Normal mitochondrial function in *Saccharomyces cerevisiae* has become dependent on inefficient splicing

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Running title: Inefficient splicing at the heart of mitochondrial function

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23 **Self-splicing introns are mobile elements that have invaded a number of highly**

24 **conserved genes in prokaryotic and organellar genomes. Here, we show that**

25 **deletion of these selfish elements from the *Saccharomyces cerevisiae***

26 **mitochondrial genome is stressful to the host. A strain without mitochondrial**

27 **introns displays hallmarks of the retrograde response, with altered**

28 **mitochondrial morphology, gene expression and metabolism impacting growth**

29 **and lifespan. Deletion of the complete suite of mitochondrial introns is**

30 **phenocopied by overexpression of the splicing factor Mss116. We show that, in**

31 **both cases, abnormally efficient transcript maturation results in excess levels of**

32 **mature *cob* and *cox1* host mRNA. Thus, inefficient splicing has become an**

33 **integral part of normal mitochondrial gene expression. We propose that the**

34 **persistence of *S. cerevisiae* self-splicing introns has been facilitated by an**

35 **evolutionary lock-in event, where the host genome adapted to primordial**

36 **invasion in a way that incidentally rendered subsequent intron loss deleterious.**

37

38 Mobile genetic elements frequently compromise host fitness[1], corrupting genetic

39 information or disturbing adaptive gene expression patterns, sometimes to lethal

40 effect. Despite this, mobile genetic elements are ubiquitous in most eukaryotic

41 genomes[2]. How do these selfish elements persist in a genomic environment where –

42 even in the absence of selection – mutational forces constantly work to erode them?

43 Although mobile elements can donate motifs (or domains) that are co-opted into host

44 regulatory pathways (or genic sequence) over time[3], deletions usually whittle away all

45 but the core beneficial motif. The components that once mediated mobility, such as

46 the reverse transcriptases of long interspersed nuclear elements (LINEs), are typically

47 lost. Thus, functional selfish elements that remain mobile are thought to persist over

48   evolutionary time not by virtue of sporadic beneficial effects for the host, but because

49   they replicate and spread to other sites in the genome faster than they are deleted[3].

50   The element survives, not where it originally invaded but as a descendant copy

51   elsewhere in the genome.

52

53   An interesting exception in this regard are self-splicing introns, which populate some

54   highly expressed genes in archaea, bacteria, and organellar genomes of fungi and

55   plants[4]. In contrast to other mobile elements, self-splicing introns do not spawn a

56   large pool of copies that disperse across the genome to escape mutational erasure.

57   Rather, owing to highly specific homing sites, each intron is typically confined to a

58   single location in the host genome and evolutionary persistence seems to rely on

59   continued re-invasion, either from other individuals in the same population[5] or across

60   species boundaries[6-8]. Self-splicing introns can spread despite considerable fitness

61   costs to the host[9]. However, in practice, fitness costs might be relatively low as the

62   host RNA is intact and fully functional once the intron has been spliced out. As far as

63   we know, self-splicing introns do not contribute positively to host fitness and, naively,

64   one would expect that deleting these introns from the genome would be beneficial or,

65   at worst, make no difference to the host.

66

67   Here, we investigate the consequences of deleting all 13 self-splicing introns from the

68   *S. cerevisiae* mitochondrial genome, where they reside in three host genes: the 21S

69   ribosomal RNA gene Q0158 (which harbours a single group I intron named *omega*)

70   and two protein-coding genes, *cox1* (group I: aI3, aI4, aI5α, aI5β; group II: aI1, aI2,

71   aI5γ) and *cob* (group I: bI2, bI3, bI4, bI5; group II: bI1), both encoding components

72   of the electron transport chain. Note here, that we use "self-splicing introns" as a

73   convenient shorthand to describe the complete collection of group I and group II

74   introns, even though timely splicing *in vivo* often depends on one or several *trans*-

75   factors (see below)[4]. We show that, contrary to expectations, removing these introns

76   has dramatic consequences for mitochondrial physiology and function, triggering

77   changes in nuclear gene expression that affect organismal growth and lifespan. Our

78   results demonstrate that the presence of mitochondrial self-splicing introns has

79   become integral to normal mitochondrial gene expression and that, curiously, normal

80   mitochondrial function in *S. cerevisiae* has come to require inefficient splicing. Our

81   findings have implications for understanding how self-splicing introns and mobile

82   elements more generally can survive over evolutionary time without providing an

83   adaptive benefit to the host.

84

85   **Results**

86

87   *Removal of mitochondrial introns is associated with a multi-faceted stress phenotype*

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89   To test whether the removal of self-splicing mitochondrial introns affects host

90   physiology and fitness, we compared two *S. cerevisiae* strains that are isogenic with

91   regard to their nuclear genomes (except for a single marker gene, *ura3*, see Methods)

92   but differ with respect to mitochondrial intron content. The control strain a161 (WT)

93   contains the full complement of seven *cox1* and five *cob* introns, whereas strain a161-

94   U7 (I_0) carries an intronless mitochondrial genome, which was originally constructed

95   by Seraphin and co-workers via serial recombination of natural yeast isolates that lack

96   individual mitochondrial introns[10].

97

98   Contrary to a model where self-splicing introns are dispensable parasitic passengers,

99   we find that I_0 exhibits stark phenotypic differences to the control strain. When the

100 two strains are cultured in isolation, exponential growth on glucose-supplemented

101 YPD medium is ~30% slower for I_0 compared to WT (Figure 1a). I_0 also fares poorly

102 when pitted directly against WT in competitive fitness assay (Figure 1-figure

103 supplement 1). Chronological life span (CLS, see Methods), on the other hand, is

104 almost two-fold longer for I_0 (Figure 1b). At the cellular level, I_0 displays increased

105 mitochondrial mass and volume and a mitochondrial morphology characterized by a

106 large network of branched tubules of homogeneous diameter (Figure 1c-e). Transcript

107 levels of mitofusin (*fzo1*) and the GTPase *mgm1*, key nuclearly encoded regulators of

108 mitochondrial fusion, are strongly upregulated (qPCR, *t*-test, *fzo1*: 4.02-fold, p=0.002;

109 *mgm1*: 5.24-fold, p=3.19×10^{-5}, Figure 2a) while levels of *dnm1* and *fis1*, which

110 orchestrate mitochondrial fission, are only moderately induced (qPCR, *t*-test, *dnm1*:

111 1.67-fold, p=0.026; *dnm1*: 1.81-fold, p=0.085), suggesting that the changes in

112 mitochondrial morphology result from increased fusion rather than impaired fission.

113 The I_0 strain also exhibits a 2.7-fold increase in mitochondrial DNA copy number

114 (qPCR, *t*-test P=1.09×10^{-7}). At the same time, there are no significant differences in

115 mitochondrial   inner   membrane   potential,   as   measured   by   3,3'-

116 Dihexyloxacarbocyanine iodide [DiOC6(3)] fluorescence (Figure 1f), suggesting that

117 mitochondria are functional despite grossly altered morphology. This notion is further

118 supported by the observation that I_0 retains the capacity to grow on glycerol, a non-

119 fermentable carbon source (Figure 1-figure supplement 2), as previously reported for

120 a different nuclear background[11]. In fact, biomarkers of mitochondrial metabolism

121 point to increased mitochondrial activity, with higher oxygen consumption (Figure

122 1g) and cellular ATP levels (Figure 1h) during exponential growth. Despite increased

123   activity, levels of mitochondrial superoxide are reduced (Figure 1i), likely reflecting a

124   >7.5-fold upregulation of the mitochondrial ROS-scavenger *sod2* (qPCR, *t*-test

125   P=0.003, Figure 2a). Thus, intron removal challenges but does not terminally

126   compromise mitochondrial function.

127

128   *Removal of mitochondrial introns triggers the retrograde response*

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130   The phenotypic changes we observe suggest an involvement of the retrograde

131   response, as do upregulation of *cit2*, upregulation of the two rate-limiting members of

132   the TCA cycle, *cit1* and *idh1*, and upregulation of both mitochondrially (*cox1*, *cox2*,

133   *atp6*) and nuclearly (*cox4*, *atp1, sdh1, sdh2*) encoded parts of the respiratory chain

134   (Figure 2a). Indeed, deletion of *rtg2*, the transcriptional master regulator of the

135   retrograde response and a sensor of mitochondrial dysfunction, suppresses the I_0

136   phenotype (Figure 3). Tubular structure is lost and large spherical shapes become

137   prominent (Figure 3a), suggesting distinct defects in the maintenance of

138   mitochondrial ultrastructure[12,13]. Deletion of *rtg2* in WT strains, where the retrograde

139   response is not activated, has no significant effect on mitochondrial volume and

140   morphology, oxygen consumption and ATP levels (Figure 3a-d). The extension of

141   CLS is abrogated in the absence of *rtg2*, becoming shorter even than the wildtype

142   (Figure 3e). Upon deletion of *hap4*, the transcriptional activator of nuclearly encoded

143   components of the respiratory chain (as well as the TCA cycle enzymes under normal

144   conditions), mitochondrial morphology reverts back to the wild-type state (Figure 4a-

145   e). We conclude that an intact retrograde response, including upregulation of nuclear

146   components of the respiratory chain, is necessary to generate the mitochondrial

147   phenotype observed in the I_0 strain.

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Promoter attenuation of cox1 *and* cob *reverses the phenotype*

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To determine the ultimate molecular trigger(s) of the retrograde response, we examined how intron removal affects host gene expression. As previous work had found the *omega* intron to be "optional" – present in some yeast strains but absent in others without obvious phenotypic effects[14,15] – we focused on *cox1* and *cob*. For both these genes, mRNA levels are strongly elevated in I_0 (10.9-fold and 5.8-fold for *cox1* and *cob,* respectively; Figure 2a) but also in $\Delta rtg2$ (Figure 3f) and $\Delta hap4$ (Figure 4f), suggesting that this is a direct effect of intron removal rather than a downstream consequence of activating the retrograde response. To investigate whether elevated *cox1* and/or *cob* transcript levels might underpin the wider transcriptional, metabolic and phenotypic changes, we introduced attenuating point mutations into the promoters of *cox1* and *cob* (see Methods). We find that simultaneous attenuation of both promoters in I_0 fully reverses the I_0-characteristic suit of morphological and molecular phenotypes (see I_0pp throughout Figure 5). Attenuation of either *cob (I_0cobp)* or *cox1* *(I_0cox1p)* in isolation only partially reverses the phenotype, although I_0cobp has a larger relative effect than I_0cox1p (Figure 5).

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The effects of intron removal are phenocopied by overexpression of Mss116

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Next, we sought to establish why the wholesale deletion of self-splicing introns leads to increased abundance of the host transcripts. We considered two main possibilities. First, in deleting introns, we might have inadvertently removed DNA-/RNA-level regulatory elements that affect expression of the host genes. Alternatively, the act of

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173 short-circuiting the splicing process itself might interfere with normal expression.

174 Specifically, we hypothesized that normal levels of transcription might be tuned to

175 accommodate a certain proportion of transcripts that fail to splice correctly. Group II

176 introns in particular are known for low splicing efficiency, even in the presence of

177 auxiliary proteins[16]. As a corollary, a large fraction of pre-mRNAs might be targeted

178 and degraded by mitochondrial quality control, either because splicing is erroneous

179 (mis-splicing) or does not occur in a timely manner so that the transcript is shunted

180 into degradation (kinetic coupling). In I_0, splicing does not occur so that erroneous

181 splicing products do not arise. As a result, production of functional *cox1/cob* mRNAs

182 might overshoot its target and trigger a system-wide response, for example because

183 altered COX1/COB levels upset dosage balance amongst respiratory complexes. To

184 test this abnormally-efficient-maturation hypothesis and simultaneously rule out that

185 mitochondrial stress is caused by the removal of DNA-/RNA-level regulatory

186 elements, we sought to alter splicing efficiency by orthogonal means. To this end, we

187 overexpressed the nuclearly encoded DEAD box RNA helicase Mss116, which

188 promotes splicing of all *S. cerevisiae* mitochondrial introns by remodeling or

189 stabilizing splice-relevant RNA structures in an ATP-dependent manner[16,17]. We

190 confirmed overexpression and mitochondrial localization of Mss116 by flow

191 cytometry and immunofluorescence, respectively, using an N-terminal His-tagged

192 version of the protein (Figure 1-figure supplement 3), and then characterized the

193 effects of Mss116 overexpression in the Y258 strain using an untagged version of the

194 protein. Remarkably, the Mss116 overexpression strain (*Mss116$_{OE}$*) phenocopies I_0.

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196 *Mss116$_{OE}$* exhibits increased generation times, extended chronological life span,

197 lower competitive fitness, increased mitochondrial fusion, 2.9-fold increased mtDNA

198   copy number (qPCR, t-test P=5.5×10^{-7}), elevated oxygen consumption, and altered

199   ATP and ROS production (Figure 1, Figure 1-figure supplement 1). In addition, we

200   observe longer replicative life span (RLS) in $Mss116_{OE}$ (Figure 1-figure supplement

201   4), a more direct proxy of ageing that we were not able to measure accurately in I_0,

202   where separating mother and daughter cells in a timely fashion proved challenging

203   (see Methods). Overexpression of a DEAD box mutant of Mss116 ($Mss116^{E268K}$),

204   which lacks ATPase and therefore helicase activity, does not phenocopy I_0 (Figure 1).

205   This suggests that the role of Mss116 in splicing – which relies on helicase activity –

206   is critical rather than a recently suggested ATP-independent role in transcription

207   elongation[18]. More generally, the fact that $Mss116_{OE}$ – which encodes a full

208   complement of introns – phenocopies I_0 indicates that the stress phenotype in these

209   strains is not caused by missing DNA-level functionality and instead points towards a

210   critical role for splicing. Overexpressing Mss116 in I_0 did not reveal additional

211   phenotypes and the strain behaved like I_0 (Figure 2-figure supplement 1) further

212   supporting the notion that intron deletion and Mss116 overexpression act through the

213   same pathway.

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215   *Cells are stressed because of abnormally efficient transcript maturation*

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217   We suspected that phenotypic effects of Mss116 overexpression (and intron deletion)

218   are linked to altered transcript maturation of the host genes, *cob* and *cox1*. However,

219   we first wanted to rule out an alternative hypothesis. In both $Mss116_{OE}$ and I_0,

220   Mss116 is in excess relative to need (in $Mss116_{OE}$ because Mss116 is overexpressed,

221   in I_0 because its usual targets – the introns – are absent). Phenotypic effects could

222   therefore be caused by excess Mss116 interacting with RNAs that it would not

223 normally target or not target to the same extent. This is conceptually related to the

224 idea that splicing dynamics can change simply as a result of altered competition

225 between mRNAs for access to the spliceosome[19]. To test this hypothesis, we deleted

226 Mss116 in the I_0 strain. We observe the same suite of phenotypes we see in I_0 (Figure

227 2-figure supplement 2), demonstrating that neither absolute nor relative excess of

228 Mss116 are responsible for the stress phenotype described above.

229

230 Having ruled out this hypothesis, we then focused on characterizing the effects of

231 Mss116 overexpression on *cob/cox1* splicing dynamics in greater detail. Using qPCR,

232 we first measured the levels of total *cox1* and *cob* transcripts as well as individual

233 introns at steady state (Figure 2a, 240 mins after Mss116 induction, see Figure 2-

234 figure supplement 3). For *cox1,* we additionally monitored exon and intron (aI2, aI5β)

235 levels using RNA fluorescent *in situ* hybridization (RNA FISH, Figure 2b, see

236 Methods). We find that most mitochondrial introns are strongly depleted in $Mss116_{OE}$

237 compared to the empty vector control and relative to exons. The relative depletion of

238 individual introns is somewhat variable and one intron – aI5α - is equally abundant in

239 $Mss116_{OE}$ and the Y258 WT (Figure 2a). We then examined shifts in the abundance

240 of pre-mRNA and mature mRNA over the course of Mss116 induction, using

241 different primer combinations to monitor unspliced RNAs and spliced exon-exon

242 junctions. For both *cox1* and *cob,* Mss116 overexpression shifts the balance between

243 unspliced (or partially spliced) pre-mRNA transcripts, which dominate the uninduced

244 steady state, towards mature mRNA transcripts (Figure 2c), while total transcript

245 abundance (mRNA plus pre-mRNA) remains largely unchanged. These observations

246 are consistent with a model where nascent transcriptional output is unchanged in

247   *Mss116$_{OE}$* compared to the empty vector control and differential steady state levels

248   are the result of post-transcriptional events.

249

250   Based on these findings, we suggest that *Mss116$_{OE}$* phenocopies *I$_0$* because

251   eliminating introns at the DNA level (*I$_0$*) and facilitating accurate and efficient

252   excision at the RNA level (*Mss116$_{OE}$*) both result in abnormally efficient transcript

253   maturation. That is, fewer transcripts are eliminated by mitochondrial quality control

254   because splicing is erroneous or does not proceed in a timely manner, resulting in a

255   greater number of mature *cox1/cob* mRNAs. For reasons that remain to be elucidated,

256   increased transcript levels are then perceived as stressful and trigger the retrograde

257   response, culminating in a multifaceted stress phenotype. We speculate in this regard

258   that elevated protein levels of COB (*I$_0$*: 9.3-fold; *Mss116$_{OE}$*: 5.0-fold upregulation, as

259   determined by quantitative label-free mass spectrometry) and COX1 (*I$_0$*: 11.6-fold;

260   *Mss116$_{OE}$*: 12.1-fold upregulation) might interfere with proper assembly and function

261   of complex III and complex IV, respectively, and therefore constitute a deleterious

262   dosage imbalance phenotype.

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264

265   **Discussion**

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267   It is now well documented that disruption of splicing homeostasis can impact normal

268   physiological function and lead to cellular stress and disease[20]. There is also

269   increasingly detailed mechanistic knowledge of how proteins involved in splicing can

270   alter growth and ageing via a metabolic route, exemplified by the recent finding that

271   splicing factor 1 is a modulator of dietary restriction-induced longevity in

272 *Caenorhabditis elegans*[21]. The classic model here is that loss of splicing homeostasis

273 – through genetic, developmental, or environmental perturbation – leads to

274 deleterious shifts in splice isoform production or precipitates increased production of

275 erroneous transcripts that tax the quality control system and/or have direct cytotoxic

276 effects. In other words, the disease/stress state is a high-error state. Our results are

277 unusual in that they suggest that normal splicing can be associated with high error

278 rates and that, therefore, splicing homeostasis can also be disturbed by increasing

279 splicing efficacy.

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281 Further research will be required to tease apart how individual mitochondrial introns

282 affect the overall burden from failed splicing in this system. It is evident from

283 population genomic analysis of different *S. cerevisiae* strains that some mitochondrial

284 introns are fixed across extant populations whereas others exhibit presence/absence

285 polymorphism[14]. This seems to suggest that the removal of at least some introns in

286 isolation is insufficiently stressful to be purged by natural selection. At the same time,

287 studies of *suv3,* the second DEAD box RNA helicase present in yeast mitochondria,

288 suggest that the deleterious effect of deleting individual introns – while possibly

289 idiosyncratic – is at least partially cumulative: deletion of *suv3,* a component of the

290 mitochondrial degradosome, decreases levels of mature *cox1/cob* mRNA and

291 compromises respiratory capacity, but less so where more introns had been removed

292 from the mitochondrial DNA[22]. Importantly – at least for the combinations tested –

293 severity was found to depend on the number but not identity of the *cox1* or *cob*

294 introns present.

295

296 In addition to providing a new insight into post-transcriptional gene regulation in

297 mitochondria, our findings have implications for understanding the evolutionary

298 persistence of self-splicing introns and perhaps mobile elements more generally. The

299 phenotypic effects we observe run counter to the notion that self-splicing introns are

300 low-cost passengers and instead demonstrate that at least some of these selfish

301 elements are firmly embedded in the organization of mitochondrial gene expression

302 such that removing them upsets proper expression of their host genes. We suggest that

303 our observations can be explained by an evolutionary lock-in model where the

304 primordial colonization of an intron-free *cox1/cob* ancestor by a self-splicing intron

305 led to a drop in *cox1/cob* mRNA levels and favoured compensatory mutations that

306 increased *cox1/cob* transcription to restore mRNA abundances back to their original

307 levels. When we forcibly remove these introns, however, this hard-wired upregulation

308 turns maladaptive. There no longer is a pool of transcripts targeted for degradation

309 leading to excess levels of mature mRNA. In principle, it is also possible that the

310 initial invader spliced very efficiently and imposed no cost but subsequently co-

311 evolved with host gene expression in a ratchet-like fashion, whereby incidental

312 greater-than-required levels of the host gene allowed recurrent small decreases in

313 splicing efficiency. However, since self-splicing introns can spread despite substantial

314 fitness costs[9], we do not actually need to evoke a cost-free ancestral event. We

315 suggest that evolutionary lock-ins of this type might provide an unappreciated

316 mechanism to facilitate the longer-term persistence of genetic parasites, especially in

317 large host populations where evolution is not mutation-limited. We also note that this

318 argument might in principle extend to nuclear introns: if, for a given dosage-sensitive

319 gene, failure to splice is common and transcription levels are set to compensate, intron

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320   loss might be deleterious and prevented by purifying selection even though the intron

321   makes no adaptive contribution to gene regulation.

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323

324

325   **Methods**

326   **Strains and growth conditions**

327   Strain a161 (also known as ID41-6/161, or sometimes simply 161), described by

328   Wenzlau et al.[23], and the intronless a161-U7 (I_0) were gifts from Alan Lambowitz.

329   These strains were used previously to show that splicing of group I and II introns is

330   Mss116-dependent[17]. a161 and a161-U7 are isogenic except for the mitochondrial

331   genome and a single marker gene (a161: MATa ade1 lys1; a161-U7: MATa ade1 lys1

332   ura3).

333   Strain Y258 and the pBG1805 plasmid bearing *Mss116* for overexpression were

334   purchased from Thermo Scientific (Dharmacon). *Mss116^{E268K}* was purchased from

335   DNA 2.0 and cloned into pBG1805 using standard cloning techniques[24]. Mss116 and

336   Mss116^{E268K} were overexpressed in the Y258 nuclear background. The expression of

337   Mss116 and Mss116^{E268K} was induced from the plasmids using 2% galactose (final),

338   added at OD 0.2.

339   All strains were grown on YPD medium with 2% (w/v) glucose at 30°C with shaking.

340   All experiments were performed on exponentially growing cells: cells were grown to

341   OD 0.6-0.7 for WT, I_0, ΔHap4, and ΔRtg2 and to OD 0.9-1.0 for *Mss116$_{OE}$* and

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342     $Mss116^{E268K}$, harvested by centrifugation at $4000 \times g$ for 5 minutes, washed and

343   further treated as required.

344   In order to test growth on glycerol, strains were grown in YPD medium until

345   saturation, at 30 °C with shaking. Stationary cells were serially diluted and 5 μL drops

346   plated onto YPEG agar plates (containing 3% ethanol and 3% glycerol). Growth was

347   observed after 4 days.

348   **Gene deletion**

349   Deletion of *hap4* and *rtg2* was performed as previously described[25], using a

350   hygromycin cassette for selection in the WT background and a nourseothricine

351   cassette in the I_0 background. Primers used for the deletions are listed in

352   Supplementary File 1.

353   **Measurement of splicing kinetics in *Mss116$_{OE}$***

354   Overnight cultures of *Mss116$_{OE}$* were diluted to OD 0.1. Galactose (2% final) was

355   added at OD 0.2 to induce the expression of Mss116, and this is designated as time 0.

356   Aliquots of the culture were harvested at 30, 60, 90, 120, 135, 150, 180, 210 and 240

357   minutes post-induction. The cells were pelleted and used for RNA isolation and

358   cDNA preparation for qPCR. Multiple primer pairs were used to monitor unspliced

359   intron-exon fragments and spliced exon-exon junctions (Figure 2-figure supplement

360   4, Supplementary File 1). Additional aliquots were harvested at 60, 150, 300, 360,

361   420, and 540 minutes post-induction and were used to measure Mss116 expression

362   levels by flow cytometry (see below).

363

Insertion of point mutations into the promoter regions

In order to introduce promoter-attenuating mutations into mitochondrial DNA, we followed the protocol described in[26] for the integration of altered mtDNA sequences by homologous double crossovers. Briefly, a mutant fragment of mtDNA (in this case promoter sequences) flanked by WT mtDNA sequence is first transformed into a *rho⁰* strain, which is then mated with a recipient *rho⁺* strain. Upon mating, mitochondria from the two strains fuse and recombination between the two mtDNAs produces recombinant *rho⁺* strains in which the new mtDNA sequence is integrated by double crossover. For transformation, tungsten powder was used as a carrier of DNA (Tungsten M-10 Microcarriers #1652266, BioRad). Bombardment was performed using the Biolistic PDS-1000/He particle delivery system (BioRad). Cells were transformed with linear DNA fragments obtained by ligation of each mutated promoter region with 500bp of up- and downstream flanking DNA (Supplementary File 1). SacI and SalI restriction sites were added by PCR for ligation between the 3'-end of the upstream flanking region and the 5'-end of the promoter sequence, and 3'-end of the promoter sequence and 5'-end of the downstream flanking region, respectively. The mutations introduced here (highlighted in Figure 5-figure supplement 1) have been previously shown to reduce the strength of *cox1* and *cob* promoters[27].

Chronological lifespan measurement

All strains were grown to saturation as described above and pelleted at $4000 \times g$ for 5min. Cells were then washed twice and resuspended in sterile deionized water (10^6

387   cells in 10mL in order to avoid cell growth on the debris of dead cells) and incubated

388   at 30°C with shaking. Every 2-3 days, cells were serially diluted and plated onto YPD

389   plates in order to evaluate cell growth.

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391   **Replicative lifespan measurement**

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393   Replicative lifespan (RLS) was determined by micromanipulation for $Mss116_{OE}$ and

394     $Mss116^{E268K}$ in the Y258 background, as well as for $Mss116_{OE}$ in the $\Delta hap4$ and $\Delta rtg2$

395   nuclear backgrounds, counting the number of daughters produced by individual

396   mother cells. We were unable to reliably determine RLS for the I_0 strain as daughter

397   and mother cells could not be separated in a timely manner, which is critical for RLS

398   measurements. For unknown reasons, and perhaps specific to the nuclear background,

399   cells were unusually sticky so that the first daughter often could not be separated from

400   the mother until the mother had already produced other buds, making it difficult to

401   track mother/daughter identity over time, an essential prerequisite for reliably

402   determining replicative lifespan. Cells were incubated at 30°C on YPD (WT) or -

403   URA (mutants) plates for the duration of the experiment. Using a microscope

404   equipped with a microdissection apparatus suitable for *S. cerevisiae* (Singer

405   Instruments), cells were transferred to defined places on agar plates and virgin

406   daughter cells collected. Each cell was monitored continuously over several days

407   every 60 - 90min until all mother cells stopped budding. The total number of daughter

408   cells was noted for each mother cell. The total number of monitored mother cells is as

409   follows: 90 cells for the empty vector control, 86 cells for $Mss116_{OE}$, and 91 cells for

410   the $Mss116^{E268K}$. The measurements were pooled from 3 independent experiments.

411

Respiration measurement

Oxygen uptake was monitored polarographically using an oxygraph equipped with a Clark-type electrode (Oxygraph, Hansatech, Norfolk, UK). Cells were harvested during exponential growth phase, spun and resuspended in growth medium (as above) at the density of 30×10^6 cells/mL. 500μL of culture were transferred to an airtight 1.5mL oxygraph chamber. Cells were assayed in conditions closely similar to the ones in a flask culture (30°C and stirring). Oxygen content was monitored for at least 4min. To ensure that the observed oxygen consumption was due to the mitochondrial activity, complex III inhibitor antimycin (final concentration 10μg/mL) was routinely added to the cultures and compared to the rate observed without antimycin.

Competition assay

Competition experiments were carried out between I_0 (a161-U7) and its control strain (a161) as well as between $Mss116_{OE}$ and its corresponding empty vector control strain. Prior to the competition, we plated 5×10^9 cells of each mutant (I_0 or $Mss116_{OE}$) strain on YPD agar plates with 200 μg/mL geneticin, followed by a 4-day incubation at 30°C, to select for spontaneous geneticin resistance. In doing so, we can subsequently determine relative fitness in a relatively simple fashion using a plating method (rather than, for example, sequencing barcodes). The two strains to be competed were then grown independently on YPD medium in 2% glucose until saturation. The next day, an equal number of cells from each WT culture (geneticin-sensitive) and each mutant (geneticin-resistant) were mixed in fresh YPD medium, 2% glucose so that each was diluted 200x. In mid-exponential phase, OD 0.4-0.6

435   (approximately 5-6 hours after dilution), aliquots of cells were harvested, and serial

436   dilutions were plated on YPD-agar plates without geneticin. Next, dilutions with

437   between 50 and 200 colonies were replica-plated on YPD-agar plates with geneticin

438   (200 µg/mL). Colonies were counted on both types of plates and the ratio

439   of geneticin-resistant (mutant) to geneticin-sensitive (WT) colonies was calculated as

440   a measure of relative fitness.

441

442   **Flow cytometry**

443   Flow cytometry was carried out on a Becton-Dickinson FACSCalibur machine

444   equipped with a 488nm Argon laser and a 635nm red diode laser.

445

446   **Measurement of the Mss116 overexpression level**

447   The expression level of Mss116 in the *Mss116$_{OE}$* strain was measured by using a

448   rabbit polyclonal anti-His tag antibody (Abcam, ab137839, 1:10000) and secondary

449   IgG goat anti-rabbit labeled with Alexa 488 (Thermo Fisher Scientific, A11034,

450   1:2000). The signal obtained by flow cytometry (mean fluorescence over 10000 cells)

451   was compared to the Mss116 tagged with GFP (Thermo Scientific) endogenous

452   expression level estimated by using flow cytometry measurement based on the GFP

453   signal. The mean fluorescence intensity in *Mss116$_{OE}$* was normalized to the mean

454   fluorescence intensity detected in wild type cells with endogenous expression of

455   Mss116.

456   **Mass spectrometry - sample processing**

457 Isolated mitochondrial fractions containing 100µg of protein were loaded onto

458 Microcon 30kD centrifugal filters (Merck Millipore, MRCF0R030). Samples were

459 then digested using a Filter Aided Sample Preparation (FASP) protocol[28]. Briefly,

460 samples were concentrated on the filter unit by centrifugation and buffer exchanged

461 using sequential washing and centrifugation with 8M urea, 100mM TRIS/HCL buffer

462 (pH8.5). Proteins were reduced and alkylated sequentially with 10mM Dithiothreitol

463 and 50mM Iodoacetamide (in 8M urea buffer), respectively. Samples were buffer

464 exchanged to remove salts using sequential washing with 50mM ammonium

465 bicarbonate (AmBic). Trypsin Gold (Promega, V5280) was added to the samples in

466 50mM ammonium bicarbonate to an approximate 1:50, protease:protein ratio.

467 Digestions were incubated at 37°C overnight (17h). Digest extracts were recovered

468 from FASP filters via centrifugation and acidified with 1% trifluoroacetic acid (TFA).

469 Acidified protein digests were desalted using Glygen C18 spin tips (Glygen Corp,

470 TT2C18.96) according to the manufacturer's recommendation and peptides eluted

471 with 60% acetonitrile, 0.1% formic acid (FA). Eluents were then dried using a

472 vacuum centrifuge.

473

474 **Mass spectrometry - liquid chromatography-tandem mass spectrometry (LC-**

475 **MS/MS) analysis**

476

477 Protein digests were redissolved in 0.1% TFA by shaking (1200rpm) for 30min and

478 sonication on an ultrasonic water bath for 10min, followed by centrifugation

479 (14,000rpm, 5°C) for 10min. LC-MS/MS analysis was carried out in technical

480 duplicates (1µg on column) and separation was performed using an Ultimate 3000

481 RSLC nano liquid chromatography system (Thermo Scientific) coupled to a Orbitrap

482   Velos mass spectrometer (Thermo Scientific) via an Easy-Spray source. For LC-

483   MS/MS analysis protein digests were injected and loaded onto a trap column

484   (Acclaim PepMap 100 C18, 100μm × 2cm) for desalting and concentration at

485   8μL/min in 2% acetonitrile, 0.1% TFA. Peptides were then eluted on-line to an

486   analytical column (Easy-Spray Pepmap RSLC C18, 75μm × 50cm) at a flow rate of

487   250nL/min. Peptides were separated using a 120 minute gradient, 4-25% of buffer B

488   for 90 minutes followed by 25-45% buffer B for another 30 minutes (composition of

489   buffer B – 80% acetonitrile, 0.1% FA) and subsequent column conditioning and

490   equilibration. Eluted peptides were analysed by the mass spectrometer operating in

491   positive polarity using a data-dependent acquisition mode. Ions for fragmentation

492   were determined from an initial MS1 survey scan at 30,000 resolution, followed by

493   CID (Collision Induced Dissociation) of the top 10 most abundant ions. MS1 and

494   MS2 scan AGC targets were set to 1e6 and 3e4 for maximum injection times of

495   500ms and 100ms respectively. A survey scan m/z range of 350 – 1500 was used,

496   normalised collision energy set to 35%, charge state screening enabled with +1 charge

497   states rejected and minimal fragmentation trigger signal threshold of 500 counts.

498

499   **Mass spectrometry - raw data processing**

500

501   Data was processed using the MaxQuant software platform (v1.5.6.0), with database

502   searches carried out by the in-built Andromeda search engine against the Uniprot

503   *S.cerevisiae* database (version 20160815, number of entries: 6,729). A reverse decoy

504   database approach was used at a 1% false discovery rate (FDR) for peptide spectrum

505   matches. Search parameters included: maximum missed cleavages set to 2, fixed

506   modification of cysteine carbamidomethylation and variable modifications of

507 methionine oxidation, asparagine deamidation and N-terminal glutamine to

508 pyroglutamate conversion. Label-free quantification was enabled with an LFQ

509 minimum ratio count of 2. 'Match between runs' function was used with match and

510 alignment time limits of 1 and 20 minutes respectively. Data have been deposited in

511 the PRIDE repository [project accession number PXD008785].

512

513 **Assessment of mitochondrial membrane potential and mass**

514 Variations of the mitochondrial transmembrane potential ($\Delta\Psi$m) were studied using

515 3,3-dihexyloxacarbocyanine iodide [DiOC6(3)]. This cyanine cationic dye

516 accumulates in the mitochondrial matrix as a function of $\Delta\Psi$m[29]. Cells (1×10^6/mL)

517 were incubated in 1mL culture medium containing 40nM DiOC6(3) for 30min in the

518 dark at 30°C with constant shaking. DiOC6(3) membrane potential-related

519 fluorescence was recorded using FL1 height. A total of 10,000 cells were analyzed for

520 each curve. The collected data was analyzed using FlowJo software version 7.2.5 to

521 determine the mean green fluorescence intensity after each treatment. The results are

522 expressed as a percentage of mean fluorescence of the control strain. As a negative

523 control, in each experiment, we preincubated aliquots of cells with carbonyl-cyanide

524 4-(trifluoromethoxy)- phenylhydrazone (FCCP, Sigma) and antimycin (Sigma) at

525 100μM and 5μg/mL, respectively, 10min before fluorescent dye staining, which leads

526 to a collapse of mitochondrial membrane potential.

527 To measure mitochondrial mass, we used 10-N-Nonyl acridine orange (NAO), a dye

528 that binds to cardiolipin, a phospholipid specifically present on the mitochondrial

529 membrane[29]. Cells (1×10^6/mL) were incubated in 1 mL culture medium containing

530 100nM NAO for 30min in the dark at 30°C with constant shaking, followed by

531   analysis on FACSCalibur flow cytometer with the same photomultiplier settings as

532   used for DiOC6(3).

533

534   Evaluation of the mitochondrial morphology and protein import machinery

535   To image mitochondrial morphology, strains were transformed with a MitoLoc

536   plasmid[30] (a gift from Markus Ralser) according to a previously described protocol[31],

537   with the only difference that the cells were incubated with the plasmid overnight at

538   room temperature. Microscope slides were prepared as follows: 150μL of YPD media

539   containing 2% agarose was placed on a preheated microscope slide and cooled, before

540   applying yeast cells to obtain a monolayer. The cells were centrifuged at $4000 \times g$ for

541   3min, and resuspended in 50μL YPD. Once dry, the cover slip was placed, sealed, and

542   mounted on a temperature-controlled Nikon Ti-E Eclipse inverted/UltraVIEW VoX

543   (Perkin Elmer) spinning disc confocal setup, driven by Volocity software (version

544   6.3; Perkin Elmer). Images were recorded through a 60xCFI PlanApo VC oil

545   objective (NA 1.4) using coherent solid state 488nm and 543nm diode lasers with a

546   DPSS module, and a 1000×1000 pixel 14-bit Hamamatsu (C9100-50) electron-

547   multiplied, charge-coupled device (EMCCD). The exposure time was 100ms for GFP

548   and 300ms for mCherry, at 5–10% laser intensity. The number of cells with cytosolic

549   mCherry accumulation was counted manually. More than 1000 cells were examined

550   for each strain. Images were analysed using ImageJ software with the MitoLoc

551   plugin.

552

553   ROS measurement

554 Cells were incubated in the dark with 5μM MitoSOX™ red mitochondrial superoxide

555 indicator (Molecular Probes) for 10min at 30°C and subsequently analyzed by flow

556 cytometry. Fluorescence (excitation/emission maxima of 510/580nm) of 10,000 cells

557 resulting from the intracellular red fluorescence was measured in the FL2 channel.

558 The collected data was analyzed using FlowJo software version 7.2.5 for Microsoft

559 (TreeStar, San Carlos, CA, USA) to determine the mean green fluorescence intensity

560 after each treatment. The results are expressed as the mean fluorescence across 10,000

561 cells.

562

563 **RNA extraction**

564 Total RNA was isolated using the NucleoSpin RNA kit (Macherey&Nagel) according

565 to the manufacturer's instructions for up to 3×10^8 yeast cells, which includes

566 incubation with 50–100U of zymolyase for 1hr at 30°C. The quality of the resulting

567 total RNA was tested on 1% agarose gels.

568

569 **Genomic DNA isolation**

570 Cells from saturated cultures (approximately 10^9 cells) of WT Y258, WT a161, I_0,

571 and $Mss116_{OE}$ were harvested by centrifugation and washed, as described above. Cell

572 wall was digested for 1h at 30°C in the presence of 50–100U of zymolyase.

573 Spheroplasts were then resuspended in 500μL of cell lysis buffer (75mM NaCl,

574 50mM EDTA, 20mM HEPES pH 7.8, 0.2% SDS). Next, 10μL of 20mg/mL

575 proteinase K was added and the mixture incubated for 2h at 50°C. DNA was

576   precipitated by the addition of isopropanol at room temperature, followed by

577   centrifugation at 4°C and 11000g for 30min. The DNA pellet was then washed with

578   ice-cold 70% ethanol, air-dried and resuspended in 50μL of DNase free water at

579   55°C.

580

581   Quantitative real-time PCR (qPCR)

582   cDNA was synthesized from 1000ng of total RNA using the iScript™ cDNA

583   Synthesis Kit (Biorad). The cDNA was diluted 100-fold, mixed with primer pairs for

584   each gene and SYBRgreen (BioRad). All primer pairs were designed to have a

585   melting temperature of 60°C and are listed in Supplementary File 1. The qPCR

586   reaction was run on a QuantFlexStudio 6 (Life Technologies) using 40 cycles, after

587   which the melting curves for each well were determined. Final fold change values

588   were estimated relative to the UBC6 gene in the control strain replicates.

589   mtDNA copy number was assessed by qPCR using genomic DNA as template and

590   primers against *cox1* and *cox3* (mtDNA) and *rpl32* (nuclear DNA, for normalization).

591

592   Single cell generation time measurement

593   Individual cells (approximately 100 for each strain) were placed on agar plates of

594   appropriate growth medium, as described above, using a micromanipulator. Next, an

595   image of each original mother cell was taken every 10 minutes for 8-9 hours. The

596   images were then analysed and division time of each cell was extracted.

597

598

599

RNA-FISH and imaging

601

Yeast cultures were grown as described above, fixed with 37% formaldehyde for
45min at room temperature, digested with 2.5μL of zymolyase (Zymo Research,
2000 U) at 30°C for 60min and permeabilized with 70% ethanol overnight at 4°C.
Cells were hybridized in the dark at 30°C using Stellaris RNA-FISH probes
(Biosearch Technologies). 45 probes targeting intron aI2 and 40 probes targeting
intron aI5b *cox1* were coupled to Quasar 670 dye (red). 43 probes targeting *cox1*
exons were coupled to Quasar 570 dye (green). Yeast cells were placed on
microscope slides with Vectashield Mounting Medium and imaged with an Olympus
IX70 wide-field fluorescence microscope. A series of z-stacks was acquired with a
step size of 0.3μm. The images were analysed using Image J. The number of green
(exon), red (intron) and yellow (colocalized) foci was manually counted and
normalized per 100 cells in each of three biological replicates. At least 300 cells were
analyzed per replicate per strain.

615

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Competing financial interests

The authors declare that no competing financial interests exist.

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Figure legends

718

Figure 1. Phenotypic effects of deleting all self-splicing introns from the *S. cerevisiae* mitochondrial genome.

Deletion of mitochondrial introns (I_0) or overexpression of Mss116 (*Mss116$_{OE}$*) (a) reduces growth rates, (b) extends chronological lifespan, (c) increases mitochondrial mass, measured as NAO fluorescence, (d,e) increases mitochondrial volume, (g)

724 oxygen consumption, and (h) ATP levels but (i) decreases superoxide levels,

725 measured as MitoSOX fluorescence. (f) Mitochondrial inner membrane potential does

726 not differ significantly between strains. WT a161 and WT Y258 are control strains for

727 I_0 and $Mss116_{OE}$, respectively, as described in the text. The $Mss116^{E268K}$ strain

728 harbours a mutant version of Mss116 that lacks ATPase activity. As a visual guide,

729 strains are coloured consistently throughout. Bar heights display the mean of three

730 biological replicates, each calculated as the mean of three technical replicates. Error

731 bars are standard errors of the mean. ***P < 0.001; **P < 0.01; *P < 0.05 (ANOVA

732 plus post hoc).

733

734 **Figure 2. RNA abundance changes associated with intron removal.**

735 (a) qPCR measurements of selected genes, comparing focal strains (I_0, $Mss116_{OE}$,

736 $Mss116^{E268K}$) to their isogenic control strains (left panel). For both *cox1* (central

737 panel) and *cob* (right panel), intron levels are specifically reduced upon

738 overexpression of $Mss116_{OE}$ but not $Mss116^{E268K}$, while mature mRNA levels

739 increase. Heat maps display mean values of log-fold changes observed across three

740 biological replicates (each averaged over three technical replicates). UBC6 was used

741 for normalization.

742 (b) RNA-FISH confirms elimination/reduction of introns aI2 and aI5β from the *cox1*

743 transcript pool. Exon (green), intron (red) and co-localized (green/red) puncta were

744 counted in more than 300 cells. The bar chart shows the number of signals per 100

745 cells. Bar heights display the mean of three biological replicates (each averaged over

746 three technical replicates). Error bars are standard error of the mean. ***P < 0.001;

747 **P < 0.01; *P < 0.05 (ANOVA plus post hoc). White lines mark cell boundaries.

748   White arrows mark examples of exonic puncta that do not co-localize with intronic

749   puncta.

750   (c) qPCR time series of pre-mRNA and mature mRNA levels following induction of

751   Mss116. Mature mRNA for *cox1* and *cob* was quantified using primer pairs (f_{xT}, f_{bT})

752   overlapping the terminal exon-exon junctions. Pre-mRNA was quantified using a

753   series of primer pairs (f_{x1-5}, f_{b1-5}). For each pair, one primer is located in exonic, the

754   other in intronic sequence, as detailed in Figure 2-figure supplement 4. Each circle

755   (shades of blue for the pre-mRNA and red for the mature transcript) represents the

756   mean value from three biological replicates (each averaged over three technical

757   replicates). UBC6 was used for normalization.

758

759   **Figure 3. The intronless phenotype requires a functional retrograde response.**

760   (a) Mitochondrial morphology is altered and (b) mitochondrial volume, (c) oxygen

761   consumption, (d) ATP levels, and (e) chronological lifespan are reduced when *rtg2* is

762   deleted in the I_0 or $Mss116_{OE}$ background. This contrasts sharply with I_0 and

763    $Mss116_{OE}$ where *rtg2* is intact (see Figure 1). Bar heights display the mean of three

764   biological replicates (each averaged over three technical replicates). Error bars are

765   standard error of the mean. ***$P < 0.001$; **$P < 0.01$; *$P < 0.05$ (ANOVA plus post

766   hoc). (f) Transcriptional responses in different strains where *rtg2* has been deleted, as

767   measured by qPCR. Heat maps display mean values of log-fold changes observed

768   across three biological replicates (each averaged over three technical replicates).

769   UBC6 was used for normalization.

770

771   **Figure 4. Hap4 is required for the intronless phenotype.**

772   (a) Mitochondrial morphology, (b) mitochondrial volume, (c) oxygen consumption,

773   (d) ATP levels, and (e) chronological lifespan do not differ between I_0/$Mss116_{OE}$ and

774   their corresponding control strains if *hap4* has been deleted. Bar heights display the

775   mean of three biological replicates (each averaged over three technical replicates).

776   Error bars are standard error of the mean.   ***P < 0.001; **P < 0.01; *P < 0.05

777   (ANOVA plus post hoc). (f) Transcriptional responses in different strains where *hap4*

778   has been deleted, as measured by qPCR. Heat maps display mean values of log-fold

779   changes observed across three biological replicates (each averaged over three

780   technical replicates). UBC6 was used for normalization.

781

782   **Figure 5. Dampening RNA levels of *cox1* and *cob* by reducing promoter activity**

783   **partially rescues the intronless phenotype.**

784   (a) qPCR measurements in strains where either the *cob* promoter (cobp), the *cox1*

785   promoter (cox1p) or both (pp) have been attenuated via targeted mutations. Heat maps

786   display mean values of log-fold changes observed across three biological replicates

787   (each averaged over three technical replicates). UBC6 was used for normalization.

788   (b) Oxygen consumption and (c) mitochondrial morphology and (d) volume in

789   response to promoter attenuation. Bar heights display the mean of three biological

790   replicates (each averaged over three technical replicates). Error bars are standard error

791   of the mean. Asterisks to indicate statistical significance are omitted for clarity. All

792   comparisons between single- and double-promoter mutants and the corresponding

793   parent strains are significant at P<0.001 (ANOVA plus post hoc).

794
795
796   **Supplementary Figure Legends**

797

798 **Figure 1-figure supplement 1.** Competitive fitness is decreased in I_0 (competed

799 against WT a161) and $Mss116_{OE}$ (competed against the empty vector control WT

800 Y258). Bar height represents the mean of three biological replicates. Error bars are

801 standard error of the mean. ***$P < 0.001$; **$P < 0.01$; *$P < 0.05$ (ANOVA plus post

802 hoc). See Methods for details of how competitions were carried out and relative

803 fitness determined.

804

805 **Figure 1-figure supplement 2.** I_0 and $Mss116_{OE}$ show no qualitative difference in

806 growth on (a) glucose and (b) glycerol. Numbers on top of each panel represent the

807 decimal dilution of the stationary culture that is plated as a spot of 5 μl.

808

809 **Figure 1-figure supplement 3.** (a) GFP-tagged Mss116 localizes to mitochondria,

810 whether expressed at endogenous levels (left image) or upon overexpression in the

811 Y258 background (right image). (b) The expression level of Mss116 increases

812 approximately 2.5-fold in the $Mss116_{OE}$ strain, as measured by flow cytometry. Bar

813 heights display the mean of three biological replicates, each of them representing

814 mean of three technical replicates. Error bars are standard error of the mean. ***$P <$

815 0.001; **$P < 0.01$; *$P < 0.05$ (ANOVA plus post hoc).

816

817 **Figure 1-figure supplement 4.** Median and maximum replicative lifespan of

818 $Mss116_{OE}$ is extended compared to the empty vector control and $Mss116^{E268K}$. The

819 total number of monitored mother cells is as follows: 90 cells for the empty vector

820 control, 86 cells for $Mss116_{OE}$, and 91 cells for the $Mss116^{E268K}$. Measurements were

821 pooled across 3 independent experiments.

822

823 **Figure 2-figure supplement 1.** (a) Comparison of qPCR measurements in I_0 and I_0

824 overexpressing Mss116. Heat maps display mean values of log-fold changes observed

825 across three biological replicates (each averaged over three technical replicates).

826 UBC6 was used for normalization. (b) ATP level, (c) oxygen consumption and (d)

827 ROS level do not change upon overexpression of Mss116 in the I_0 genetic background

828 ($I_0 + Mss116_{OE}$) compared to I_0.

829

830 **Figure 2-figure supplement 2.** Mss116 deletion does not affect I_0 phenotypes,

831 highlighted by (a) the transcript levels (measured by qPCR) of relevant genes, (b)

832 mitochondrial morphology, and (c) mitochondrial volume. The heat map displays

833 mean values of log-fold changes observed across three biological replicates (each

834 averaged over three technical replicates). UBC6 was used for normalization. In panel

835 (c), bar heights display the mean of three biological replicates (each averaged over

836 three technical replicates). Error bars are standard error of the mean. ***$P < 0.001$;

837 **$P < 0.01$; *$P < 0.05$ (ANOVA plus post hoc).

838

839 **Figure 2-figure supplement 3.** Expression level of Mss116 during 9 hours after

840 induction using 2% galactose. Circles represent mean value of three biological

841 replicates (each performed as technical replicate). Error bars are standard error of the

842 mean.

843

844 **Figure 2-figure supplement 4.** Schematic presentation of the fragments of *cox1* and

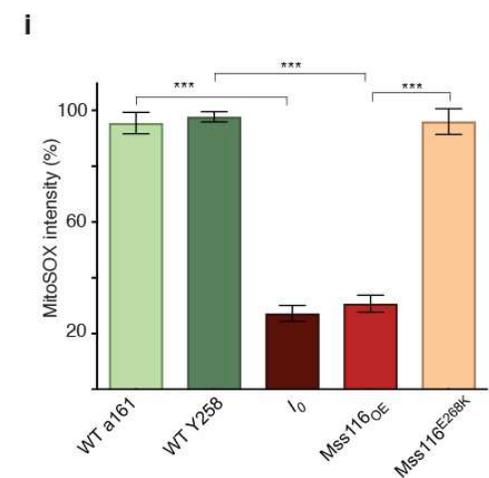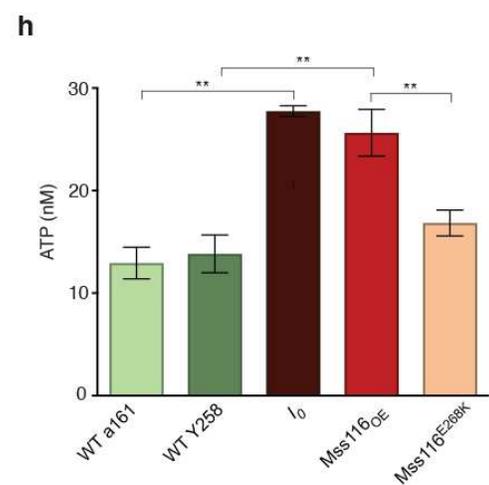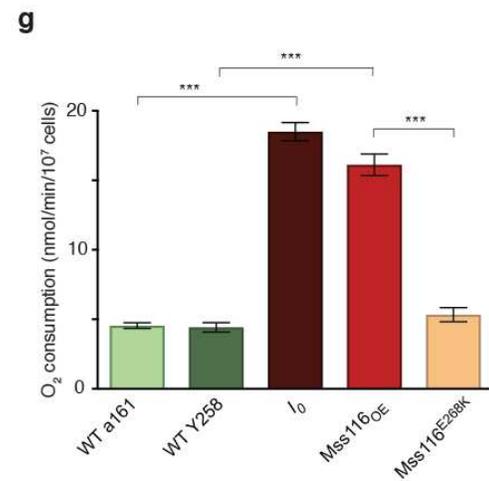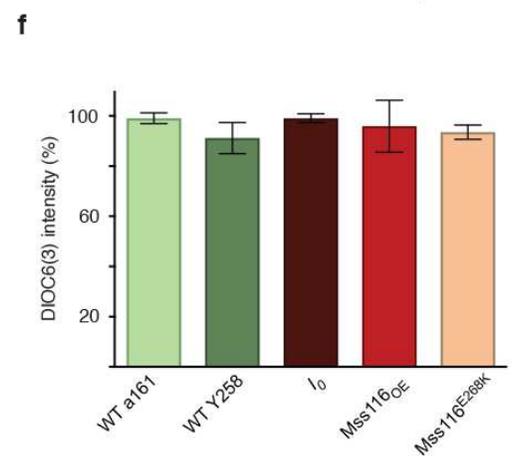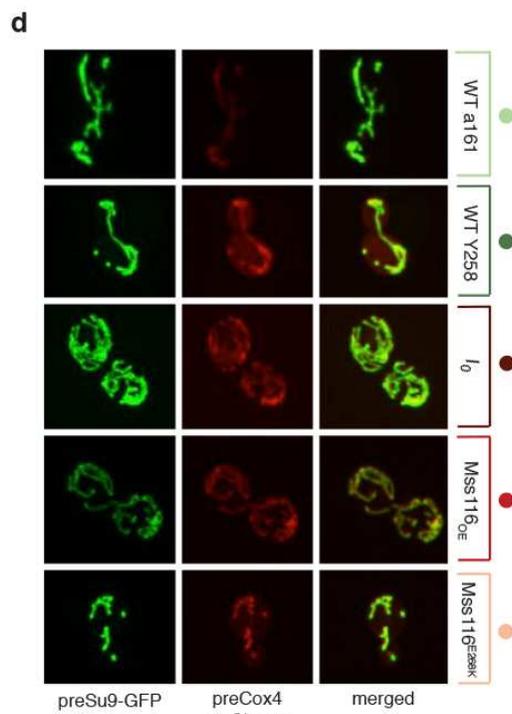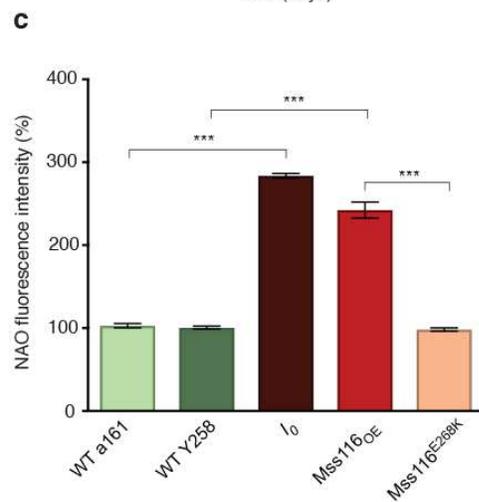845 *cob* amplified to determine the pre-mRNA and mature mRNA levels.
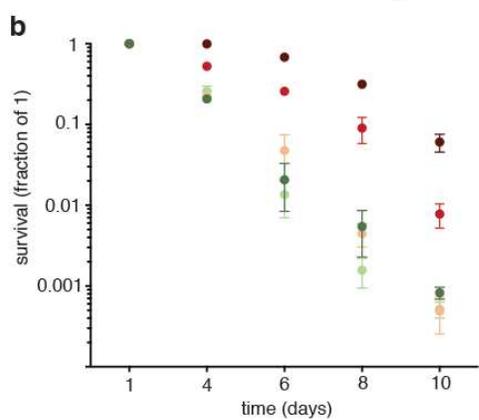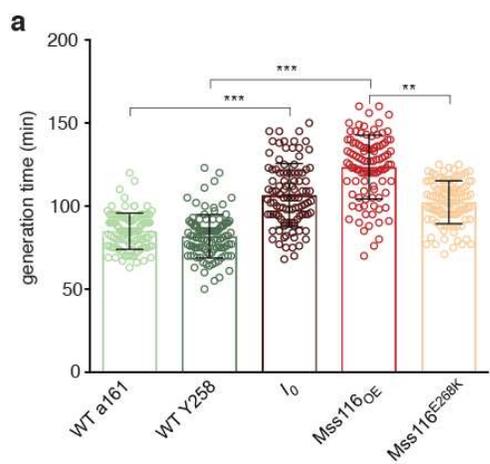
846

847 **Figure 5-figure supplement 1.** *Related to Figure 5.* Schematic representation of

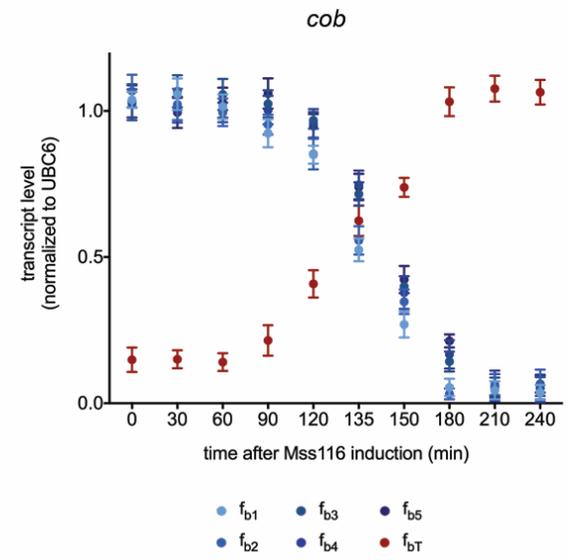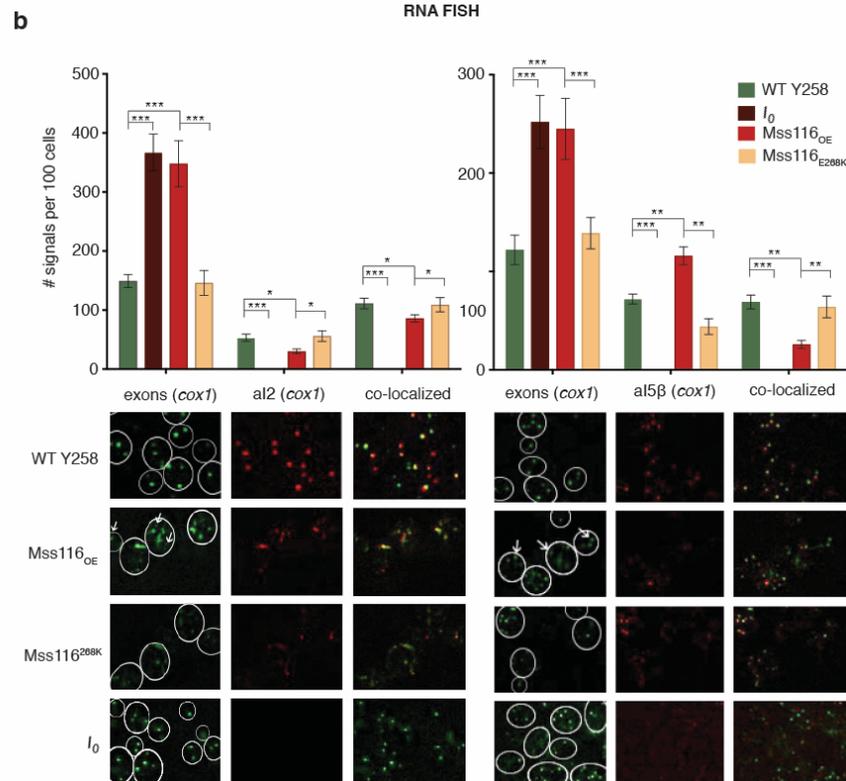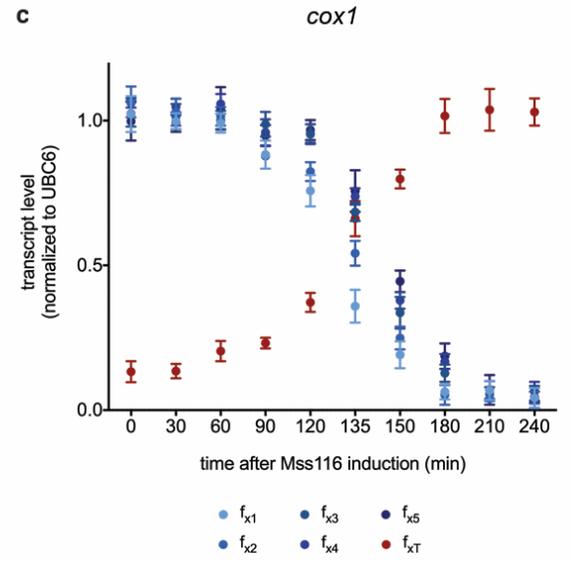848 mutated positions in the promoter regions of *cox1* and *cob*.

849

850

851

852

a generation time (min) — WT a161, WT Y258, I_0, Mss116$_{OE}$, Mss116^{E268K}

b survival (fraction of 1) vs time (days)

c NAO fluorescence intensity (%) — WT a161, WT Y258, I_0, Mss116$_{OE}$, Mss116^{E268K}

d preSu9-GFP, preCox4-mCherry, merged — WT a161, WT Y258, I_0, Mss116$_{OE}$, Mss116^{E268K}

e mean mitochondrial volume (pixel^3) — WT a161, WT Y258, I_0, Mss116$_{OE}$, Mss116^{E268K}

f DIOC6(3) intensity (%) — WT a161, WT Y258, I_0, Mss116$_{OE}$, Mss116^{E268K}

g O_2 consumption (nmol/min/10^7 cells) — WT a161, WT Y258, I_0, Mss116$_{OE}$, Mss116^{E268K}

h ATP (nM) — WT a161, WT Y258, I_0, Mss116$_{OE}$, Mss116^{E268K}

i MitoSOX intensity (%) — WT a161, WT Y258, I_0, Mss116$_{OE}$, Mss116^{E268K}

a

cob
cox1
cox2
cox4
sdh1
sdh2
atp1
atp6

respiratory chain & oxidative phosphorylation

fold-change vs WT

idh1
cit1

TCA cycle

cit2

retrograde response

sod1
sod2

antioxidant protection

fzo1
mgm1

mitochondrial fusion

dnm1
fis1

mitochondrial fission

qPCR

cox1 mRNA

al1
al2
al3
al4
al5α
al5β
al5γ

introns

cob mRNA

bl1
bl2
bl3
bl4
bl5

b

RNA FISH

signals per 100 cells

exons (cox1)   al2 (cox1)   co-localized

WT Y258
I_0
Mss116$_{OE}$
Mss116$_{E268K}$

exons (cox1)   al5β (cox1)   co-localized

WT Y258

Mss116$_{OE}$

Mss116^{268K}

I_0

c

cox1

transcript level (normalized to UBC6)

time after Mss116 induction (min)

f_{x1} f_{x3} f_{x5}
f_{x2} f_{x4} f_{xT}

cob

transcript level (normalized to UBC6)

time after Mss116 induction (min)

f_{b1} f_{b3} f_{b5}
f_{b2} f_{b4} f_{bT}

a

preSu9-GFP | preCox4-mCherry | merged

WT Y258
Δrtg2 Y258
Δrtg2 I_0
Δrtg2 Mss116$_{OE}$

b

mean mitochondrial volume (pixel³)

WT a161, WT Y258, *Δrtg2* a161, *Δrtg2* Y258, *Δrtg2* I_0, *Δrtg2* Mss116$_{OE}$

c

O_2 consumption (nmol/min/10⁷ cells)

WT a161, WT Y258, *Δrtg2* a161, *Δrtg2* Y258, *Δrtg2* I_0, *Δrtg2* Mss116$_{OE}$

d

ATP (nM)

WT a161, WT Y258, *Δrtg2* a161, *Δrtg2* Y258, *Δrtg2* I_0, *Δrtg2* Mss116$_{OE}$

e

survival (fraction of 1)

time (days)

WT a161
WT Y258
Δrtg2 a161
Δrtg2 Y258
Δrtg2 I_0
Δrtg2 Mss116$_{OE}$

f

Δrtg2 Y258, *Δrtg2* a161, *Δrtg2* I_0, *Δrtg2* Mss116$_{OE}$

cob
cox1
cox2
cox4
sdh1
sdh2   respiratory chain & oxidative phosphorylation
atp1
atp6
idh1   TCA cycle
cit1
cit2   retrograde response
sod1   antioxidant protection
sod2
fzo1   mitochondrial fusion
mgm1
dnm1   mitochondrial fission
fis1

fold-change vs WT

a

preSu9-GFP	preCox4-mCherry	merged	
			WT Y258
			$\Delta hap4$ Y258
			$\Delta hap4\ I_0$
			$\Delta hap4$ Mss116$_{OE}$

b

mean mitochondrial volume (pixel^3)

WT a161, WT Y258, $\Delta hap4$ a161, $\Delta hap4$ Y258, $\Delta hap4\ I_0$, $\Delta hap4$ Mss116$_{OE}$

c

O$_2$ consumption (nmol/min/10^7 cells)

WT a161, WT Y258, $\Delta hap4$ a161, $\Delta hap4$ Y258, $\Delta hap4\ I_0$, $\Delta hap4$ Mss116$_{OE}$

d

ATP (nM)

WT a161, WT Y258, $\Delta hap4$ a161, $\Delta hap4$ Y258, $\Delta hap4\ I_0$, $\Delta hap4$ Mss116$_{OE}$

e

survival (fraction of 1)

time (days)

- WT a161
- WT Y258
- $\Delta hap4$ a161
- $\Delta hap4$ Y258
- $\Delta hap4\ I_0$
- $\Delta hap4$ Mss116$_{OE}$

f

$\Delta hap4$ Y258, $\Delta hap4$ a161, $\Delta hap4\ I_0$, $\Delta hap4$ Mss116$_{OE}$

cob, cox1, cox2, cox4, sdh1, sdh2, atp1, atp6, idh1 — respiratory chain & oxidative phosphorylation

cit1 — TCA cycle

cit2 — retrograde response

sod1, sod2 — antioxidant protection

fzo1, mgm1 — mitochondrial fusion

dnm1, fis1 — mitochondrial fission

fold-change vs WT

a

	WT a161 pp	WT Y258 pp	I₀ pp	Mss116_OE pp	I₀ cox1 p	I₀ cob p	Mss116_OE cox1 p	Mss116_OE cob p	
cob									
cox1									
cox2									respiratory chain & oxidative phosphorylation
cox4									
sdh1									
sdh2									
atp1									
atp6									
idh1									TCA cycle
cit1									retrograde response
cit2									
sod1									antioxidant protection
sod2									
fzo1									mitochondrial fusion
mgm1									
dnm1									mitochondrial fission
fis1									

fold-change vs WT

b O₂ consumption (nmol/min/10⁷ cells)

c preSu9-GFP / preCox4-mCherry / merged

d mean mitochondrial volume (pixel³)

strain competing against its control

a 2% glucose   **b** glycerol

-1 -2 -3 -4 -5 -6

WT a161
WT Y258
I_0

-1 -2 -3 -4 -5 -6

$Mss116_{OE}$
$Mss116^{E268K}$
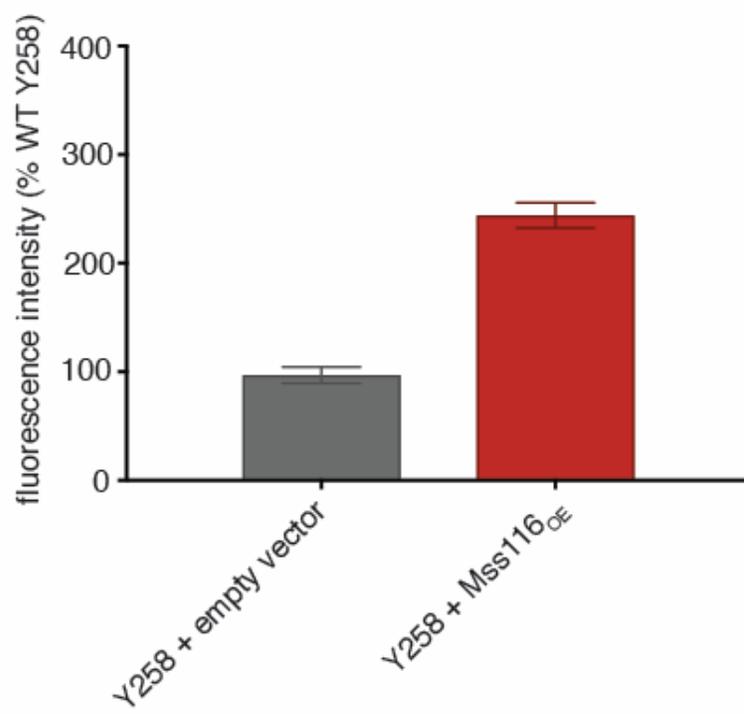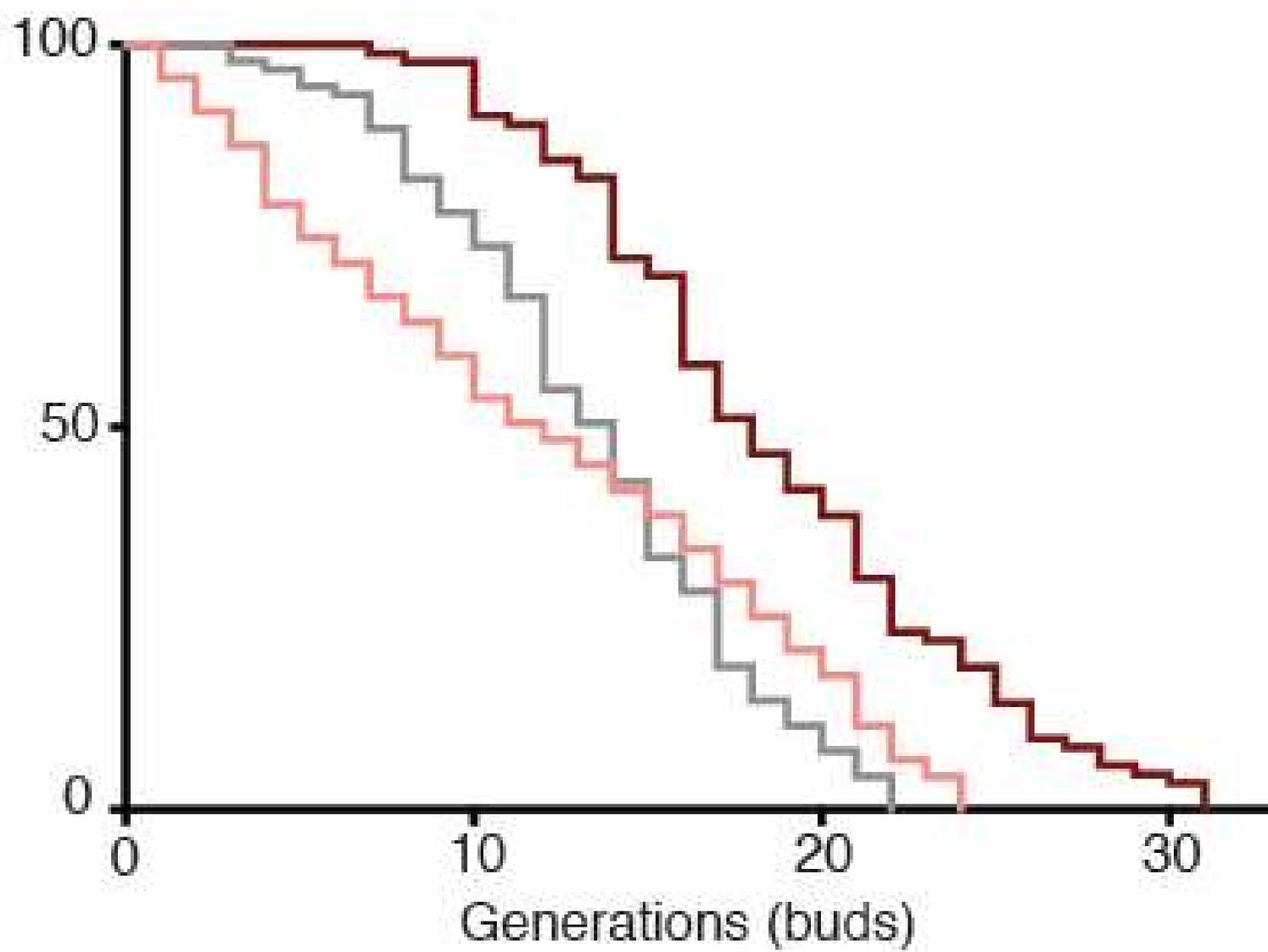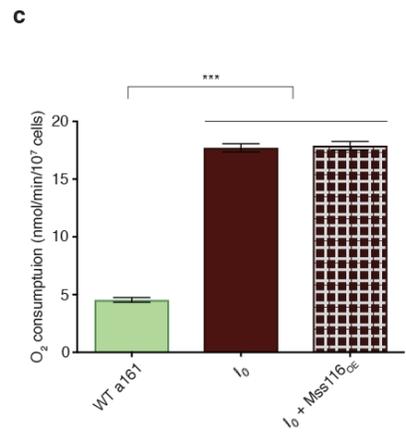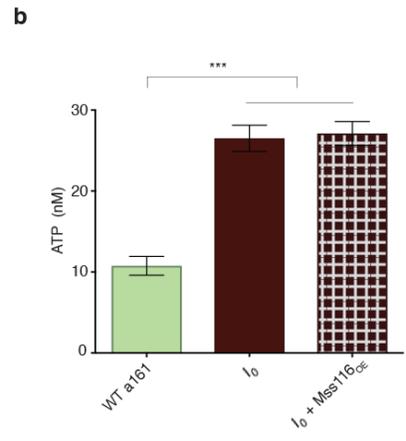
a

Mss116-GFP

ICC alpha-His-tag
Mss116$_{OE}$

b

- WT Y258   — Mss116$_{OE}$

— Mss116^{E268K}

a

	I_0	I_0 + Mss116$_{OE}$
cob		
cox1		
cox2		respiratory chain
cox4		&
sdh1		oxidative
sdh2		phosphorylation
atp1		
atp6		
idh1		TCA cycle
cit1		
cit2		retrograde response
sod1		antioxidant protection
sod2		
fzo1		mitochondrial fusion
mgm1		
dnm1		mitochondrial fission
fis1		

fold-change
vs
WT

b

ATP (nM)

WT a161 I_0 I_0 + Mss116$_{OE}$

***

c

O_2 consumptuion (nmol/min/10^7 cells)

WT a161 I_0 I_0 + Mss116$_{OE}$

***

d

MitoSOX fluorescnec intensity (%)

WT a161 I_0 I_0 + Mss116$_{OE}$

***

a

	I_0	I_0 ΔMss116	I_0 ΔMss116+ Mss116^{E268K}

COB				
COX1				respiratory chain
COX2				
COX4				&
SDH1				
SDH2				oxidative phosphorylation
ATP1				
ATP6				
SOD1				antioxidant protection
SOD2				
IDH1				TCA cycle
CIT1				
CIT2				retrograde response
FZO1				mitochondrial fusion
MGM1				
DNM1				mitochondrial fission
FIS1				

2 4 6 8

b

preSu9-GFP   preCox4-mCherry   merged

WT a161
I_0
I_0 ΔMss116
I_0 ΔMss116+ Mss116^{E268K}

c

mean mitochondrial volume (pixel^3)

WT a161 I_0 ΔMss116 I_0 ΔMss116 I_0 + Mss116^{E268K}

mRNA

f_{bT}

exon1-exon2 junction

exon$_5$-exon$_6$ junction

f_{xT}

exon1-exon2 junction

exon$_7$-exon$_8$ junction

pre-mRNA

Fw1

Fw2

cob

RC1   RC2   RC3   RC4   RC5

f_{b1} f_{b2} f_{b3} f_{b4} f_{b5}

Fw1

Fw2

cox1

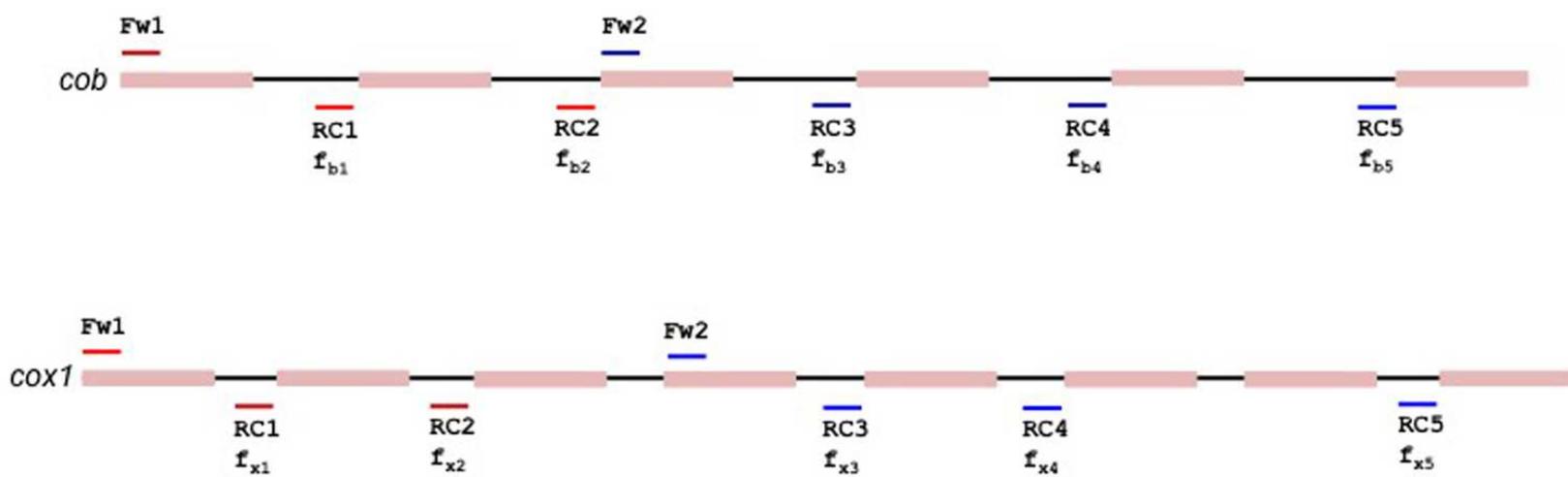RC1   RC2   RC3   RC4   RC5

f_{x1} f_{x2} f_{x3} f_{x4} f_{x5}

cox1 promoter

ATTGATATAAGTAATAGATA

↓

A

beginning of *cox1* gene

ATGGTACAAAGATGATTA

cob promoter

TATTATATAAGTAATATATA

↓

A

beginning of *cob* gene

ATGGCATTTAGAAAATCAAAT