

Mitochondrial-nuclear DNA interactions regulate nuclear transcription.

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Abstract

The nuclear and mitochondrial organelles must maintain a communication system in order to respond effectively to environmental conditions. Previous studies have identified mitochondrial DNA inside the nucleus and interacting with the nuclear chromosomes. How this transfer occurs and what the function of the mitochondrial DNA is once inside the nucleus remains unclear. Here, we comprehensively map DNA-DNA interactions between the mitochondrial and nuclear genomes (Mito-nDNA) using Genome Conformation Capture in *Saccharomyces cerevisiae* cells grown under three different metabolic conditions (glucose, galactose and glycerol lactate). Global differences are observed for the numbers and types of Mito-nDNA interactions according to the metabolic state of the cell. Two interactions between the mitochondrial and nuclear genomes (*COX1-MSY1* and *Q0182-RSM7*) showed significant reductions in the absence of mitochondrial encoded reverse transcriptase machinery. Moreover, the *COX1-MSY1* inter-organelle DNA interaction significantly reduced the transcript levels of the nuclear *MSY1* gene while the *Q0182-RSM7* interaction also caused an approximately 30 % reduction in the *RSM7* transcript levels. We conclude that Mito-nDNA interactions are biologically relevant and our results argue for a role for reverse transcription in inter-organelle DNA mediated communication.

Supplementary files

Online supplementary data consists of the following: Supplementary Figures, supplementary methods, supplementary statistics, supplementary interaction tables and the aligned sequence files.

Introduction

Mitochondria have a central role within the metabolic systems of cells. In yeast, as in other organisms, the mitochondrial organelle is the site of oxidative phosphorylation and the citric acid cycle. As such, yeast mitochondria are essential for both the fermentative and respiratory pathways that yeast use to metabolize different carbon sources. Unlike most other organisms, yeast can employ both respiration and fermentation simultaneously (*i.e.* respiro-fermentation) when grown on fermentable carbon sources such as glucose or galactose, at a concentration above 0.8mM (Lagunas 1986; Otterstedt et al. 2004). *Saccharomyces cerevisiae* mitochondria contain an 85,779bp genome, which encodes a subset of the electron transport chain components (Foury et al. 1998). This genome is essential for respiratory growth on non-fermentable carbon sources like glycerol lactate (Lagunas 1976), as the subset of electron transport chain components it encodes are required to generate a proton gradient across the inner mitochondrial membrane, ultimately driving ATP synthesis. In contrast, a functioning electron transport chain, and thus the mitochondrial genome, is non-essential when cells grow solely by fermentation.

The mitochondrial genome is widely recognized as having drastically reduced in size over the course of evolution of the mitochondrial–host symbiosis, to the point that ~98% of the genes required for mitochondrial function are presently encoded within the nuclear chromosomes (Timmis et al. 2004). Consequently, mechanisms must exist to co-ordinate and control the expression of the nuclear- and mitochondrial genome- encoded genes required to maintain and control mitochondrial function (Butow and Avadhani 2004; Liu and Butow 1999). Intriguingly, despite the fact that the majority of mitochondrial genes have transferred to the nuclear genome; the transfer of mitochondrial DNA (mtDNA) to the yeast nucleus remains an on-going process with mtDNA being used to repair double stranded breaks in yeast nuclear chromosomes under certain conditions (Ricchetti et al. 1999). Additionally, unstable mitochondrial plasmids have been observed to transfer into the yeast nucleus (Thorsness and Fox 1990; Thorsness and Fox 1993) in a nuclear gene (*e.g.* *YME1*, *YME2*) dependent manner (Campbell and Thorsness 1998; Shafer et al. 1999; Thorsness and Fox 1993). The nuclear functions of these transferred mtDNAs are unknown, however elevated mitochondria to nucleus DNA migration rates correlate with accelerated chronological aging in yeast (Cheng and Ivesa 2010).

Distal regulatory regions (*e.g.* enhancers) loop within chromosomes in order to interact with the promoter region of the genes which they control (Tolhuis et al. 2002). In fact, enhancers can also control promoters on different chromosomes (Spilianakis and Flavell 2004; Spilianakis et al. 2005). These types of intra- and inter-chromosomal interactions can be captured using proximity-based ligation methodologies (*e.g.* Genome Conformation Capture (GCC)) that incorporate high resolution (*i.e.* ~2 Å (Fujita and Wade 2004)) cross-linking of interacting DNA strands, restriction digestion (*e.g.* *MspI*), and ligation to identify DNA sequences that interact at a frequency greater than background (O'Sullivan 2010b; Rodley et al.

2009). We have previously observed that nucleic acids of mitochondrial origin interact with nuclear loci (hereinafter referred to as Mito-nDNA interactions) in *S. cerevisiae* (Rodley et al. 2009). The inter-organellar, Mito-nDNA interactions formed a subset of the interactions that were identified; however, preliminary data indicated that they may respond to the metabolic status of the yeast cells, reflecting a possible role in mitochondrial-nuclear coordination (Rodley et al. 2009). GCC, being a proximity-based ligation methodology, does not inform on the nature of the contacts or the identity of the complexes that may or may not maintain them (Dekker et al. 2002; Duan et al. 2010; Laine et al. 2009; Singh and Hampsey 2007; Tolhuis et al. 2002). However, inter- and intra-chromosomal interactions have been hypothetically and experimentally linked with transcription and transcriptional memory (Brickner 2010; Osborne et al. 2007). Moreover, the quality and quantity of mitochondrial DNA has been shown to affect patterns of nuclear transcription (Parikh et al. 1987; Parikh et al. 1989) and replication (Blank et al. 2008) in yeast.

Here we identify the Mito-nDNA interactions occurring in *S. cerevisiae* during growth on glucose, galactose (*i.e.* respiration-fermentation), and glycerol lactate (*i.e.* solely respiration) using GCC. Inter-condition comparisons indicated that both the frequency and identities of inter-organellar interactions were dramatically different between conditions. Interactions between mitochondrial genes (*i.e.* *COX1* and *Q0182*, a dubious mitochondrial ORF) and nuclear encoded loci (*i.e.* *MSY1* and *RSM7*, respectively), are shown to be dependent upon a functional electron transport chain and mitochondrial encoded reverse transcriptase machinery. Finally, the levels of the nuclear encoded *MSY1* and *RSM7* gene transcripts are increased when the interaction frequency is reduced by the knockout of mitochondrial reverse transcriptase activity. Our results argue for a role for reverse transcription in inter-organellar DNA mediated communication.

Results

We previously captured Mito-nDNA interactions in *S. cerevisiae* cells grown in glucose by GCC (Rodley et al. 2009). A detailed investigation of one of these Mito-nDNA interactions demonstrated that it was carbon source dependent. Therefore, we hypothesized that Mito-nDNA interactions would alter, on a global scale, according to the cells metabolic state, and in particular, the carbon source used for growth. Thus, we generated comprehensive maps of the Mito-nDNA interactions in *S. cerevisiae* by GCC, using the *MspI* restriction enzyme, during exponential growth in media containing glucose, galactose, or glycerol lactate. GCC networks were constructed from 36bp paired end Illumina Genome Analyzer sequence reads (total reads; glucose 56,167,792, galactose 48,419,385, glycerol lactate 49,134,906).

Statistical and experimental methods were used to determine if the Mito-nDNA interaction patterns could have been generated by experimental noise alone, which would be expected to produce random pairings of fragments from the two genomes. *In silico* simulations (100,000) were performed (Rodley et al. 2009) to determine the maximum count of a particular interaction that would be observed under this noise model, given the same number of sequences, interactions and fragments as in the experimental data. The maximum count over 100,000 simulations was 6 for the glycerol lactate condition, and 5 for the other two conditions. In our real dataset the maximum count we observe for any pairing is 14 for the glucose condition, 32 for galactose and 41 for glycerol lactate. Therefore, we conclude that the interaction patterns cannot be attributed to noise alone under any of the conditions, in each case with a p-value less than 10^{-5} . Secondly, we performed analyses to determine what frequency individual interactions have to achieve before they are deemed to be present at a level above experimental noise (Supplementary Statistics). As a result, we identified 8678 statistically significant interactions occurring between the nuclear and mitochondrial genomes during glucose growth, 1780 during galactose growth, and 8153 during growth in glycerol lactate. Biological repeats for each condition were highly correlated for statistically significant interactions ($R^2=0.78$, 0.93 , 0.93 , respectively; Supplementary Figure 1 and Supplementary Statistics). Accordingly, sequences from biological repeats were combined and reanalyzed.

To experimentally control for spurious inter-molecular ligation events, during the GCC process, samples were spiked with two ligation controls during library preparation. The first ligation control consisted of PCR products (Supplementary Table 2) that were added (1:1 ratio with the nuclear genome copy number) before the GCC ligation step. These controls were designed to estimate the frequency of random inter-molecular ligation events during GCC library preparation. A maximum of 47 separate ligation events were observed, none of which occurred at levels above the statistically defined experimental noise. The second ligation control consisted of the addition of pUC19 plasmid to the sample following the GCC ligation in order to control for random ligation events during preparation of the samples for sequencing, at the

sequencing centre. We observed a maximum of six interactions between pUC19 and the rest of the genome; again none of these interactions were above the statistically defined experimental noise. In conclusion, the fact that the high copy number rDNA and mitochondrial DNA elements do not show significant levels of random inter-molecular interactions with our internal control sequences is empirical evidence that the interactions we observe result from intra-molecular ligation events. Therefore, random ligation events during sample preparation do not account for the interactions we observe.

Significant interactions were separated into two pools, those which occur between the mtDNA and the nuclear ribosomal DNA repeats (Mito-rDNA), and those between mtDNA and unique nuclear loci (Mito-nDNA; Table 1). The rDNA repeats form part of the nucleolus and encode the rRNA component of the cytosolic ribosomes. The rDNA repeats constitute ~9.8% of the yeast genome; yet, the Mito-rDNA interactions constitute 95.8%, 52.4%, and 84.5% of the total interactions between the nuclear and mitochondrial genomes in glucose, glycerol lactate, and galactose, grown cells, respectively. There does not appear to be an interaction “hotspot”, with Mito-rDNA interactions evenly spread across the 9.1kb rDNA repeat (data not shown). Hence, Mito-rDNA interactions are over-represented within the data-set and are carbon source dependent (Table 1).

We determined whether nuclear chromosome length correlated with the number of Mito-nDNA interactions identified for that chromosome. The number of Mito-nDNA interactions per nuclear chromosome is highly correlated with chromosome length in the glycerol lactate condition, but not in glucose or galactose (Figure 1). This discrepancy is mainly due to the deviation of chromosome X from the trend during growth in glucose and galactose. Intriguingly, the increase in mtDNA interactions with chromosome X is accounted for by a single *MspI* fragment that encompasses the promoter region and partial coding sequence for two divergent ORFs: one uncharacterized ORF (YJR115W) and *RSM7* which encodes a mitochondrial small subunit ribosomal protein. Interestingly, numerous mtDNA *MspI* fragments, including fragments surrounding or overlapping the *COX1*, *COX3*, *VAR1* and *SCE1* genes, interact with this one “hotspot” on chromosome X. Thus, indicating the presence of hotspots for both interacting partners. The presence of hotspots for interactions within the mitochondrial genome was supported by the finding that the mitochondrial regions that are involved in the interactions are not uniformly distributed across the mitochondrial genome (Supplementary Figure 2).

Yeast mitochondrial escape mutants (YME) (Thorsness and Fox 1993) have been previously implicated in an elevated rate of transfer of unstable mitochondrial plasmids to the yeast nucleus (Campbell and Thorsness 1998; Shafer et al. 1999; Thorsness and Fox 1990; Thorsness and Fox 1993). Therefore, we predicted that the YME pathway was the source of mtDNA fragments interacting with the nuclear genome, and thus mutations within this pathway would result in an increase in the frequency of inter-organelle DNA interactions. To test this prediction, we used quantitative Chromosome Conformation

Capture (3C) to compare the interaction frequency between the *COX1* gene (Mt: 24872–26193bp) and the nuclear encoded *MSY1* gene (Chr XVI; 365496–365760bp), hereinafter denoted *COX1-MSY1*, in *S. cerevisiae* YME knockout mutants (*i.e.* $\Delta yme1$, $\Delta yme2$). The *COX1-MSY1* interaction was chosen for further investigation. Contrary to expectations, we observed a significant decrease in the frequency of the *COX1-MSY1* interaction in the $\Delta yme1$ strain as compared to the wild-type (T-test [Paired $P(T \leq t)$ one-tail, $n=4$] $p=0.010$; Figure 2). Deletion of a functionally unconnected nuclear gene (*ade2*) did not significantly affect the *COX1-MSY1* interaction frequency (t-test [paired $P(T \leq t)$ one-tail, $n=4$] $p=0.103$; Figure 2). These results suggest that the source of the mtDNA that participates in the Mito-nDNA interactions is not the unstable mitochondrial plasmids which have previously been identified as escaping the mitochondria for the nuclear compartment.

Deletion of *yme1* results in an elevated rate of mitochondrial turn-over as well as an abnormal globular mitochondrial morphology (Campbell et al. 1994; Campbell and Thorsness 1998). The α -factor mediated arrest of yeast cells in the G1 phase of the cell cycle also causes fragmentation of the lattice-like mitochondrial network (Neutzner and Youle 2005), a phenotype that is similar to that observed in *yme1* deletion strains (Campbell et al. 1994). Interestingly, we observed a similar reduction in the *COX1-MSY1* interaction frequency upon α -factor induced synchronization (Supplementary Figure 4). Therefore, it is possible that the fragmented mitochondrial phenotype contributes to the reduction in the *COX1-MSY1* interaction frequency we observed in the *yme1* deletion strain. Moreover, deletion of *yme2*, which does not affect mitochondrial morphology but rather is involved in the maintenance of the mitochondrial nucleoid (Hanekamp et al. 2002; Park et al. 2006), results in a relatively unchanged *COX1-MSY1* interaction frequency when compared to the wild-type (T-test [Paired $P(T \leq t)$ one-tail, $n=4$] $p=0.377$; Figure 2). By contrast, *MDV1* is involved in mitochondrial fission with deletion strains being unable to correctly fragment mitochondria (Fekkes et al. 2000; Naylor et al. 2006). Therefore, if the phenotype were directly due to mitochondrial fragmentation, one would predict that strains carrying the *mdv1* deletion would show the opposite phenotype to the $\Delta yme1$ strain. However, the *COX1-MSY1* interaction frequency measured in the $\Delta mdv1$ strain was intermediate between that observed for the wild-type and $\Delta yme1$ strains, and not significantly different from either (T-test [Paired $P(T \leq t)$ one-tail, $n=4$] $wt-\Delta mdv1$ $p=0.143$, $\Delta mdv1-\Delta yme1$ $p = 0.210$; Figure 2). Therefore, it is unlikely that mitochondrial fragmentation is directly responsible for the observed changes in *COX1-MSY1* interaction frequency.

We postulated that an abnormal mitochondrial morphology, coupled with elevated mitochondrial turnover would result in a disturbance of the mitochondrial ATP synthesis pathway and a reduction in the *COX1-MSY1* interactions frequency. We tested the inter-organelle *COX1-MSY1* interaction for ATP dependence. Yeast cells were treated with an electron transport chain uncoupling agent, 2,4-Dinitrophenol (DNP), at a concentration (5mM) that inhibits respiration but allows fermentation

(Supplementary Figure 5). Quantitative 3C analyses were performed to monitor the effect of increasing DNP treatment times on the *COX1-MSY1* interaction (Figure 3). We observed a significant time-dependent decrease in the frequency of the *COX1-MSY1* interaction in the presence of DNP (t-test $p < 0.05$; Figure 3A). However, an interaction between two nuclear loci (nDNA-nDNA; Chr VII: 868673-873686bp - Chr IX: 172565-173311bp) was also shown to be affected by treatment with DNP (Figure 3B). The observed dependence of the nDNA-nDNA interaction on a proton gradient across the mitochondrial membrane, and thus mitochondrial ATP synthesis, suggests that formation of these DNA-DNA interactions is ATP dependent. Hence, while we knew that the transfer event was different (Figure 2) from the unstable plasmid transfer described previously (Thorsness and Fox 1993), we were unable to rule out ATP dependence.

The mitochondrial *COX1* gene contains group II introns that encode functional splicing, reverse transcriptase and endonuclease machinery (Eskes et al. 1997; Yang et al. 1996; Zimmerly et al. 1995a; Zimmerly et al. 1995b). Therefore, we predicted that the *COX1-MSY1* interaction involved a *COX1* complementary DNA (cDNA) produced by mitochondrial encoded reverse transcriptase. We obtained a *S. cerevisiae* strain which had all group II introns removed (strain 161-U7 GII-0), and a strain with all group II introns removed with the exception of *al5y* (strain 161-U7 GII-0 *al5y*; Figure 4A; (Huang et al. 2005)). Crucially, *al5y* does not encode any reverse transcriptase machinery. The *al5y* intron overlaps the *COX1* region which interacts with *MSY1* (Figure 4A). We performed quantitative 3C analyses to establish the frequency of the inter-organelle *COX1-MSY1* interaction in these strains growing with a respiro-fermentative metabolism (*i.e.* using glucose or galactose as sole C-source). The inter-organelle *COX1-MSY1* interaction was shown to be partially dependent upon the presence of the group II introns and thus the reverse transcriptase machinery (Figure 4B). The lack of a measurable *COX1-MSY1* interaction within strain GII-0, which does not contain the fluorogenic probe or reverse primer binding site (Figure 4A and 4B), confirmed that the *COX1-MSY1* interaction does not involve a nuclear-mitochondrial sequence (NUMT). An independent interaction involving a dubious mitochondrial ORF (*Q0182*; mtDNA [65783-65903bp]) and the nuclear encoded *RSM7* (Chr X [638756-640423bp]) was also shown to be partially dependent upon the presence of the group II introns (Figure 4C). The dubious mitochondrial ORF involved in this interaction does not contain any group II introns, and thus does not encode any of the components involved in reverse transcriptase activity. These results suggest that the nucleic acids of mitochondrial origin which participate in the Mito-nDNA interaction are reverse transcribed from RNA intermediates prior to transfer to the nucleus as cDNAs.

The number of significant (Supplementary Methods) Mito-nDNA interactions increased by >10-fold in respiring (*i.e.* glycerol lactate grown) cells, as opposed to glucose or galactose grown cells (Table 1). This increase was not due to a higher number of sequence reads for the respiring sample. Thus, a greater number of unique nuclear loci connect to mtDNA during respiratory growth when the mitochondria are

most active. This result, coupled with the need for a functional electron transport chain and reverse transcriptase machinery, led us to hypothesize that the Mito-nDNA interactions are functional in nature, and specifically that they are capable of controlling the transcript levels of the nuclear loci with which they interact. We performed quantitative reverse transcriptase PCR (qRT-PCR) to determine the transcript levels of the nuclear encoded *MSY1* and *RSM7* genes in WT cells, the mitochondrial group-II intron knockout mutant (161-U7 GII-0), and strain 161-U7 GII-0 Δ 5y (Figure 4A). We found that the transcript level of the *MSY1* gene is significantly higher (t-test, two-sample unequal variance, one-tail, $n=2$, $p=0.0007$) in strain 161-U7 GII-0 (Figure 5A), which does not contain the probe site and therefore has no detectable *COX1-MSY1* interaction (Figure 4A and B), thus identifying the maximum transcript level in the absence of detectable inter-organelle interactions. Critically, removal of the type II introns, except Δ 5y, (*i.e.* strain 161-U7 GII-0 Δ 5y; Figure 4A), caused a similar increase in *MSY1* transcript levels (Figure 5A). The mean level of *RSM7* transcripts is also higher in both the 161-U7 GII-0 and 161-U7 GII-0 Δ 5y strains than WT (Figure 5B). By contrast deletion of *MRS1*, which is involved in mitochondrial group I intron splicing (Bassi et al. 2002), had no effect on either *MSY1* or *RSM7* transcript levels (Figure 5C), or the *COX1-MSY1* interaction frequency (Supplementary Figure 6). Thus, alterations to mitochondrial reverse transcriptase activity lower the frequency of Mito-nDNA interactions and increase the levels of the nuclear encoded transcripts. Hence we conclude that, at least in the case of the *COX1-MSY1*, and to a lesser extent *Q0182-RSM7* interactions, cDNA mediated Mito-nDNA interactions are involved in the regulation of the nuclear transcripts.

Discussion

Despite the fact that inter-organelle nucleic acid transfer events are clearly an on-going (Adams et al. 2002; Adams et al. 1999; Brennicke et al. 1993; Campbell and Thorsness 1998; Farrelly and Butow 1983; Hazkani-Covo et al. 2010; Richly and Leister 2004; Shirafuji et al. 1997; Thorsness and Fox 1990; Thorsness and Fox 1993) and predominantly unidirectional process (Shirafuji et al. 1997; Tarasov and Entelis 1992), it has been proposed that there is no function for recent transfer events (Cavalier-Smith 1987). The observation that the number of mitochondrial interactions per nuclear chromosome is highly correlated with the nuclear chromosome length, with the exception of Chromosomes X (during growth on glucose and galactose) and XII, appears to support this hypothesis. However, the variation exhibited by Chromosome X is almost entirely explained by an increase in Mito-nDNA interaction frequency at a single locus: *RSM7*. These data correlate with the fact that *RSM7* encodes a conditionally essential (*i.e.* respiration) small subunit mitochondrial ribosomal protein that is induced during the switch from fermentation to respiration (DeRisi et al. 1997; Roberts and Hudson 2006), the metabolic condition in which it has the least interactions with mitochondrial DNA. Similarly, the *COX1-MSY1* inter-organelle interaction, couples a conditionally essential (respiration) nuclear encoded tyrosyl-tRNA synthetase to mitochondrial activity, and specifically *COX1* transcript processing. Again this interaction regulates the

transcript levels of the nuclear *MSY1* gene. Therefore, in combination with the observation that a reduction in interaction frequency correlates with an increase in the amount of nuclear encoded *RSM7* and *MSY1* transcripts, we propose that the transfer events are functional and form part of the mitochondrial – nuclear communication system.

Proximity-based ligation methodologies (e.g. GCC) only identify the sequences that are interacting, and do not provide information: 1) on whether or not the entire mitochondrial genome or fragments thereof are transferred to the nucleus; or 2) on the percentage of a population that contain a particular interaction or the absolute level of that interaction within a particular cell. However, our combined 3C and transcript analyses of *S. cerevisiae* 161-U7 GII-0 Δ 5 γ , in which the Δ 1 and Δ 2 introns present within the *COX1* gene have been deleted (Huang et al. 2005; Moran et al. 1995), go some way to addressing these issues. Firstly, deletion of the Δ 1 and Δ 2 introns caused a 40 - 60% reduction in the frequency of the inter-organelle *COX1-MSY1* and *Q0182-RSM7* interactions. These results confirm a role for a mitochondrial encoded RNA intermediate in these interactions. The incomplete ablation of the interactions following the deletion of the *COX1* Δ 1 and Δ 2 introns could result from: 1) the presence of other retrotransposon or retroviral encoded reverse-transcriptase of either mitochondrial or nuclear origin within the mitochondrial matrix, 2) cytoplasmic or nuclear reverse transcription of mRNA released from damaged mitochondria, or 3) the transfer of mitochondrial mini-circles (Thorsness and Fox 1993). Mechanisms 1) and 2) are most likely given the identification of remnants of nuclear derived *cop*ia-, *gypsy*- and LINE-like retrotransposon elements within *Arabidopsis* mitochondria (Knoop et al. 1996; Marienfeld et al. 1999), the finding that transcription does not affect mitochondrial mini-circle transfer in yeast (Shafer et al. 1999; Thorsness and Fox 1993), and the reductions in interaction frequency we observed with the Δ *yme1* mutant. Secondly, the combined analyses of *S. cerevisiae* 161-U7 GII-0 Δ 5 γ identified a significant population effect on nuclear transcript levels correlating with the reduction in the number of mitochondrial to nuclear DNA interactions caused by deletion of the Δ 1 and Δ 2 introns.

It is unclear whether the mtDNA that participates in the Mito-nDNA interactions is transferred by a direct connection between the mitochondrial and nuclear organelles or by uptake from the cytoplasm. Uptake from the cytoplasm is feasible given the high success rates attained for yeast transformation (Costanzo and Fox 1988; Ito et al. 1983), and the finding that the uptake of unstable mitochondrial plasmids occurs by vacuole mediated release into the cytoplasm (Campbell and Thorsness 1998; Thorsness and Fox 1990). However, Ricchetti *et al.* demonstrated that the mtDNA mediated repair of nuclear double strand breaks is independent of Δ *yme1* mutations (Ricchetti et al. 1999) and therefore occurs through another, possibly direct, transport mechanism. Direct transport from the mitochondria to nuclear compartments could occur as a result of a tethering/transport complex that physically links mitochondria to the endoplasmic reticulum (Kornmann et al. 2009), which is formed from the outer-leaflet of the nuclear membrane (Shibata et al. 2010).

The mtDNA fragments involved in the Mito-nDNA interactions that we observed were not evenly distributed across the mitochondrial genome. The finding that there is no bias for ORFs over non-coding regions appears to argue against the transfer occurring through reverse-transcribed cDNAs. However, yeast mitochondrial genes are transcribed as polycistronic transcripts (reviewed in (Costanzo and Fox 1990)), from 14 ATATAAGTA consensus promoters and possibly another 5 non-consensus promoter sites (Christianson and Rabinowitz 1983). Hence, as with other genomes, a large percentage of the mitochondrial genome is physically transcribed (Barth et al. 1999; Holec et al. 2006; Montoya et al. 1982).

We observed a large number of metabolism dependent, inter-organelle interactions between mitochondrial genomic loci and the nuclear rDNA repeats. These interactions can be explained in terms of the control of cryptic RNA polymerase II (PolII) transcription from within the rDNA repeats (Butow et al. 1988; Mayan and Aragon 2010; O'Sullivan 2010a; Parikh et al. 1989). Previous observations indicate that the mechanism may involve the formation of *SIR2* dependent inter- and intra-rDNA repeat loops (O'Sullivan et al. 2009) that contact the replication fork block site, present within the repeats, and in so doing regulate cryptic rDNA PolII transcription (Mayan and Aragon 2010; O'Sullivan 2010a). Hypothetically, the formation of the Mito-rDNA interactions antagonize the formation of these rDNA loops and thus regulate the rate of cryptic rDNA PolII transcription (Mayan and Aragon 2010; O'Sullivan 2010a) and replication. Such a mechanism fits with observations that cryptic rDNA transcription (Parikh et al. 1987; Parikh et al. 1989) and replication (Blank et al. 2008) are dependent upon the mitochondrial genome. Furthermore, the replication effect was antagonized by *SIR2* (Blank et al. 2008). Alternatively, it is possible that the nucleolus sequesters cDNAs derived from the mitochondria in a cell cycle- or environmentally-dependent manner. This situation would be analogous to the role the nucleolus is postulated to play in sequestering nuclear proteins involved in the cell cycle (Cerutti and Simanis 2000) and environmental responses (reviewed in (Boisvert et al. 2007)).

Our results establish a role for Mito-nDNA interactions in inter-organelle signaling and the control of nuclear transcript levels. These results are consistent with previous findings that nuclear transcription is positively and negatively altered in response to the presence or absence of mitochondrial genome sequences in yeast (Parikh et al. 1987; Parikh et al. 1989). It is clear that there is considerable redundancy in the pathways that coordinate the carbon controlled expression of genes, with regulation occurring at all stages of expression both in the nucleus and in the mitochondria themselves (Costanzo and Fox 1990; Schuller 2003; Towpik 2005). We propose that a fraction of mitochondrial RNAs are reverse-transcribed and these cDNAs are transported to the nucleus where they regulate nuclear functions, including transcription and replication. While we have shown a repressive role for Mito-nDNA interactions in the control of nuclear transcript levels, there is no reason to assume that all interactions are repressive. We further argue that the targets of these interactions differ depending on metabolism

specific nuclear encoded factors (e.g. transcription factors (Schuller 2003)), and the steady state levels of the mitochondrial RNAs, which are ATP dependent (Amiott and Jaehning 2006). In the case of the glucose and galactose dependent *COX1-MSY1* and *Q0182-RSM7* interactions, this communication acts to reinforce the catabolite dependent repression of mitochondrial translation (Figure 6).

The mechanism by which Mito-nDNA interactions control transcript levels is unknown. It is possible that it is mediated by physical interaction between the mitochondrial derived cDNA and the nuclear locus or by more indirect means. Group II introns are not ubiquitous within the mitochondrial genomes of higher eukaryotes. Despite this, it is not essential that the reverse transcriptase be encoded within the mitochondrial genome given that nuclear encoded retrotransposons have been identified within mitochondria (Knoop et al. 1996; Marienfeld et al. 1999). Therefore, further work is warranted in higher eukaryotes to determine the universal significance of these interactions.

In conclusion, we have shown that the inter-organelle DNA-DNA interactions are capable of altering the transcript levels of nuclear genes. Moreover, our results argue for a role for reverse transcription in inter-organelle DNA mediated communication.

Materials and Methods

Strains and growth conditions

Saccharomyces cerevisiae strains (Supplementary Table 1) were stored (-80°C) and cultured (30°C, 160rpm) on synthetic complete (SC) medium containing amino acid supplements and glucose (2% w/v) (Kaiser 1994), glycerol lactate (2% glycerol v/v 2% lactic acid v/v with 0.05% glucose w/v), or galactose (2% w/v). For Genome Conformation Capture (GCC) and Chromosome Conformation Capture (3C) analyses, strains were recovered from -80°C on SC glucose (2% w/v) agar (2%) plates for 48 hours prior to starter culture inoculation. Starter cultures were grown (30°C, 160rpm, 16h) in SC glycerol lactate or glucose medium containing amino acid supplements, as indicated. Test cultures were inoculated, from the starter cultures into SC media (containing the indicated carbon source), grown (30°C, 160rpm) and harvested at an optical density (OD₆₀₀) of 0.6. Mitochondrial uncoupling was achieved by the addition of 2,4-Dinitrophenol (5mM final concentration) for 45, 90, or 180 minutes (Supplementary Figure 5). Cell cycle arrest was achieved by treatment (180mins, 30°C, 160rpm) with cell cycle inhibitors (*i.e.* α -factor (3.4 μ m), nocodazole (15 μ gml⁻¹), or hydroxyurea (100mM)). Cell cycle arrest was confirmed by microscopy.

Genome Conformation Capture (GCC)

GCC was performed according to (Rodley et al. 2009). Briefly, chromatin was prepared from 15 sets of 10⁸ (*i.e.* a total of 1.36x10⁹) cross-linked cells. Chromatin was digested with *MspI* (Fermentas) and ligated (T4 ligase; Invitrogen). Crosslinks were reversed in the presence of proteinase K (final concentration 7-11ug, Roche). Samples were treated with RNase A (final concentration 10 μ gml⁻¹) prior to purification by phenol:chloroform (1:1 v/v, three times) and column extraction (Zymo Clean and Concentrator, Zymo Research). Paired-end sequencing (36 bp) was performed on 5 μ g DNA using the Illumina Genome Analyzer platform (Allan Wilson Centre, Massey University, New Zealand & Friedrich Miescher Institute for Biomedical Research, Basel, Switzerland).

External controls were added at two steps in the GCC protocol to control for random ligation events. The first ligation control, a linear DNA fragment with a free *MspI* site at one end (Supplementary Methods), was added in a 1:1 ratio with the nuclear genome prior to the addition of ligase. The second ligation control (1x10⁶ molecules of pUC19) was included prior to RNase A treatment as a control for the sequencing step ligation.

GCC Network Assembly

Network assembly was performed using Topography v1.19 (available on request (Rodley et al. 2009)). The SOAP (Li et al. 2008) algorithm was used to position paired end sequences and single ends, which

contain an *MspI* restriction site, onto the *S. cerevisiae* reference genome (Supplementary Methods). No mismatches were allowed.

Bioinformatic analyses

Bioinformatic and statistical analyses (see Supplementary Methods) were performed on chromosomal interactions involving the nuclear and mitochondrial genomes for which the sequences mapped uniquely onto the reference genome. Connections with the ribosomal DNA (rDNA), 2 micron plasmid and mitochondrial genomes were considered as unique because they could be positioned to a ~1 MB region of Chromosome XII, the 6318 bp 2 micron plasmid or the ~85 kbp mitochondrial genome, respectively (Rodley et al. 2009). All statistical analyses involving 2 micron plasmid, mitochondrial, or rDNA sequences included copy number corrections (Supplementary Methods). Other repetitive elements, such as LTRs and tRNAs, were omitted from the analysis.

Chromosome Conformation Capture (3C)

3C samples were prepared as previously described (O'Sullivan et al. 2004). Quantitative 3C analyses (Rodley et al. 2009) were performed using FAM labeled BHQ Probes (BioSearch Technologies; Supplementary Table 2) and Taqman[®] Gene Expression Master Mix (Applied Biosystems) on an ABI Prism 7000 Sequence Detection System (SDS7000). Chromosomal coordinates for the interactions under investigation are listed in Supplementary Methods. Samples (2 μ l in triplicate) were analyzed in a final reaction volume of 20 μ l using primers listed in Supplementary Table 2. Assays were performed using a 3-stage program (50°C, 2:00 min; 95°C, 10:00 min; 45x[95°C, 0:15 sec; 60°C, 1:00 min]).

Dedicated interaction standards (concentration from: 2 ng μ l⁻¹ – 2x10⁻¹⁵ g μ l⁻¹) were prepared by PCR amplification (from *S. cerevisiae* BY4741) of the interacting regions, followed by *MspI* digestion and ligation of the two interacting partner fragments. Mitochondrial and nuclear genome (*i.e.* *GAL1*) copy number were determined by qPCR (Supplementary Table 2) using Sybr-green and a five stage program (50°C, 2:00 min; 95°C, 2:00 min; 40x [95°C, 0:15 sec; 59.5°C, 0:30 sec; 72°C, 0:30 sec]; 55°C, 1:00; followed by a dissociation analysis) on an ABI Prism 7000 Sequence Detection System (SDS7000). An *S. cerevisiae* BY4741 genomic DNA sample (concentration from: 2 ng μ l⁻¹ – 7.78125x10⁻⁴ ng μ l⁻¹) was used as a control for all Sybr-green assays.

For comparison, all samples were presented as a percentage of wild-type, following standardization for: 1) the amount of α 15 γ intron-containing DNA (*i.e.* mitochondrial copy number); or 2) the number of nuclear genomes (determined using the single copy *GAL1* locus; O'Sullivan et al. 2009); Primer sequences are listed in Supplementary Table 2. This standardization was performed to correct for alterations to mitochondrial genome stability and the rates of appearance of rho⁻ or rho⁰ strains. This is critical as inter-organelle interactions are dependent upon the presence of the mitochondrial genome (see

161-U7 GII-0 results below). The method of standardization depends upon the interaction being investigated (*i.e.* *COX1-MSY1* interactions were standardized by mitochondrial genome copy number while nuclear-nuclear locus interactions were standardized by *GAL1* copy number). No significant differences were observed when inter-organelle interactions were standardized by mitochondrial or nuclear copy number (data not shown).

RNA extraction

Total RNA was extracted from *S. cerevisiae* grown in SC (Glucose) to an OD₆₀₀ of 0.600. Briefly, cells were harvested (4,000 rpm, 4°C, 2 min) and washed with AE buffer (4,000 rpm, 4°C, 2 min; 50mM Sodium Acetate, 10mM EDTA, pH 5.3). The cell pellet was suspended in phenol/chloroform/isoamyl alcohol (400 µl, 24/24/1) and glass beads (400 µl). Cells were lysed in a bead mill (SPEX sample prep 2010, Geno/Grinder; 1,750 rpm, 8 x 30 sec cycles with 60 sec resting intervals at 4°C). Lysed cells were frozen (-80°C, 15 min), thawed and pelleted (15,000 rpm, 5 min, 4°C). The aqueous phase was extracted twice with phenol/chloroform/isoamyl alcohol (400 µl, 24/24/1). Total RNA was pelleted (15,000 rpm, 10 min, 4°C), following addition of 2/3s volume of 8M LiCl and freezing (-20°C, 2h). RNA was washed (70% ethanol), and the pellet air-dried. Total RNA was suspended (60°C, 10min) in 80µl of DECP treated water (Invitrogen). DNA was removed from the total RNA samples (5µg, 20 µl) by treatment with 1µl of TURBO DNase (TURBO DNA-free™ Kit, Ambion) as per manufacturer's instructions. Samples were centrifuged (10,000g, 1.5 min) and the supernatant was retained. Total RNA concentration was measured using a Nano-drop and 50 µl samples (50ng/µl) were stored at -80°C.

Quantitative Reverse Transcription-PCR

qRT-PCR standards were amplified from *S. cerevisiae* BY4741 genomic DNA (Supplementary Table 2). PCR products were purified (Zymo DNA clean and concentrator™-5 kit according to manufacturer's instructions). The concentration of each qRT-PCR standard was determined by Nano-drop and used to make dilutions ranging from 4.0 - 4.0X10⁻⁵ ng/µl. qRT-PCR reactions were performed using One Step SYBR® Ex Taq™ qRT-PCR Kit according to the manufacturer's instructions (TaKaRa). The qRT-PCR was run with the following protocol: 42°C, 5min; 95°C, 10sec; 40x [95°C, 5sec; 60°C, 31sec] 95°C, 15sec; 60°C, 1min; 95°C, 15sec. All transcript levels were standardized to nuclear *ACT1* and expressed as percentage of wild-type (set at 100%) +/- standard error of the mean.

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Author Contributions

CR Designed experiments, prepared GCC samples, performed laboratory experiments, bioinformatic analyses, statistical analyses, and wrote the manuscript. RSG prepared and analyzed 3C and RNA samples. BJ performed statistical analyses and advised on statistical analyses. JOS Designed experiments and helped write the manuscript.

Figure Legends

Table 1: Inter-organelle interactions are carbon source dependent. There was a >10 fold increase in the number of Mito-nDNA interactions during growth in glycerol lactate (respiration) as compared to growth in glucose and galactose (respiro-fermentation). Growth on galactose resulted in less Mito-nDNA and Mito-rDNA interactions combined, compared to the other two conditions. Statistically significant DNA-DNA interactions were divided according to whether the mtDNA was interacting with nuclear rDNA, or with unique nuclear loci. Corrections for the copy numbers of the rDNA repeats and the mitochondrial genome were incorporated into the significance calculations (Supplementary Statistics).

Figure 1: The number of Mito-nDNA interactions correlates with chromosome length, except chromosome X. Statistically significant Mito-nDNA interactions, occurring above the expected noise level (selected to have a false positive rate of between 1 and 3 %), have been summed for each nuclear chromosome and expressed as a percentage of the total number of interactions for the particular sample before being plotted according to chromosome length in base pairs. Interactions included in this analysis are between the mitochondrial genome and nuclear chromosomes, with the 2-micron plasmid and rDNA interactions removed. The length of chromosome XII has been reduced to account for the rDNA interactions being removed.

Figure 2: Deletion of *yme1* causes a significant reduction in the frequency of the mitochondrial-nuclear *COX1-MSY1* interaction. Interaction frequency between the mitochondrial *COX1* and nuclear *MspI* fragments was assayed by quantitative 3C (Supplementary Methods) in wild-type (*S. cerevisiae* BY4741), $\Delta yme1$ (BY4741 $\Delta yme1$), $\Delta yme2$ (BY4741 $\Delta yme2$), $\Delta ade2$ (BY4741 $\Delta ade2$) and $\Delta mdv1$ (BY4741, $\Delta mdv1$) strains. Interaction values were corrected for mitochondrial genome copy number (see Methods) and are expressed as percentages of wild-type (set at 100%) +/- standard error of the mean (n=4). Deletion of an unconnected gene (*ade2*) did not significantly affect interaction frequency. T-tests (paired P(T<=t) one-tail, n=4) were performed to determine the significance of observed variations: wild-type: $\Delta yme1$ p = 0.01; wild-type: $\Delta yme2$ p = 0.377; wild-type: $\Delta ade2$ p = 0.103; wild-type: $\Delta mdv1$ p = 0.143; $\Delta yme1$: $\Delta mdv1$ p = 0.210. Only $\Delta yme1$ demonstrated a significant difference.

Figure 3: A functional electron transport chain is required to maintain the interaction between the mitochondrial *COX1* and nuclear *MSY1* loci. Uncoupling of the electron transport chain was achieved by 2,4-dinitrophenol (5mM) treatment of exponentially growing *S. cerevisiae* in synthetic complete media, containing glucose or galactose, for the indicated time (Supplementary Figure 5). *COX1-MSY1* A) and nDNA-nDNA B) interaction frequencies were determined by quantitative 3C analyses using fluorescent probes (see Supplementary Methods). Interaction values in A) were corrected for mitochondrial genome

copy number while those in B) were corrected for nuclear genome copy number (see Methods). Interaction values were expressed as percentages of the untreated sample (set at 100%) \pm standard error of the mean (n=3).

Figure 4: Mito-nDNA interactions require active mitochondrial reverse transcriptase machinery. A) Illustration of *COX1* gene arrangement in the WT (161-U7), intron $\alpha 15\gamma$ (161-U7 GII-0 $\alpha 15\gamma$), and no mitochondrial group II introns (161-U7 GII-0) strains. The region of *COX1* that participates in the *COX1-MSY1* interaction is indicated (qPCR probe). Strain 161-U7 GII-0 was included as a control to rule out a nuclear sequence, originating from a mitochondrial integration within the nuclear genome (NUMT), being responsible for the observed interaction. B) *COX1-MSY1* interaction frequencies for mitochondrial reverse transcriptase mutants, illustrated in A), grown in glucose or galactose. C) *Q0182-RSM7* interaction frequencies for mitochondrial reverse transcriptase mutant 161-U7 GII-0 $\alpha 15\gamma$, illustrated in A), grown in glucose or galactose. Interaction frequencies are expressed as percentages of the wild-type *S. cerevisiae* strain 161-U7 for each carbon source (set at 100%) \pm standard error of the mean (n=3). Interaction values in B) and C) were corrected for nuclear genome copy number to facilitate direct comparison.

Figure 5: Knocking out mitochondrial encoded reverse transcriptase activity results in increased transcript levels of nuclear genes that are involved in Mito-nDNA interactions. A) Nuclear encoded *MSY1* transcript levels were determined by qRT-PCR in WT (strain 161-U7), 161-U7 GII-0 (lacks both the mitochondrial group II introns and the *COX1* interacting region; Figure 4A), and 161-U7 GII-0 $\alpha 15\gamma$ (contains the interacting region and lacks the group II introns; Figure 4A) cells. B) Nuclear encoded *RSM7* transcript levels were determined by qRT-PCR in: WT (strain 161-U7); 161-U7 GII-0; and 161-U7 GII-0 $\alpha 15\gamma$ cells. Neither 161-U7 GII-0 nor 161-U7 GII-0 $\alpha 15\gamma$ has any alteration within the *Q0182* open reading frame. C) Deletion of *MRS1* (BY4741 $\Delta mrs1$), a nuclear gene involved in splicing mitochondrial type-I introns, has no effect on i) *MSY1* or ii) *RSM7* transcript levels. All transcript levels were standardized to nuclear *ACT1* and expressed as percentage of wild-type (set at 100%) \pm standard error of the mean (n=2).

Figure 6: Cartoon depicting the role for inter-organelle communication in refining mitochondrial translation levels. Transcription levels of the nuclear encoded *MSY1* and *RSM7* genes are modulated by interactions with mitochondrial cDNAs, which are produced from stable non-translated mitochondrial RNA populations. Other Mito-nDNA interactions could also contribute to this feed-back system. While negative feedback is emphasized in this model, feed-forward systems are also predicted.

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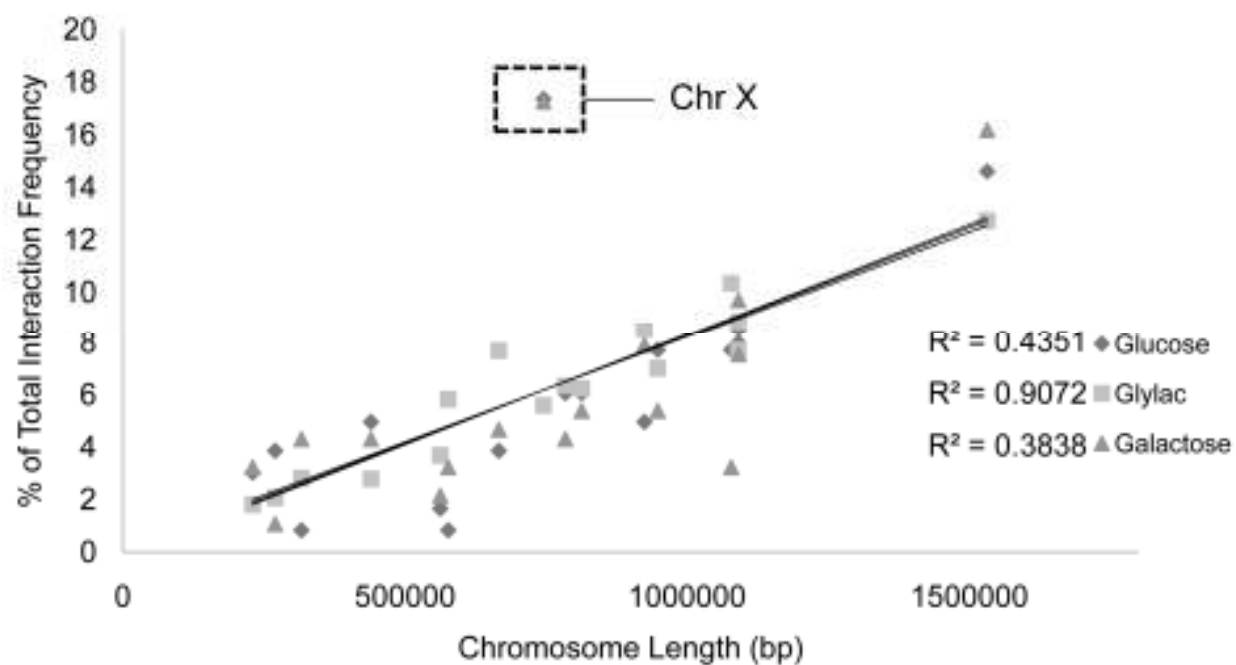


Figure 1

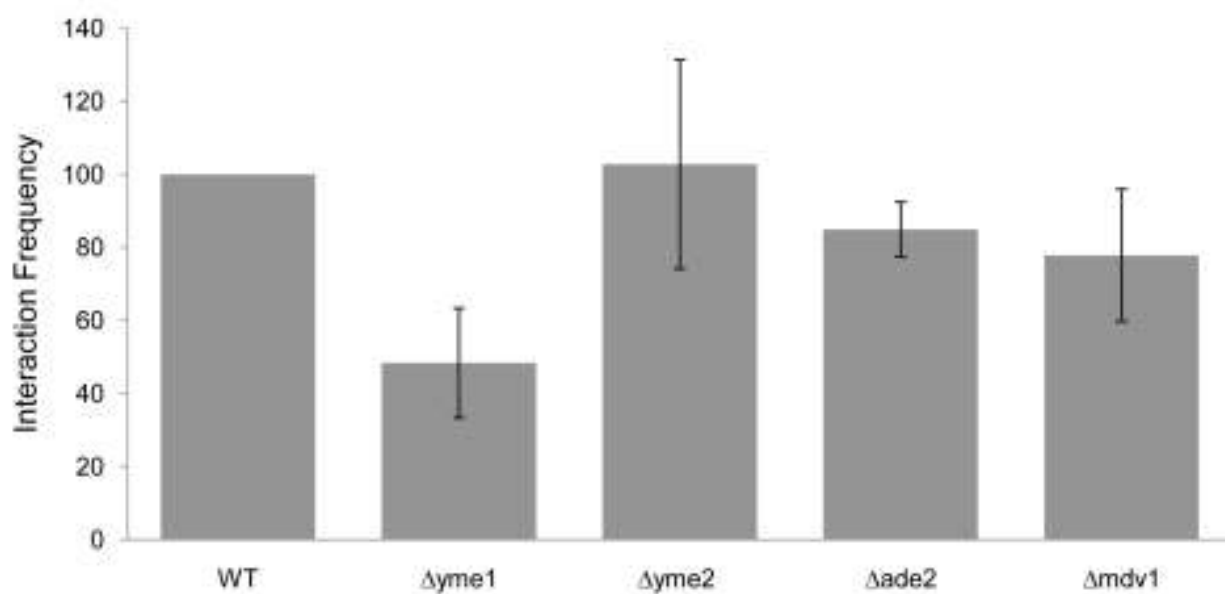
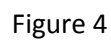
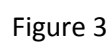


Figure 2



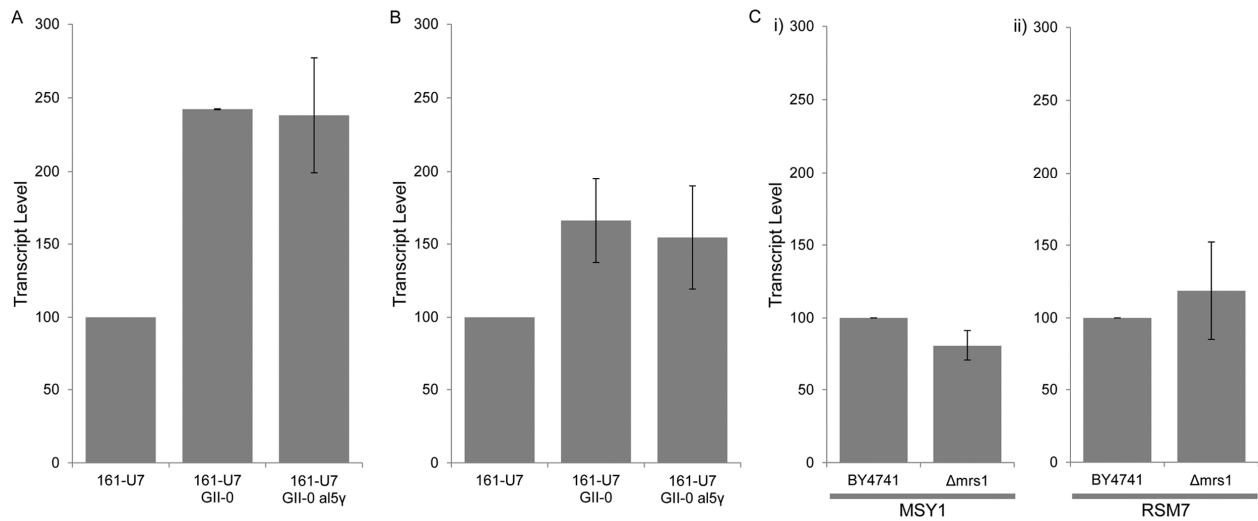


Figure 5

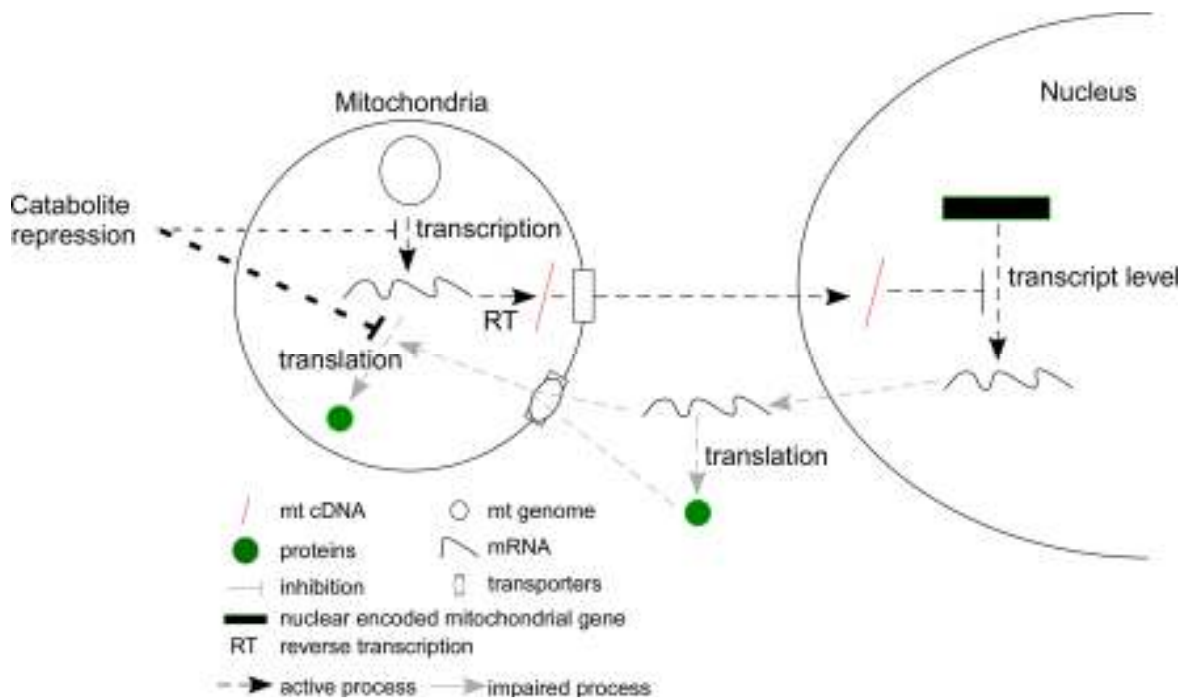


Figure 6

Table 1:

	Glucose	Glycerol Lactate	Galactose
Mito-gDNA Interactions	363	3879	278
Mito-rDNA Interactions	8315	4274	1512
Total	8678	8153	1780

Table 1: Inter-organelle interactions are carbon source dependent. There was a >10 fold increase in the number of Mito-gDNA interactions during growth in glycerol lactate (respiration) as compared to growth in glucose and galactose (respiro-fermentation). Growth on galactose resulted in less Mito-gDNA and Mito-rDNA interactions combined, compared to the other two conditions. Statistically significant DNA-DNA interactions were divided according to whether the mtDNA was interacting with nuclear rDNA, or with unique nuclear loci. Corrections for the copy numbers of the rDNA repeats and the mitochondrial genome were incorporated into the significance calculations (Supplementary Statistics).

