copy number plasmids
509 are indicated.

510 Two-sided Student's t-test for all panels. *p < 0.05; **p < 0.01; ***p < 0.001

512 **SUPPLEMENTAL TABLE LEGENDS**

513 **Table S1. Puf3p Target mRNA Analysis, Related to Figure 2**

514 (A) Key describing each column in sheets (tables) (B) – (D) of this data set: Gene systematic name, Gene
515 standard name, RNA Tagging Class, Tagging U1 TRPM log₂, Tagging PBE, CLIP peak height, CLIP *P*-
516 value, CLIP Class, Number of peaks, Peak location, CLIP PBE, Method. (B) Table of Puf3p mRNA targets,
517 including a summary of RNA Tagging and HITS-CLIP data for each target. (C) Table of all mRNAs
518 identified as a Puf3p-bound mRNA in either approach, including a summary of RNA Tagging and HITS-
519 CLIP data for each mRNA. (D) Table of all genes, highlighting mRNAs identified as a Puf3p-bound mRNA
520 in either approach, including a summary of RNA Tagging and HITS-CLIP data for each mRNA. (E) Table
521 of all genes, highlighting which method identified them as putative Puf3p targets (HITS-CLIP, RNA
522 Tagging, PAR-CLIP, or RIP-seq). It includes a list of the aggregate 2,018 mRNAs identified as putative
523 Puf3p targets.

524 **Table S2. Transomic Puf3p Target Analysis, Related to Figure 2**

525 (A) Key describing each column in sheets (tables) (B) – (D) of this data set: Gene systematic name, Gene
526 standard name, Pathway, Protein effect (cis, trans, or unclear), RNA Tagging Class, Tagging U1 TRPM
527 log₂, CLIP peak height, CLIP *P*-value, CLIP Class, Number of peaks, Peak location, Method, Proteomics
528 log₂(KO/WT) in fermentation, Proteomics *P*-value in fermentation, and 25% sig protein change. (B) Table
529 of Puf3p cis targets, including a summary of RNA Tagging, HITS-CLIP, and proteomics data for each
530 target. (C) Table of Puf3p trans effect proteins, including a summary of the proteomics data for each
531 protein. (D) Table of all genes and all proteins in the transomic analysis, including a summary of RNA
532 Tagging, HITS-CLIP, and proteomics data for each molecule.

533 **Table S3. Puf3p Cis Target GO Term Analysis, Related to Figure S4**

534 (A) Table of Gene Ontology (GO) terms enriched in the Puf3p cis target set. Includes GO terms, *P*-values,
535 –log₁₀(*P*-values), genes in the Puf3p cis target set that overlap with those in the GO term set, and GO term
536 ID number. (B) Table of Gene Ontology (GO) terms enriched in the set of trans effect proteins with
537 increased protein abundance in yeast that lack Puf3p. Includes GO terms, *P*-values, –log₁₀(*P*-values),
538 genes in the Puf3p trans effect set that overlap with those in the GO term set, and GO term ID number.

539

540 **ACKNOWLEDGEMENTS**

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547 **AUTHOR CONTRIBUTIONS**

548 C.P.L., J.A.S., M.P.W., and D.J.P. conceived of the project and its design and wrote the manuscript. J.A.S.
549 and A.J. prepared samples and performed biochemical experiments. P.D.H. and G.W. acquired MS data.
550 C.P.L., J.A.S., A.J., P.D.H., G.M.W., N.W.K., J.J.C., M.P.W., and D.J.P. analyzed data.

551 **METHODS**

552 **CONTACT FOR REAGENT AND RESOURCE SHARING**

553 Further information and requests for resources and reagents should be directed to and will be fulfilled by
554 the Lead Contact David Pagliarini (dpagliarini@morgridge.org).

555 **EXPERIMENTAL MODEL AND SUBJECT DETAILS**

556 **Yeast strains**

557 The parental (wild type, WT) *Saccharomyces cerevisiae* strain for this study was the haploid MATalpha
558 BY4742. Single gene deletion (Δ gene) derivatives of BY4742 were obtained through the gene deletion
559 consortium (via Thermo, Cat#YSC1054). Gene deletions were confirmed by proteomics (significant [$p <$
560 0.05] and selective decrease in the encoded protein) or PCR assay.

561 *Coq5 PBE mutant strain.* The PBE in *coq5* was identified by analysis of the HITS-CLIP data
562 (Wilinski et al., 2017), in which *coq5* had a single peak centered over the sequence: CTGTACATA. Using
563 the haploid MATalpha BY4742 as the parental strain, a yeast strain with a mutant Puf3p-binding element
564 (PBE) in the 3' untranslated region (UTR) of the *coq5* gene was generated using the *Delitto Perfetto*
565 method (Storici and Resnick, 2006). Briefly, the 282 nucleotides immediately downstream of the *coq5*
566 coding sequence were replaced with the KanMx4-KIUra3 cassette. Thus, the "*coq5* Δ 3'UTR" strain was
567 created. To enable generation of the endogenous PBE mutant, the *coq5* ORF + 3' UTR was cloned in
568 p426gpd. The PBE sequence was mutagenized by PIPE cloning. The sequence-confirmed plasmid,
569 containing mutagenized PBE, served as the PCR template in a reaction that generated the cassette that
570 seamlessly replaced KanMx4-KIUra3 with the *coq5* 3' UTR sequence harboring the CTGT --> GACA PBE
571 mutation. Two separate yeast strains with identical *coq5* PBE mutants were independently generated in
572 this manner. One of the two strains maintained a *coq5* 3' UTR identical to that of the WT strain outside of
573 the mutated PBE. In the second strain, the PBE change was scarless, with the exception of the deletion
574 of a single 3' UTR thymidine nucleotide in a stretch of 10 thymidine nucleotides beginning 184 nucleotides
575 upstream of the desired PBE mutation. As shown in the figures of this report, both of these independently
576 generated strains shared the same biological phenotypes.

577 **METHOD DETAILS**

578 **Yeast cultures**

579 *General culture procedures and media components.* Yeast were stored at -80 °C as glycerol stocks and
580 initially cultured on selective solid media plates at 30 °C. Biological replicates were defined as separate
581 yeast colonies after transformation and plating onto solid selective media. Experiments were conducted in
582 biological triplicate ("n = 3") to afford statistical power. Cell density of liquid media cultures was determined
583 by optical density at 600 nm (OD₆₀₀) as described (Hebert et al., 2013). Media components included yeast
584 extract ('Y') (Research Products International, RPI), peptone ('P') (RPI), agar (Fisher), dextrose ('D') (RPI),
585 glycerol ('G') (RPI), uracil drop out (Ura⁻) mix (US Biological), histidine drop out (His⁻) mix (US Biological),
586 and G418 (RPI). YP and YPG solutions were sterilized by automated autoclave. G418 and dextrose were
587 sterilized by filtration (0.22 μ m pore size, VWR) and added separately to sterile YP or YPG. YPD+G418
588 plates contained yeast extract (10 g/L), peptone (20 g/L), agar (15 g/L), dextrose (20 g/L), and G418 (200
589 mg/L). YPD media (rich media fermentation cultures) contained yeast extract (10 g/L), peptone (20 g/L),

590 and dextrose (20 g/L). YPGD media (rich media respiration cultures) contained yeast extract (10 g/L),
591 peptone (20 g/L), glycerol (30 g/L) and dextrose (1 g/L). Synthetic Ura⁻ media were sterilized by filtration
592 (0.22 μ m pore size). Ura⁻,D media contained Ura⁻ mix (1.92 g/L), yeast nitrogen base (YNB) (6.7 g/L, with
593 ammonium sulfate and without amino acids), and dextrose (20 g/L). Ura⁻,GD media contained Ura⁻ mix
594 (1.92 g/L), YNB (6.7 g/L), glycerol (30 g/L), and dextrose (1 g/L). Ura⁻,D+4HB media contained Ura⁻ mix
595 (1.92 g/L), YNB (6.7 g/L), dextrose (20 g/L), and 4-HB (100 μ M). Ura⁻,GD+4HB media contained Ura⁻ mix
596 (1.92 g/L), YNB (6.7 g/L), glycerol (30 g/L), dextrose (1 g/L), and 4-HB (100 μ M).

597 *Puf3 rescue cultures.* WT or Δ puf3 yeast were transformed with plasmids encoding Puf3p
598 [p423(2 μ)-PUF3 or p413(CEN)-PUF3] and cultured on His⁻,D plates. Starter cultures (3 mL His⁻,D+4HB)
599 were inoculated with an individual colony of yeast and incubated (30 °C, 230 rpm, 10–15 h). For CoQ
600 quantitation, His⁻,D+4HB (fermentation) or His⁻,GD+4HB (respiration) media (100 mL media at ambient
601 temperature in a sterile 250 mL Erlenmeyer flask) was inoculated with 2.5×10^6 yeast cells and incubated
602 (30 °C, 230 rpm). Samples of the His⁻,D+4HB cultures were harvested 13 h after inoculation, a time point
603 that corresponds to early fermentation (logarithmic) growth. Samples of His⁻,GD+4HB cultures were
604 harvested 25 h after inoculation, a time point that corresponds to early respiration growth. For each growth
605 condition, 1×10^8 yeast cells were pelleted by centrifugation (3,000 g, 3 min, 4 °C), the supernatant was
606 removed, and the cell pellet was flash frozen in N₂(l) and stored at -80 °C prior to lipid extractions. For
607 relative growth rate measurements, analogous cultures (initial density of 5×10^6 cells/mL) were incubated
608 in a sterile 96 well plate with an optical, breathable cover seal (shaking at 1096 rpm). Optical density
609 readings were obtained every 10 min.

610 *Protein overexpression cultures.* WT yeast were transformed with plasmids encoding Coq5p,
611 Coq5p-FLAG, Coq8p, Coq9p, Hfd1p, Yjr120w, or Hem25p (p426[2 μ]-GPD plasmids) and cultured on Ura⁻
612 ,D plates. Starter cultures (3 mL Ura⁻,D+4HB) were inoculated with an individual colony of yeast and
613 incubated (30 °C, 230 rpm, 10–15 h). For CoQ quantitation, Ura⁻,D+4HB (fermentation) or Ura⁻,GD+4HB
614 (respiration) media (100 mL media at ambient temperature in a sterile 250 mL Erlenmeyer flask) was
615 inoculated with 2.5×10^6 yeast cells and incubated (30 °C, 230 rpm). Samples of the Ura⁻,D+4HB cultures
616 were harvested 13 h after inoculation. Samples of Ura⁻,GD+4HB cultures were harvested 25 h after
617 inoculation. For each growth condition, 1×10^8 yeast cells were pelleted by centrifugation (3,000 g, 3 min,
618 4 °C), the supernatant was removed, and the cell pellet was flash frozen in N₂(l) and stored at -80 °C prior
619 to lipid extractions. For relative growth rate measurements, analogous cultures (initial density of 5×10^6
620 cells/mL) were incubated in a sterile 96 well plate with an optical, breathable cover seal (shaking at 1096
621 rpm). Optical density readings were obtained every 10 min. Growth rates were determined by fitting a linear
622 equation to the linear growth phase and determining the slope of the line.

623 *Cultures for microscopy.* BY4742 *S. cerevisiae* overexpressing C-terminally FLAG-tagged genes
624 from p416gpd_FLAG plasmids were cultured in Ura⁻,D media (30 °C, 3 mL starter cultures, ~14 h). From
625 these starter cultures, 1.25×10^6 cells were used to inoculate Ura⁻,GD media (50 mL) (respiration culture
626 condition). After incubating 25 hours (30 °C, 230 r.p.m), 1×10^8 cells were removed from the culture by
627 pipetting and immediately fixed with formaldehyde for microscopy as described below.

628 Yeast Transformations

629 Yeast were transformed with plasmids using a standard lithium acetate protocol (Gietz et al., 1992). Briefly,
630 BY4742 yeast were cultured in YEPD (50 mL) to a density of 2×10^7 cells/mL. Cells were pelleted and
631 washed twice with water. For each transformation, added PEG 3350 (50% w/v, 240 μ L), lithium acetate (1
632 M, 36 μ L), boiled salmon sperm DNA (5 mg/mL, 50 μ L), water (30 μ L), and plasmid (4 μ L) to a pellet
633 containing 1×10^8 cells, mixed by vortexing, and incubated (42 °C, 45 min). The transformed cells were
634 pelleted, resuspended in water (100 μ L), and plated on selective media.

635 **DNA Constructs**

636 Yeast gene constructs were generated by amplifying the *S. cerevisiae* genes *fmp10*, *mpm1*, *rdl2*, and
637 *ynr040w* from strain BY4742 genomic DNA with primers containing HindIII recognition sequence (forward)
638 and Sall recognition sequence (reverse). Similarly, *hem25* and *yjr120w* were amplified with BamHI
639 (forward) and EcoRI (reverse) primers. *Coq5* (from strain W303) was amplified with Spel (forward) and
640 Sall or Xhol (reverse) primers. PCR reactions contained 1× Accuprime PCR mix, 1 μM forward primer, 1
641 μM reverse primer, ~250 ng template, and 1× Accuprime Pfx (Invitrogen cat#12344024). After an initial 2
642 min denaturation at 95 °C, reactions were exposed to 5 cycles of 95 °C for 15 seconds, 55 °C for 30
643 seconds, and 68 °C for 2 minutes followed by 30 cycles of 95 °C for 15 seconds, 60 °C for 30 seconds,
644 and 68 °C for 2 minutes. Amplicons were purified using a PCR purification kit (Thermo cat#K0702) and
645 digested with the appropriate restriction enzymes and again subjected to PCR purification. Amplified genes
646 were cloned into restriction enzyme digested yeast expression vectors (p426gpd and/or p416gpd_FLAG).
647 The plasmid p416gpd_FLAG was generated by digesting p416gpd with Xhol and MluI and inserting a
648 double stranded oligonucleotide containing the Flag tag nucleotide sequence and processing Xhol and
649 MluI ends. For *puf3*, p423(2μ)-PUF3 and p413(CEN)-PUF3 expression plasmids were constructed in
650 modified p423(2μ) or p413(CEN) backgrounds in which the plasmid promoter and terminators had been
651 removed. The *puf3* gene, including 1,000 upstream nts and 457 downstream nts, was amplified from
652 BY4742 genomic DNA using a standard Phusion DNA polymerase PCR. Amplified products were cleaned
653 via a PCR purification kit (ThermoFisher), digested with the Sall and KpnI restriction enzymes under
654 standard conditions, and cloned into the appropriate plasmid. All recombinants were confirmed by DNA
655 sequencing. Cloning was previously reported for p426-GPD-*coq8* (Stefely et al., 2015), p426-GPD-*coq9*
656 (Lohman et al., 2014), p426-GPD-*hfd1* (Stefely et al., 2016a).

657 **HITS-CLIP Class Definition**

658 Puf3p-bound RNAs identified via HITS-CLIP (Wilinski et al., 2017) were sorted by the number of RNAs
659 detected in their CLIP peak (“peak height”) from most to least. Classes were then defined as follows: the
660 top 10% were designated class I; 11–40% class II; 41–70% class III; and 71–100% class IV. Classes were
661 defined to be of comparable size to the analogous RNA Tagging class to facilitate cross-method
662 comparisons (Lapointe et al.; Lapointe et al., 2015). As a control, the signal detected for these mRNAs
663 [$\log_2(\text{TRPM})$ for RNA Tagging and $\log_2(\text{peak height})$ for HITS-CLIP] was correlated across methods
664 (Spearman’s $\rho = 0.42$, $p < 10^{-12}$), but not with mRNA abundance ($p > 0.01$).

665 **MEME Analyses**

666 For all analyses, the 3' untranslated regions (UTRs) was defined as the longest observed isoform for a
667 gene (Xu et al., 2009) or 200 nts downstream of the stop codon if not previously defined. MEME was run
668 on a local server with the command: `meme [input.txt] -oc [outputdirectory] -dna -mod zoops -evt 0.01 -`
669 `nmotifs 10 -minw 8 -maxw 12 -maxsize 1000000000000`.

670 **Definition of Puf3p mRNA Targets**

671 Puf3p HITS-CLIP data were obtained from (Wilinski et al., 2017). Only peaks that were assigned to
672 annotated genes were considered: 467 in total, with 15 genes identified by two peaks. RNA Tagging data
673 were obtained from (Lapointe et al.; Lapointe et al., 2015). All 476 reported RNA Tagging targets were
674 considered. RNA Tagging classes were obtained from (Lapointe et al.) because they were generated using
675 an improved strategy than in the initial report (Lapointe et al., 2015). Genes with mRNAs identified by both
676 approaches, visualized by Venn diagrams, were designated as “Puf3p target mRNAs”. Protein-RNA
677 network maps were constructed using Cytoscape (v. 3.2.1) and the ‘Organic’ ‘yFiles Layouts’ option.

678 **Definition of Puf3p Cis Target Proteins**

679 We defined “Puf3p cis target proteins” (interchangeably referred to as “Puf3p cis targets”) as: proteins
680 encoded by Puf3p mRNA targets with at least a 25% significant ($p < 0.05$) alteration in protein abundance
681 in yeast that lack *puf3* relative to WT yeast grown in fermentation culture conditions. The proteomic data
682 included 165 proteins encoded by Puf3p mRNA targets (out of 269 total), and 91 proteins were designated
683 as Puf3p cis target proteins (“Puf3p cis targets”) out of the 160 proteins with at least a 25% significant
684 alteration in protein abundance. To ensure rigorous definition, we excluded 20 proteins with significantly
685 altered protein abundances because they were identified as Puf3p-bound mRNAs only via a single method,
686 thus confounding their assignment.

687 **Definition of Puf3p Trans Targets**

688 For the proteome, Puf3p trans effects were defined as proteins that were *not* encoded by Puf3p mRNA
689 targets with at least a 25% significant ($p < 0.05$) alteration in protein abundance in yeast that lack *puf3*
690 relative to WT yeast grown in fermentation culture conditions. For the metabolome and lipidome, Puf3p
691 trans effects were defined as metabolites or lipids, respectively, with at least a 25% significant ($p < 0.05$)
692 alteration in abundance in yeast that lack *puf3* relative to WT yeast grown in fermentation culture
693 conditions.

694 **Gene Ontology Analyses**

695 Analyses were conducted using YeastMine, from the *Saccharomyces* Genome database
696 (<http://yeastmine.yeastgenome.org>), with the default settings (Holm-Bonferroni correction).

697 **Gene Property Analyses**

698 To test for characteristic properties of Puf3p cis targets, Puf3p trans effect proteins, and mitochondrial
699 proteins, we compared our data against numerous publicly available data sets. Briefly, all proteins
700 quantified in $\Delta puf3$ yeast ($n = 3152$, fermentation growth conditions, Y3K data set (Stefely et al., 2016a))
701 were assigned to one or more of the following categories where appropriate: Puf3p cis targets ($n = 91$, as
702 defined in this report), Puf3p trans effect proteins ($n = 49$, as defined in this report), mitochondrial proteins
703 (Jin et al., 2015) ($n = 715$), and all profiled proteins ($n = 3152$) in fermenting $\Delta puf3$ yeast. For each gene
704 property analysis, each protein was assigned a qualitative or quantitative value as reported in a publicly
705 available data set if a corresponding value was reported therein. Protein overexpression toxicity was
706 assigned using data from (Gelperin et al., 2005). Enrichment in protein toxicity amongst cis, trans, and
707 mitochondrial proteins was calculated relative to all proteins. Statistical significance of these enrichments
708 was determined via a Fisher’s exact test. Protein abundance, in fermentation and respiration conditions,
709 was assigned by taking the average \log_2 label free quantitation (LFQ) value from 36 replicates of WT yeast
710 grown as part of the Y3K study (Stefely et al., 2016a). Data sets have been reported previously for
711 cotranslational import of mitochondrial proteins (Williams et al., 2014), protein abundance changes across
712 the diauxic shift (Stefely et al., 2016b), and average respiration deficiency response (RDR) protein
713 abundance change (change in protein abundance in respiration deficient yeast compared to respiration
714 competent yeast) (Stefely et al., 2016a). For these quantitative variable analyses, statistical significance
715 was calculated using a Student’s *t*-test (two-tailed, homostatic).

716 **Lipid Extractions**

717 Frozen pellets of yeast (10^8 cells) were thawed on ice and mixed with glass beads (0.5 mm diameter, 100
718 μL). $\text{CHCl}_3/\text{MeOH}$ (2:1, v/v, 4 °C) (900 μL) and CoQ_{10} (10 μL , 10 μM , 0.1 nmol) were added and vortexed
719 (2 × 30 s). HCl (1 M, 200 μL , 4 °C) was added and vortexed (2 × 30 s). The samples were centrifuged
720 (5,000 *g*, 2 min, 4 °C) to complete phase separation. 555 μL of the organic phase was transferred to a

721 clean tube and dried under Ar_(g). The organic residue was reconstituted in ACN/IPA/H₂O (65:30:5, v/v/v)
722 (100 µL) for LC-MS analysis.

723 **LC-MS Lipid Analysis**

724 LC-MS analysis was performed on an Acquity CSH C18 column held at 50 °C (100 mm × 2.1 mm × 1.7
725 µm particle size; Waters) using a Vanquish Binary Pump (400 µL/min flow rate; Thermo Scientific). Mobile
726 phase A consisted of 10 mM ammonium acetate in ACN/H₂O (70:30, v/v) containing 250 µL/L acetic acid.
727 Mobile phase B consisted of 10 mM ammonium acetate in IPA/ACN (90:10, v/v) with the same additives.
728 Mobile phase B was held at 40% for 6.0 min and then increased to 60% over 3.0. Mobile phase B was
729 further increased to 85% over 0.25 min and then to 99% for over 1.25 min. The column was then
730 reequilibrated for 3.5 min before the next injection. Ten microliters of sample were injected by a Vanquish
731 Split Sampler HT autosampler (Thermo Scientific). The LC system was coupled to a Q Exactive mass
732 spectrometer by a HESI II heated ESI source kept at 325 °C (Thermo Scientific). The inlet capillary was
733 kept at 350 °C, sheath gas was set to 25 units, and auxiliary gas to 10 units, and the spray voltage was
734 set to 3,000 V. The MS was operated in positive and negative parallel reaction monitoring (PRM) mode
735 acquiring scheduled, targeted PRM scans to quantify key CoQ intermediates. Phospholipids were
736 quantified and identified using a negative dd-Top2 scanning mode.

737 *LC-MS Lipid Data Analysis.* CoQ intermediate data were processed using TraceFinder 4.0 (Thermo
738 Fisher Scientific). Discovery lipidomic data were processed using an in-house software pipeline and
739 Compound Discoverer 2.0 (Thermo Fisher Scientific).

740 **Fluorescence Microscopy**

741 Yeast (1×10⁸ cells) transformed with various FLAG tagged constructs were removed from cultures by
742 pipetting and immediately fixed with formaldehyde (4% final concentration, gentle agitation on a nutator, 1
743 h, ~23 °C). The fixed cells were harvested by centrifugation (1000 g, 2 min, ~23 °C), washed three times
744 with 0.1 M potassium phosphate pH 6.5 and once with K-Sorb buffer (5 mL, 1.2 M sorbitol, 0.1 M KP_i, pH
745 6.5), and re-suspended in K-Sorb buffer (1 mL). An aliquot of the cells (0.5 mL) was mixed with K-Sorb-
746 BME (0.5 mL, K-Sorb with 140 mM BME) and incubated (~5 min, ~23 °C). Zymolase 100T was added to
747 1 mg/mL final concentration and incubated (20 min, 30 °C). The resultant spheroplasts were harvested by
748 centrifugation (1000 g, 2 min, ~23 °C), washed once with K-Sorb buffer (1.4 mL), and re-suspended in K-
749 Sorb buffer (0.5 mL). A portion of the cells (0.25 mL) was pipetted onto a poly-D-lysine coated microscope
750 coverslip and allowed to settle onto the slides (20 min, ~23 °C). To permeabilize the cells, the supernatant
751 was aspirated from the coverslips, and MeOH (2 mL, -20 °C) was added immediately and incubated (6
752 min, on ice). The MeOH was aspirated and immediately replaced with acetone (2 mL, -20 °C) and
753 incubated (30 s, on ice). The acetone was aspirated, and the slides were allowed to air-dry (~2 min). DNA
754 was stained with Hoechst 33342 dye (1 µg/mL in PBS, 2 mL, 5 min incubation, protected from light), and
755 the cells were immediately wash with PBS. The samples were blocked with BSA ("BSA-PBS" [1% BSA in
756 PBS], 2 mL, 30 min incubation at ~23 °C), and incubated with primary antibodies (1 mg/mL stock anti-
757 FLAG primary Ab [Sigma F1804] at a 1:2000 dilution in PBS-BSA; anti-Cit1p antibody [Biomatik Anti-
758 SA160118(Ser)] at a 1:500 dilution in PBS-BSA; 1 mL, 12 h, 4 °C). The anti-Cit1p antibody used in this
759 experiment was generated against the peptide CRPKSFSTEKYKELVKKIESKN (Biomatik, AB001455,
760 Anti-SA160118(Ser), lot # A160414-SF001455, peptide 506543, rabbit RB7668, 0.52 mg/mL). The
761 samples were washed 5 times with PBS-BSA (2 mL, ~23 °C) and incubated with secondary antibodies
762 diluted in PBS-BSA [1 µg/mL working concentration for each: Goat anti-Mouse IgG (H+L) Secondary
763 Antibody, Alexa Fluor 594 conjugate (Thermo A-11005) and Goat anti-Rabbit IgG (H+L) Secondary
764 Antibody, Alexa Fluor 488 (Thermo Cat# A-11008)] (1 mL, 2 h, ~23 °C, in the dark). The samples were

765 washed 5 times with PBS-BSA (2 mL, ~23 °C) and twice with PBS (2 mL, ~23 °C). The last wash was
766 aspirated and the slides were allowed to air dry briefly in the dark. The coverslips were mounted onto slides
767 with 50% glycerol in PBS (8 µL). Fluorescence microscopy was performed on a Keyence BZ-9000
768 microscope using 100X oil immersion optics at room temperature. Line scan analysis was performed with
769 ImageJ.

770 **QUANTIFICATION AND STATISTICAL ANALYSIS**

771 **Overview of Statistical Analyses**

772 For each reported *P*-value, the statistical test used is reported in the legend for the corresponding figure
773 panel. The majority of *P*-values in this report were calculated using an unpaired, two-tailed, Student's *t*-
774 test. In a few select instances, as noted, *P*-values for hypergeometric tests and Spearman correlation
775 coefficients were calculated using the *R* software suite. Also as noted above, a Fischer's exact test was
776 used for a few select qualitative gene set analyses. For yeast experiments, all instances where *n* replicates
777 are reported had *n* biological replicates. As detailed above, for the gene and protein set analyses, *n*
778 indicates the number of genes or proteins in each set: Puf3p cis targets (*n* = 91), Puf3p trans effect proteins
779 (*n* = 49), mitochondrial proteins (*n* = 715), and all profiled proteins (*n* = 3152) in fermenting *Δpuf3* yeast.

780

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