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

Marina Rudan¹, Peter Bou Dib², Marina Musa¹, Matea Kanunnikau¹, Sandra Sobocanec³, David Rueda⁴,⁵,⁶, Tobias Warnecke⁴,⁵*, Anita Kriško¹*

¹Mediterranean Institute for Life Sciences, Meštrovićevo šetalište 45, 21000 Split, Croatia
²Universitätsmedizin Göttingen, Institut für Zellbiochemie, Humboldtallee 23, 37073 Göttingen
³Division of Molecular Medicine, Rudjer Boškovic Institute, Bijenička 54, 10000 Zagreb, Croatia
⁴MRC London Institute of Medical Sciences (LMS), Du Cane Road, London W12 0NN, United Kingdom
⁵Institute of Clinical Sciences (ICS), Faculty of Medicine, Imperial College London, Du Cane Road, London W12 0NN, United Kingdom
⁶Molecular Virology, Faculty of Medicine, Imperial College London, Du Cane Road, London W12 0NN, United Kingdom

Running title: Inefficient splicing at the heart of mitochondrial function

*co-corresponding authors
Self-splicing introns are mobile elements that have invaded a number of highly conserved genes in prokaryotic and organellar genomes. Here, we show that deletion of these selfish elements from the *Saccharomyces cerevisiae* mitochondrial genome is stressful to the host. A strain without mitochondrial introns displays hallmarks of the retrograde response, with altered mitochondrial morphology, gene expression and metabolism impacting growth and lifespan. Deletion of the complete suite of mitochondrial introns is phenocopied by overexpression of the splicing factor Mss116. We show that, in both cases, abnormally efficient transcript maturation results in excess levels of mature *cob* and *cox1* host mRNA. Thus, inefficient splicing has become an integral part of normal mitochondrial gene expression. We propose that the persistence of *S. cerevisiae* self-splicing introns has been facilitated by an evolutionary lock-in event, where the host genome adapted to primordial invasion in a way that incidentally rendered subsequent intron loss deleterious.

Mobile genetic elements frequently compromise host fitness\(^1\), corrupting genetic information or disturbing adaptive gene expression patterns, sometimes to lethal effect. Despite this, mobile genetic elements are ubiquitous in most eukaryotic genomes\(^2\). How do these selfish elements persist in a genomic environment where – even in the absence of selection – mutational forces constantly work to erode them? Although mobile elements can donate motifs (or domains) that are co-opted into host regulatory pathways (or genic sequence) over time\(^3\), deletions usually whittle away all but the core beneficial motif. The components that once mediated mobility, such as the reverse transcriptases of long interspersed nuclear elements (LINEs), are typically lost. Thus, functional selfish elements that remain mobile are thought to persist over
evolutionary time not by virtue of sporadic beneficial effects for the host, but because they replicate and spread to other sites in the genome faster than they are deleted. The element survives, not where it originally invaded but as a descendant copy elsewhere in the genome.

An interesting exception in this regard are self-splicing introns, which populate some highly expressed genes in archaea, bacteria, and organellar genomes of fungi and plants. In contrast to other mobile elements, self-splicing introns do not spawn a large pool of copies that disperse across the genome to escape mutational erasure. Rather, owing to highly specific homing sites, each intron is typically confined to a single location in the host genome and evolutionary persistence seems to rely on continued re-invasion, either from other individuals in the same population or across species boundaries. Self-splicing introns can spread despite considerable fitness costs to the host. However, in practice, fitness costs might be relatively low as the host RNA is intact and fully functional once the intron has been spliced out. As far as we know, self-splicing introns do not contribute positively to host fitness and, naively, one would expect that deleting these introns from the genome would be beneficial or, at worst, make no difference to the host.

Here, we investigate the consequences of deleting all 13 self-splicing introns from the *S. cerevisiae* mitochondrial genome, where they reside in three host genes: the 21S ribosomal RNA gene Q0158 (which harbours a single group I intron named omega) and two protein-coding genes, *cox1* (group I: aI3, aI4, aI5α, aI5β; group II: aI1, aI2, aI5γ) and *cob* (group I: bI2, bI3, bI4, bI5; group II: bI1), both encoding components of the electron transport chain. Note here, that we use “self-splicing introns” as a
convenient shorthand to describe the complete collection of group I and group II introns, even though timely splicing in vivo often depends on one or several trans-factors (see below). We show that, contrary to expectations, removing these introns has dramatic consequences for mitochondrial physiology and function, triggering changes in nuclear gene expression that affect organismal growth and lifespan. Our results demonstrate that the presence of mitochondrial self-splicing introns has become integral to normal mitochondrial gene expression and that, curiously, normal mitochondrial function in S. cerevisiae has come to require inefficient splicing. Our findings have implications for understanding how self-splicing introns and mobile elements more generally can survive over evolutionary time without providing an adaptive benefit to the host.

Results

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

To test whether the removal of self-splicing mitochondrial introns affects host physiology and fitness, we compared two S. cerevisiae strains that are isogenic with regard to their nuclear genomes (except for a single marker gene, ura3, see Methods) but differ with respect to mitochondrial intron content. The control strain a161 (WT) contains the full complement of seven cox1 and five cob introns, whereas strain a161-U7 (Io) carries an intronless mitochondrial genome, which was originally constructed by Seraphin and co-workers via serial recombination of natural yeast isolates that lack individual mitochondrial introns.
Contrary to a model where self-splicing introns are dispensable parasitic passengers, we find that \( I_0 \) exhibits stark phenotypic differences to the control strain. When the two strains are cultured in isolation, exponential growth on glucose-supplemented YPD medium is \( \sim 30\% \) slower for \( I_0 \) compared to WT (Figure 1a). \( I_0 \) also fares poorly when pitted directly against WT in competitive fitness assay (Figure 1-figure supplement 1). Chronological life span (CLS, see Methods), on the other hand, is almost two-fold longer for \( I_0 \) (Figure 1b). At the cellular level, \( I_0 \) displays increased mitochondrial mass and volume and a mitochondrial morphology characterized by a large network of branched tubules of homogeneous diameter (Figure 1c-e). Transcript levels of mitofusin (\( fzo1 \)) and the GTPase \( mgm1 \), key nuclearly encoded regulators of mitochondrial fusion, are strongly upregulated (qPCR, \( t \)-test, \( fzo1 \): 4.02-fold, \( p=0.002 \); \( mgm1 \): 5.24-fold, \( p=3.19\times10^{-5} \), Figure 2a) while levels of \( dnm1 \) and \( fis1 \), which orchestrate mitochondrial fission, are only moderately induced (qPCR, \( t \)-test, \( dnm1 \): 1.67-fold, \( p=0.026 \); \( dnm1 \): 1.81-fold, \( p=0.085 \)), suggesting that the changes in mitochondrial morphology result from increased fusion rather than impaired fission. The \( I_0 \) strain also exhibits a 2.7-fold increase in mitochondrial DNA copy number (qPCR, \( t \)-test \( P=1.09\times10^{-7} \)). At the same time, there are no significant differences in mitochondrial inner membrane potential, as measured by 3,3'-Dihexyloxacarbocyanine iodide [DiOC6(3)] fluorescence (Figure 1f), suggesting that mitochondria are functional despite grossly altered morphology. This notion is further supported by the observation that \( I_0 \) retains the capacity to grow on glycerol, a non-fermentable carbon source (Figure 1-figure supplement 2), as previously reported for a different nuclear background\(^{11} \). In fact, biomarkers of mitochondrial metabolism point to increased mitochondrial activity, with higher oxygen consumption (Figure 1g) and cellular ATP levels (Figure 1h) during exponential growth. Despite increased
activity, levels of mitochondrial superoxide are reduced (Figure 1i), likely reflecting a 7.5-fold upregulation of the mitochondrial ROS-scavenger sod2 (qPCR, t-test P=0.003, Figure 2a). Thus, intron removal challenges but does not terminally compromise mitochondrial function.

Removal of mitochondrial introns triggers the retrograde response

The phenotypic changes we observe suggest an involvement of the retrograde response, as do upregulation of cit2, upregulation of the two rate-limiting members of the TCA cycle, cit1 and idh1, and upregulation of both mitochondrially (cox1, cox2, atp6) and nuclearly (cox4, atp1, sdh1, sdh2) encoded parts of the respiratory chain (Figure 2a). Indeed, deletion of rtg2, the transcriptional master regulator of the retrograde response and a sensor of mitochondrial dysfunction, suppresses the I0 phenotype (Figure 3). Tubular structure is lost and large spherical shapes become prominent (Figure 3a), suggesting distinct defects in the maintenance of mitochondrial ultrastructure. Deletion of rtg2 in WT strains, where the retrograde response is not activated, has no significant effect on mitochondrial volume and morphology, oxygen consumption and ATP levels (Figure 3a-d). The extension of CLS is abrogated in the absence of rtg2, becoming shorter even than the wildtype (Figure 3e). Upon deletion of hap4, the transcriptional activator of nuclearly encoded components of the respiratory chain (as well as the TCA cycle enzymes under normal conditions), mitochondrial morphology reverts back to the wild-type state (Figure 4a-e). We conclude that an intact retrograde response, including upregulation of nuclear components of the respiratory chain, is necessary to generate the mitochondrial phenotype observed in the I0 strain.
Promoter attenuation of coxl and cob reverses the phenotype

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\textsuperscript{14,15} - we focused on coxl and cob. For both these genes, mRNA levels are strongly elevated in \textit{I}0 (10.9-fold and 5.8-fold for coxl and cob, respectively; Figure 2a) but also in \textit{Δrtg2} (Figure 3f) and \textit{Δhap4} (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 coxl and/or cob transcript levels might underpin the wider transcriptional, metabolic and phenotypic changes, we introduced attenuating point mutations into the promoters of coxl and cob (see Methods). We find that simultaneous attenuation of both promoters in \textit{I}0 fully reverses the \textit{I}0-characteristic suit of morphological and molecular phenotypes (see \textit{I}0pp throughout Figure 5). Attenuation of either cob (I0cobp) or coxl (I0cox1p) in isolation only partially reverses the phenotype, although I0cobp has a larger relative effect than I0cox1p (Figure 5).

The effects of intron removal are phenocopied by overexpression of Mss116

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
short-circuiting the splicing process itself might interfere with normal expression. Specifically, we hypothesized that normal levels of transcription might be tuned to accommodate a certain proportion of transcripts that fail to splice correctly. Group II introns in particular are known for low splicing efficiency, even in the presence of auxiliary proteins. As a corollary, a large fraction of pre-mRNAs might be targeted and degraded by mitochondrial quality control, either because splicing is erroneous (mis-splicing) or does not occur in a timely manner so that the transcript is shunted into degradation (kinetic coupling). In \( I_0 \), splicing does not occur so that erroneous splicing products do not arise. As a result, production of functional \( cox1/cob \) mRNAs might overshoot its target and trigger a system-wide response, for example because altered COX1/COB levels upset dosage balance amongst respiratory complexes. To test this abnormally-efficient-maturation hypothesis and simultaneously rule out that mitochondrial stress is caused by the removal of DNA-/RNA-level regulatory elements, we sought to alter splicing efficiency by orthogonal means. To this end, we overexpressed the nuclearly encoded DEAD box RNA helicase Mss116, which promotes splicing of all \( S. cerevisiae \) mitochondrial introns by remodeling or stabilizing splice-relevant RNA structures in an ATP-dependent manner. We confirmed overexpression and mitochondrial localization of Mss116 by flow cytometry and immunofluorescence, respectively, using an N-terminal His-tagged version of the protein (Figure 1-figure supplement 3), and then characterized the effects of Mss116 overexpression in the Y258 strain using an untagged version of the protein. Remarkably, the Mss116 overexpression strain (\( Mss116OE \)) phenocopies \( I_0 \).

\( Mss116OE \) exhibits increased generation times, extended chronological life span, lower competitive fitness, increased mitochondrial fusion, 2.9-fold increased mtDNA
copy number (qPCR, t-test $P=5.5 \times 10^{-7}$), elevated oxygen consumption, and altered ATP and ROS production (Figure 1, Figure 1-figure supplement 1). In addition, we observe longer replicative life span (RLS) in $Mss116OE$ (Figure 1-figure supplement 4), a more direct proxy of ageing that we were not able to measure accurately in $I0$, where separating mother and daughter cells in a timely fashion proved challenging (see Methods). Overexpression of a DEAD box mutant of Mss116 ($Mss116^{E268K}$), which lacks ATPase and therefore helicase activity, does not phenocopy $I0$ (Figure 1). This suggests that the role of Mss116 in splicing – which relies on helicase activity – is critical rather than a recently suggested ATP-independent role in transcription elongation$^{18}$. More generally, the fact that $Mss116OE$ – which encodes a full complement of introns – phenocopies $I0$ indicates that the stress phenotype in these strains is not caused by missing DNA-level functionality and instead points towards a critical role for splicing. Overexpressing Mss116 in $I0$ did not reveal additional phenotypes and the strain behaved like $I0$ (Figure 2-figure supplement 1) further supporting the notion that intron deletion and Mss116 overexpression act through the same pathway.

Cells are stressed because of abnormally efficient transcript maturation

We suspected that phenotypic effects of Mss116 overexpression (and intron deletion) are linked to altered transcript maturation of the host genes, $cob$ and $cox1$. However, we first wanted to rule out an alternative hypothesis. In both $Mss116OE$ and $I0$, Mss116 is in excess relative to need (in $Mss116OE$ because Mss116 is overexpressed, in $I0$ because its usual targets – the introns – are absent). Phenotypic effects could therefore be caused by excess Mss116 interacting with RNAs that it would not
normally target or not target to the same extent. This is conceptually related to the idea that splicing dynamics can change simply as a result of altered competition between mRNAs for access to the spliceosome. To test this hypothesis, we deleted Mss116 in the I0 strain. We observe the same suite of phenotypes we see in I0 (Figure 2-figure supplement 2), demonstrating that neither absolute nor relative excess of Mss116 are responsible for the stress phenotype described above.

Having ruled out this hypothesis, we then focused on characterizing the effects of Mss116 overexpression on cob/cox1 splicing dynamics in greater detail. Using qPCR, we first measured the levels of total cox1 and cob transcripts as well as individual introns at steady state (Figure 2a, 240 mins after Mss116 induction, see Figure 2-figure supplement 3). For cox1, we additionally monitored exon and intron (aI2, aI5β) levels using RNA fluorescent in situ hybridization (RNA FISH, Figure 2b, see Methods). We find that most mitochondrial introns are strongly depleted in Mss116 OE compared to the empty vector control and relative to exons. The relative depletion of individual introns is somewhat variable and one intron – aI5α - is equally abundant in Mss116OE and the Y258 WT (Figure 2a). We then examined shifts in the abundance of pre-mRNA and mature mRNA over the course of Mss116 induction, using different primer combinations to monitor unspliced RNAs and spliced exon-exon junctions. For both cox1 and cob, Mss116 overexpression shifts the balance between unspliced (or partially spliced) pre-mRNA transcripts, which dominate the uninduced steady state, towards mature mRNA transcripts (Figure 2c), while total transcript abundance (mRNA plus pre-mRNA) remains largely unchanged. These observations are consistent with a model where nascent transcriptional output is unchanged in
$Mss116OE$ compared to the empty vector control and differential steady state levels are the result of post-transcriptional events.

Based on these findings, we suggest that $Mss116OE$ phenocopies $I_0$ because eliminating introns at the DNA level ($I_0$) and facilitating accurate and efficient excision at the RNA level ($Mss116OE$) both result in abnormally efficient transcript maturation. That is, fewer transcripts are eliminated by mitochondrial quality control because splicing is erroneous or does not proceed in a timely manner, resulting in a greater number of mature $cox1/cob$ mRNAs. For reasons that remain to be elucidated, increased transcript levels are then perceived as stressful and trigger the retrograde response, culminating in a multifaceted stress phenotype. We speculate in this regard that elevated protein levels of COB ($I_0$: 9.3-fold; $Mss116OE$: 5.0-fold upregulation, as determined by quantitative label-free mass spectrometry) and COX1 ($I_0$: 11.6-fold; $Mss116OE$: 12.1-fold upregulation) might interfere with proper assembly and function of complex III and complex IV, respectively, and therefore constitute a deleterious dosage imbalance phenotype.

**Discussion**

It is now well documented that disruption of splicing homeostasis can impact normal physiological function and lead to cellular stress and disease$^{20}$. There is also increasingly detailed mechanistic knowledge of how proteins involved in splicing can alter growth and ageing via a metabolic route, exemplified by the recent finding that splicing factor I is a modulator of dietary restriction-induced longevity in
The classic model here is that loss of splicing homeostasis — through genetic, developmental, or environmental perturbation — leads to deleterious shifts in splice isoform production or precipitates increased production of erroneous transcripts that tax the quality control system and/or have direct cytotoxic effects. In other words, the disease/stress state is a high-error state. Our results are unusual in that they suggest that normal splicing can be associated with high error rates and that, therefore, splicing homeostasis can also be disturbed by increasing splicing efficacy.

Further research will be required to tease apart how individual mitochondrial introns affect the overall burden from failed splicing in this system. It is evident from population genomic analysis of different *S. cerevisiae* strains that some mitochondrial introns are fixed across extant populations whereas others exhibit presence/absence polymorphism. This seems to suggest that the removal of at least some introns in isolation is insufficiently stressful to be purged by natural selection. At the same time, studies of *suv3*, the second DEAD box RNA helicase present in yeast mitochondria, suggest that the deleterious effect of deleting individual introns — while possibly idiosyncratic — is at least partially cumulative: deletion of *suv3*, a component of the mitochondrial degradosome, decreases levels of mature *cox1/cob* mRNA and compromises respiratory capacity, but less so where more introns had been removed from the mitochondrial DNA. Importantly — at least for the combinations tested — severity was found to depend on the number but not identity of the *cox1* or *cob* introns present.
In addition to providing a new insight into post-transcriptional gene regulation in mitochondria, our findings have implications for understanding the evolutionary persistence of self-splicing introns and perhaps mobile elements more generally. The phenotypic effects we observe run counter to the notion that self-splicing introns are low-cost passengers and instead demonstrate that at least some of these selfish elements are firmly embedded in the organization of mitochondrial gene expression such that removing them upsets proper expression of their host genes. We suggest that our observations can be explained by an evolutionary lock-in model where the primordial colonization of an intron-free *cox1/cob* ancestor by a self-splicing intron led to a drop in *cox1/cob* mRNA levels and favoured compensatory mutations that increased *cox1/cob* transcription to restore mRNA abundances back to their original levels. When we forcibly remove these introns, however, this hard-wired upregulation turns maladaptive. There no longer is a pool of transcripts targeted for degradation leading to excess levels of mature mRNA. In principle, it is also possible that the initial invader spliced very efficiently and imposed no cost but subsequently co-evolved with host gene expression in a ratchet-like fashion, whereby incidental greater-than-required levels of the host gene allowed recurrent small decreases in splicing efficiency. However, since self-splicing introns can spread despite substantial fitness costs, we do not actually need to evoke a cost-free ancestral event. We suggest that evolutionary lock-ins of this type might provide an unappreciated mechanism to facilitate the longer-term persistence of genetic parasites, especially in large host populations where evolution is not mutation-limited. We also note that this argument might in principle extend to nuclear introns: if, for a given dosage-sensitive gene, failure to splice is common and transcription levels are set to compensate, intron
loss might be deleterious and prevented by purifying selection even though the intron makes no adaptive contribution to gene regulation.

Methods

Strains and growth conditions

Strain a161 (also known as ID41-6/161, or sometimes simply 161), described by Wenzlau et al.\textsuperscript{23}, and the intronless a161-U7 (I\textsubscript{0}) were gifts from Alan Lambowitz. These strains were used previously to show that splicing of group I and II introns is Mss116-dependent\textsuperscript{17}. a161 and a161-U7 are isogenic except for the mitochondrial genome and a single marker gene (a161: MATa ade1 lys1; a161-U7: MATa ade1 lys1 ura3).

Strain Y258 and the pBG1805 plasmid bearing \textit{Mss116} for overexpression were purchased from Thermo Scientific (Dharmacon). \textit{Mss116}\textsuperscript{E268K} was purchased from DNA 2.0 and cloned into pBG1805 using standard cloning techniques\textsuperscript{24}. Mss116 and \textit{Mss116}\textsuperscript{E268K} were overexpressed in the Y258 nuclear background. The expression of Mss116 and \textit{Mss116}\textsuperscript{E268K} was induced from the plasmids using 2\% galactose (final), added at OD 0.2.

All strains were grown on YPD medium with 2\% (w/v) glucose at 30°C with shaking. All experiments were performed on exponentially growing cells: cells were grown to OD 0.6-0.7 for WT, I\textsubscript{0}, \textDelta Hap4, and \textDelta Rtg2 and to OD 0.9-1.0 for \textit{Mss116OE} and
harvested by centrifugation at 4000 $\times$ g for 5 minutes, washed and further treated as required.

In order to test growth on glycerol, strains were grown in YPD medium until saturation, at 30 °C with shaking. Stationary cells were serially diluted and 5 μL drops plated onto YPEG agar plates (containing 3% ethanol and 3% glycerol). Growth was observed after 4 days.

**Gene deletion**

Deletion of *hap4* and *rtg2* was performed as previously described\(^2\), using a hygromycin cassette for selection in the WT background and a nourseothricine cassette in the *I\(_0\)* background. Primers used for the deletions are listed in Supplementary File 1.

**Measurement of splicing kinetics in *Mss116\text{OE}***

Overnight cultures of *Mss116\text{OE}* were diluted to OD 0.1. Galactose (2% final) was added at OD 0.2 to induce the expression of Mss116, and this is designated as time 0. Aliquots of the culture were harvested at 30, 60, 90, 120, 135, 150, 180, 210 and 240 minutes post-induction. The cells were pelleted and used for RNA isolation and cDNA preparation for qPCR. Multiple primer pairs were used to monitor unspliced intron-exon fragments and spliced exon-exon junctions (Figure 2-figure supplement 4, Supplementary File 1). Additional aliquots were harvested at 60, 150, 300, 360, 420, and 540 minutes post-induction and were used to measure Mss116 expression levels by flow cytometry (see below).
Insertion of point mutations into the promoter regions

In order to introduce promoter-attenuating mutations into mitochondrial DNA, we followed the protocol described in\textsuperscript{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 \textit{rho}\textsuperscript{0} strain, which is then mated with a recipient \textit{rho}\textsuperscript{+} strain. Upon mating, mitochondria from the two strains fuse and recombination between the two mtDNAs produces recombinant \textit{rho}\textsuperscript{+} 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). Sacl 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 \textit{cox1} and \textit{cob} promoters\textsuperscript{27}.

Chronological lifespan measurement

All strains were grown to saturation as described above and pelleted at 4000 × g for 5min. Cells were then washed twice and resuspended in sterile deionized water (10^6
cells in 10mL in order to avoid cell growth on the debris of dead cells) and incubated at 30°C with shaking. Every 2-3 days, cells were serially diluted and plated onto YPD plates in order to evaluate cell growth.

Replicative lifespan measurement

Replicative lifespan (RLS) was determined by micromanipulation for \textit{Mss116}\textsubscript{OE} and \textit{Mss116}\textsubscript{E268K} in the Y258 background, as well as for \textit{Mss116}\textsubscript{OE} in the \textit{\Delta}hap4 and \textit{\Delta}rtg2 nuclear backgrounds, counting the number of daughters produced by individual mother cells. We were unable to reliably determine RLS for the \textit{Io} strain as daughter and mother cells could not be separated in a timely manner, which is critical for RLS measurements. For unknown reasons, and perhaps specific to the nuclear background, cells were unusually sticky so that the first daughter often could not be separated from the mother until the mother had already produced other buds, making it difficult to track mother/daughter identity over time, an essential prerequisite for reliably determining replicative lifespan. Cells were incubated at 30°C on YPD (WT) or -URA (mutants) plates for the duration of the experiment. Using a microscope equipped with a microdissection apparatus suitable for \textit{S. cerevisiae} (Singer Instruments), cells were transferred to defined places on agar plates and virgin daughter cells collected. Each cell was monitored continuously over several days every 60 - 90min until all mother cells stopped budding. The total number of daughter cells was noted for each mother cell. The total number of monitored mother cells is as follows: 90 cells for the empty vector control, 86 cells for \textit{Mss116}\textsubscript{OE}, and 91 cells for the \textit{Mss116}\textsubscript{E268K}. The measurements were pooled from 3 independent experiments.
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 $30 \times 10^6$ 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 $Mss116OE$ and its corresponding empty vector control strain. Prior to the competition, we plated $5 \times 10^9$ cells of each mutant ($I_0$ or $Mss116OE$) 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
(approximately 5-6 hours after dilution), aliquots of cells were harvested, and serial dilutions were plated on YPD-agar plates without geneticin. Next, dilutions with between 50 and 200 colonies were replica-plated on YPD-agar plates with geneticin (200 \( \mu g/mL \)). Colonies were counted on both types of plates and the ratio of geneticin-resistant (mutant) to geneticin-sensitive (WT) colonies was calculated as a measure of relative fitness.

Flow cytometry

Flow cytometry was carried out on a Becton-Dickinson FACSCalibur machine equipped with a 488nm Argon laser and a 635nm red diode laser.

Measurement of the Mss116 overexpression level

The expression level of Mss116 in the \( \text{Mss116}\text{OE} \) strain was measured by using a rabbit polyclonal anti-His tag antibody (Abcam, ab137839, 1:10000) and secondary IgG goat anti-rabbit labeled with Alexa 488 (Thermo Fisher Scientific, A11034, 1:2000). The signal obtained by flow cytometry (mean fluorescence over 10000 cells) was compared to the Mss116 tagged with GFP (Thermo Scientific) endogenous expression level estimated by using flow cytometry measurement based on the GFP signal. The mean fluorescence intensity in \( \text{Mss116}\text{OE} \) was normalized to the mean fluorescence intensity detected in wild type cells with endogenous expression of Mss116.

Mass spectrometry - sample processing
Isolated mitochondrial fractions containing 100µg of protein were loaded onto Microcon 30kD centrifugal filters (Merck Millipore, MRCF0R030). Samples were then digested using a Filter Aided Sample Preparation (FASP) protocol\textsuperscript{28}. Briefly, samples were concentrated on the filter unit by centrifugation and buffer exchanged using sequential washing and centrifugation with 8M urea, 100mM TRIS/HCL buffer (pH8.5). Proteins were reduced and alkylated sequentially with 10mM Dithiothreitol and 50mM Iodoacetamide (in 8M urea buffer), respectively. Samples were buffer exchanged to remove salts using sequential washing with 50mM ammonium bicarbonate (AmBic). Trypsin Gold (Promega, V5280) was added to the samples in 50mM ammonium bicarbonate to an approximate 1:50, protease:protein ratio. Digestions were incubated at 37°C overnight (17h). Digest extracts were recovered from FASP filters via centrifugation and acidified with 1% trifluoroacetic acid (TFA). Acidified protein digests were desalted using Glygen C18 spin tips (Glygen Corp, TT2C18.96) according to the manufacturer’s recommendation and peptides eluted with 60% acetonitrile, 0.1% formic acid (FA). Eluents were then dried using a vacuum centrifuge.

**Mass spectrometry - liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis**

Protein digests were redissolved in 0.1% TFA by shaking (1200rpm) for 30min and sonication on an ultrasonic water bath for 10min, followed by centrifugation (14,000rpm, 5°C) for 10min. LC-MS/MS analysis was carried out in technical duplicates (1µg on column) and separation was performed using an Ultimate 3000 RSLC nano liquid chromatography system (Thermo Scientific) coupled to a Orbitrap
Velos mass spectrometer (Thermo Scientific) via an Easy-Spray source. For LC-
MS/MS analysis protein digests were injected and loaded onto a trap column
(Acclaim PepMap 100 C18, 100μm × 2cm) for desalting and concentration at
8μL/min in 2% acetonitrile, 0.1% TFA. Peptides were then eluted on-line to an
analytical column (Easy-Spray Pepmap RSLC C18, 75μm × 50cm) at a flow rate of
250nL/min. Peptides were separated using a 120 minute gradient, 4-25% of buffer B
for 90 minutes followed by 25-45% buffer B for another 30 minutes (composition of
buffer B – 80% acetonitrile, 0.1% FA) and subsequent column conditioning and
equilibration. Eluted peptides were analysed by the mass spectrometer operating in
positive polarity using a data-dependent acquisition mode. Ions for fragmentation
were determined from an initial MS1 survey scan at 30,000 resolution, followed by
CID (Collision Induced Dissociation) of the top 10 most abundant ions. MS1 and
MS2 scan AGC targets were set to 1e6 and 3e4 for maximum injection times of
500ms and 100ms respectively. A survey scan m/z range of 350 – 1500 was used,
normalised collision energy set to 35%, charge state screening enabled with +1 charge
states rejected and minimal fragmentation trigger signal threshold of 500 counts.

Mass spectrometry - raw data processing

Data was processed using the MaxQuant software platform (v1.5.6.0), with database
searches carried out by the in-built Andromeda search engine against the Uniprot
S.cerevisiae database (version 20160815, number of entries: 6,729). A reverse decoy
database approach was used at a 1% false discovery rate (FDR) for peptide spectrum
matches. Search parameters included: maximum missed cleavages set to 2, fixed
modification of cysteine carbamidomethylation and variable modifications of
methionine oxidation, asparagine deamidation and N-terminal glutamine to
pyroglutamate conversion. Label-free quantification was enabled with an LFQ
minimum ratio count of 2. ‘Match between runs’ function was used with match and
alignment time limits of 1 and 20 minutes respectively. Data have been deposited in
the PRIDE repository [project accession number PXD008785].

Assessment of mitochondrial membrane potential and mass

Variations of the mitochondrial transmembrane potential (ΔΨm) were studied using
3,3-dihexyloxacarbocyanine iodide [DiOC6(3)]. This cyanine cationic dye
accumulates in the mitochondrial matrix as a function of ΔΨm. Cells (1 × 10^6/mL)
were incubated in 1mL culture medium containing 40nM DiOC6(3) for 30min in the
dark at 30°C with constant shaking. DiOC6(3) membrane potential-related
fluorescence was recorded using FL1 height. A total of 10,000 cells were analyzed for
each curve. The collected data was analyzed using FlowJo software version 7.2.5 to
determine the mean green fluorescence intensity after each treatment. The results are
expressed as a percentage of mean fluorescence of the control strain. As a negative
control, in each experiment, we preincubated aliquots of cells with carbonyl-cyanide
4-(trifluoromethoxy)- phenylhydrazone (FCCP, Sigma) and antimycin (Sigma) at
100μM and 5μg/mL, respectively, 10min before fluorescent dye staining, which leads
to a collapse of mitochondrial membrane potential.

To measure mitochondrial mass, we used 10-N-Nonyl acridine orange (NAO), a dye
that binds to cardiolipin, a phospholipid specifically present on the mitochondrial
membrane. Cells (1 × 10^6/mL) were incubated in 1 mL culture medium containing
100nM NAO for 30min in the dark at 30°C with constant shaking, followed by
analysis on FACSCalibur flow cytometer with the same photomultiplier settings as
used for DiOC6(3).

Evaluation of the mitochondrial morphology and protein import machinery

To image mitochondrial morphology, strains were transformed with a MitoLoc
plasmid (a gift from Markus Ralser) according to a previously described protocol,
with the only difference that the cells were incubated with the plasmid overnight at
room temperature. Microscope slides were prepared as follows: 150μL of YPD media
containing 2% agarose was placed on a preheated microscope slide and cooled, before
applying yeast cells to obtain a monolayer. The cells were centrifuged at 4000 × g for
3 min, and resuspended in 50μL YPD. Once dry, the cover slip was placed, sealed, and
mounted on a temperature-controlled Nikon Ti-E Eclipse inverted/UltraVIEW VoX
(Perkin Elmer) spinning disc confocal setup, driven by Volocity software (version
6.3; Perkin Elmer). Images were recorded through a 60xCFI PlanApo VC oil
objective (NA 1.4) using coherent solid state 488nm and 543nm diode lasers with a
DPSS module, and a 1000 × 1000 pixel 14-bit Hamamatsu (C9100-50) electron-
multiplied, charge-coupled device (EMCCD). The exposure time was 100ms for GFP
and 300ms for mCherry, at 5–10% laser intensity. The number of cells with cytosolic
mCherry accumulation was counted manually. More than 1000 cells were examined
for each strain. Images were analysed using ImageJ software with the MitoLoc
plugin.

ROS measurement
Cells were incubated in the dark with 5μM MitoSOX™ red mitochondrial superoxide indicator (Molecular Probes) for 10min at 30°C and subsequently analyzed by flow cytometry. Fluorescence (excitation/emission maxima of 510/580nm) of 10,000 cells resulting from the intracellular red fluorescence was measured in the FL2 channel. The collected data was analyzed using FlowJo software version 7.2.5 for Microsoft (TreeStar, San Carlos, CA, USA) to determine the mean green fluorescence intensity after each treatment. The results are expressed as the mean fluorescence across 10,000 cells.

**RNA extraction**

Total RNA was isolated using the NucleoSpin RNA kit (Macherey&Nagel) according to the manufacturer’s instructions for up to 3 × 10⁸ yeast cells, which includes incubation with 50–100U of zymolyase for 1hr at 30°C. The quality of the resulting total RNA was tested on 1% agarose gels.

**Genomic DNA isolation**

Cells from saturated cultures (approximately 10⁹ cells) of WT Y258, WT a161, I₀, and Mss116OE were harvested by centrifugation and washed, as described above. Cell wall was digested for 1h at 30°C in the presence of 50–100U of zymolyase. Spheroplasts were then resuspended in 500μL of cell lysis buffer (75mM NaCl, 50mM EDTA, 20mM HEPES pH 7.8, 0.2% SDS). Next, 10μL of 20mg/mL proteinase K was added and the mixture incubated for 2h at 50°C. DNA was
precipitated by the addition of isopropanol at room temperature, followed by centrifugation at 4°C and 11000g for 30min. The DNA pellet was then washed with ice-cold 70% ethanol, air-dried and resuspended in 50μL of DNase free water at 55°C.

Quantitative real-time PCR (qPCR)

cDNA was synthesized from 1000ng of total RNA using the iScript™ cDNA Synthesis Kit (Biorad). The cDNA was diluted 100-fold, mixed with primer pairs for each gene and SYBRgreen (BioRad). All primer pairs were designed to have a melting temperature of 60°C and are listed in Supplementary File 1. The qPCR reaction was run on a QuantFlexStudio 6 (Life Technologies) using 40 cycles, after which the melting curves for each well were determined. Final fold change values were estimated relative to the UBC6 gene in the control strain replicates.

mtDNA copy number was assessed by qPCR using genomic DNA as template and primers against cox1 and cox3 (mtDNA) and rpl32 (nuclear DNA, for normalization).

Single cell generation time measurement

Individual cells (approximately 100 for each strain) were placed on agar plates of appropriate growth medium, as described above, using a micromanipulator. Next, an image of each original mother cell was taken every 10 minutes for 8-9 hours. The images were then analysed and division time of each cell was extracted.
RNA-FISH and imaging

Yeast cultures were grown as described above, fixed with 37% formaldehyde for 45 min at room temperature, digested with 2.5 μL of zymolyase (Zymo Research, 2000 U) at 30°C for 60 min 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 0.3 μm. 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.

References


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Competing financial interests

The authors declare that no competing financial interests exist.

Figure legends

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 (*Mss116OE*) (a) reduces growth rates, (b) extends chronological lifespan, (c) increases mitochondrial mass, measured as NAO fluorescence, (d,e) increases mitochondrial volume, (g)
oxygen consumption, and (h) ATP levels but (i) decreases superoxide levels, measured as MitoSOX fluorescence. (f) Mitochondrial inner membrane potential does not differ significantly between strains. WT a161 and WT Y258 are control strains for $I_0$ and $Mss116_{OE}$, respectively, as described in the text. The $Mss116^{E268K}$ strain harbours a mutant version of Mss116 that lacks ATPase activity. As a visual guide, strains are coloured consistently throughout. Bar heights display the mean of three biological replicates, each calculated as the mean of three technical replicates. Error bars are standard errors of the mean. **P < 0.01; *P < 0.05 (ANOVA plus post hoc).

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

(a) qPCR measurements of selected genes, comparing focal strains ($I_0$, $Mss116_{OE}$, $Mss116^{E268K}$) to their isogenic control strains (left panel). For both $cox1$ (central panel) and $cob$ (right panel), intron levels are specifically reduced upon overexpression of $Mss116_{OE}$ but not $Mss116^{E268K}$, while mature mRNA levels increase. Heat maps display mean values of log-fold changes observed across three biological replicates (each averaged over three technical replicates). UBC6 was used for normalization.

(b) RNA-FISH confirms elimination/reduction of introns aI2 and aI5β from the $cox1$ transcript pool. Exon (green), intron (red) and co-localized (green/red) puncta were counted in more than 300 cells. The bar chart shows the number of signals per 100 cells. Bar heights display the mean of three biological replicates (each averaged over three technical replicates). Error bars are standard error of the mean. **P < 0.01; *P < 0.05 (ANOVA plus post hoc). White lines mark cell boundaries.
White arrows mark examples of exonic puncta that do not co-localize with intronic puncta.

(c) qPCR time series of pre-mRNA and mature mRNA levels following induction of Mss116. Mature mRNA for coxl and cob was quantified using primer pairs (f\textsubscript{xT}, f\textsubscript{T}) overlapping the terminal exon-exon junctions. Pre-mRNA was quantified using a series of primer pairs (f\textsubscript{x1-5}, f\textsubscript{b1-5}). For each pair, one primer is located in exonic, the other in intronic sequence, as detailed in Figure 2-figure supplement 4. Each circle (shades of blue for the pre-mRNA and red for the mature transcript) represents the mean value from three biological replicates (each averaged over three technical replicates). UBC6 was used for normalization.

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

(a) Mitochondrial morphology is altered and (b) mitochondrial volume, (c) oxygen consumption, (d) ATP levels, and (e) chronological lifespan are reduced when rtg2 is deleted in the I\textsubscript{0} or MSS116\textsubscript{OE} background. This contrasts sharply with I\textsubscript{0} and MSS116\textsubscript{OE} where rtg2 is intact (see Figure 1). Bar heights display the mean of three biological replicates (each averaged over three technical replicates). Error bars are standard error of the mean. ***P < 0.001; **P < 0.01; *P < 0.05 (ANOVA plus post hoc). (f) Transcriptional responses in different strains where rtg2 has been deleted, as measured by qPCR. Heat maps display mean values of log-fold changes observed across three biological replicates (each averaged over three technical replicates). UBC6 was used for normalization.

Figure 4. Hap4 is required for the intronless phenotype.
Mitochondrial morphology, mitochondrial volume, oxygen consumption, ATP levels, and chronological lifespan do not differ between \( I_0/Mss116^{OE} \) and their corresponding control strains if hap4 has been deleted. Bar heights display the mean of three biological replicates (each averaged over three technical replicates). Error bars are standard error of the mean. **P < 0.001; **P < 0.01; *P < 0.05 (ANOVA plus post hoc).

Transcriptional responses in different strains where hap4 has been deleted, as measured by qPCR. Heat maps display mean values of log-fold changes observed across three biological replicates (each averaged over three technical replicates). UBC6 was used for normalization.

Figure 5. Dampening RNA levels of \( \text{cox1} \) and \( \text{cob} \) by reducing promoter activity partially rescues the intronless phenotype.

(a) qPCR measurements in strains where either the \( \text{cob} \) promoter (cobp), the \( \text{cox1} \) promoter (cox1p) or both (pp) have been attenuated via targeted mutations. Heat maps display mean values of log-fold changes observed across three biological replicates (each averaged over three technical replicates). UBC6 was used for normalization. (b) Oxygen consumption and (c) mitochondrial morphology and (d) volume in response to promoter attenuation. Bar heights display the mean of three biological replicates (each averaged over three technical replicates). Error bars are standard error of the mean. Asterisks to indicate statistical significance are omitted for clarity. All comparisons between single- and double-promoter mutants and the corresponding parent strains are significant at P<0.001 (ANOVA plus post hoc).

Supplementary Figure Legends
Figure 1-figure supplement 1. Competitive fitness is decreased in \( I_0 \) (competed against WT a161) and \( Mss116_{OE} \) (competed against the empty vector control WT Y258). Bar height represents the mean of three biological replicates. Error bars are standard error of the mean. ***\( P < 0.001; **P < 0.01; *P < 0.05 \) (ANOVA plus post hoc). See Methods for details of how competitions were carried out and relative fitness determined.

Figure 1-figure supplement 2. \( I_0 \) and \( Mss116_{OE} \) show no qualitative difference in growth on (a) glucose and (b) glycerol. Numbers on top of each panel represent the decimal dilution of the stationary culture that is plated as a spot of 5 µl.

Figure 1-figure supplement 3. (a) GFP-tagged Mss116 localizes to mitochondria, whether expressed at endogenous levels (left image) or upon overexpression in the Y258 background (right image). (b) The expression level of Mss116 increases approximately 2.5-fold in the \( Mss116_{OE} \) strain, as measured by flow cytometry. Bar heights display the mean of three biological replicates, each of them representing mean of three technical replicates. Error bars are standard error of the mean. ***\( P < 0.001; **P < 0.01; *P < 0.05 \) (ANOVA plus post hoc).

Figure 1-figure supplement 4. Median and maximum replicative lifespan of \( Mss116_{OE} \) is extended compared to the empty vector control and \( Mss116^{E268K} \). The total number of monitored mother cells is as follows: 90 cells for the empty vector control, 86 cells for \( Mss116_{OE} \), and 91 cells for the \( Mss116^{E268K} \). Measurements were pooled across 3 independent experiments.
Figure 2-figure supplement 1. (a) Comparison of qPCR measurements in $I_0$ and $I_0$ overexpressing Mss116. Heat maps display mean values of log-fold changes observed across three biological replicates (each averaged over three technical replicates). UBC6 was used for normalization. (b) ATP level, (c) oxygen consumption and (d) ROS level do not change upon overexpression of Mss116 in the $I_0$ genetic background ($I_0 + Mss116_{OE}$) compared to $I_0$.

Figure 2-figure supplement 2. Mss116 deletion does not affect $I_0$ phenotypes, highlighted by (a) the transcript levels (measured by qPCR) of relevant genes, (b) mitochondrial morphology, and (c) mitochondrial volume. The heat map displays mean values of log-fold changes observed across three biological replicates (each averaged over three technical replicates). UBC6 was used for normalization. In panel (c), bar heights display the mean of three biological replicates (each averaged over three technical replicates). Error bars are standard error of the mean. ***$P < 0.001$; **$P < 0.01$; *$P < 0.05$ (ANOVA plus post hoc).

Figure 2-figure supplement 3. Expression level of Mss116 during 9 hours after induction using 2% galactose. Circles represent mean value of three biological replicates (each performed as technical replicate). Error bars are standard error of the mean.

Figure 2-figure supplement 4. Schematic presentation of the fragments of $cox1$ and $cob$ amplified to determine the pre-mRNA and mature mRNA levels.
Figure 5-figure supplement 1. Related to Figure 5. Schematic representation of mutated positions in the promoter regions of *cox1* and *cob*.
strain competing against its control
Figure a: Heatmap showing the expression levels of various genes involved in mitochondrial functions.

Figure b: Bar graph showing ATP levels (nmol) in different conditions.

Figure c: Bar graph showing O$_2^-$ consumption (nmol/mg protein) in different conditions.

Figure d: Bar graph showing mitochondrial superoxide (MitoSOX) fluorescence intensity (%) in different conditions.