Published in final edited form as:

Mitochondrion. 2014 July ; 17: 141–149. doi:10.1016/j.mito.2014.04.014.

Potential roles for interactions between the mitochondrial and nuclear DNA throughout the cell cycle of *Schizosaccharomyces pombe*

R.S. Grand^{1,2}, R. Martienssen³, and J.M. O'Sullivan¹

¹Liggins institute, University of Auckland, Grafton Auckland 1032, NZ

²Institute of Natural and Mathematical Sciences, Massey University, Albany Auckland 0745, NZ

³Cold Spring Harbor Laboratory, 1 Bungtown Road, Cold Spring Harbor, New York 11724, USA

Abstract

Over the course of mitochondrial evolution, the majority of genes required for its function have been transferred and integrated into nuclear chromosomes. Ongoing transfer of mitochondrial DNA to the nucleus has been detected, but its functional significance has not been fully elucidated. Here by Genome Conformation Capture, we identify DNA-DNA interactions between the mitochondrial and nuclear chromosomes (mt-nDNA interactions) that vary in strength and number between the G1, G2 and M phases of the fission yeast cell cycle. Mt-nDNA interactions captured in mitotic anaphase were associated with nuclear genes required for the regulation of cell growth and energy availability. Furthermore, mt-nDNA interactions captured in the G1 phase involved high efficiency, early firing origins of DNA replication. Collectively, these results suggest functional roles for the ongoing transfer of regions of the mitochondrial genome to the nucleus.

Keywords

Mitochondria; Genome Conformation Capture (GCC); DNA replication; Cell cycle; Regulation; Communication

1. Introduction

Over the course of its evolution into an organelle, most of the ancestral genes present within the mitochondrial genome were transferred and integrated into the host cell genome (Timmis et al., 2004). These transfer events are not all historical; rather, there is evidence for ongoing transfer of mitochondrial DNA to the nucleus in various organisms including the budding yeast, *Saccharomyces cerevisiae* (Ricchetti et al., 2004, 1999; Rodley et al., 2012, 2009; Thorsness and Fox, 1993, 1990). However, the functional roles of the mitochondrial regions, once in the nuclear compartment, have not been fully elucidated.

Data availability

All sequencing data and processed GCC and transcriptome files are available on Gene Expression Omnibus (GEO) accession number GSE52287.

Genome Conformation Capture (GCC) is a proximity based ligation method that has recently been used to identify DNA-DNA interactions between the mitochondrial and nuclear DNA (mt-nDNA interactions) in *S. cerevisiae* (Rodley et al., 2012, 2009). These interactions involve specific regions of the mitochondrial genome that migrate to the nucleus, vary depending on the energetic state of the cell, and are linked to the regulation of transcript levels of nuclear encoded mitochondrial genes (Rodley et al., 2012, 2009). Interestingly, just increasing the amount of mitochondrial DNA present in *S. cerevisiae*, by overexpression of the mitochondrial maintenance protein Abf2p, promotes nuclear DNA replication through a mechanism that involves reducing the Sir2 mediated deacetylation of specific autonomously replicating sequence (ARS) sites (Blank et al., 2008). These observations suggest a functional role for fragments of mtDNA in the regulation of nuclear genes and DNA replication.

Mitochondrial DNA sequences are also often found inserted into eukaryotic nuclear chromosomes (NUclearMiTochondrial sequences (NUMTs))(Lenglez et al., 2010; Ricchetti et al., 2004). NUMTs can form through the use of mitochondrial DNA sequences to repair double-strand DNA breaks in *S. cerevisiae* (Ricchetti et al., 1999) and *S. pombe* (Lenglez et al., 2010), therefore, mitochondrial DNA must be present within the nuclear environment. The mitochondrial sequences that constitute these NUMTs are proposed to have nuclear functions. For example, in the Budding yeast, NUMTs are rich in ARS consensus motifs that promote nuclear DNA replication (Blank et al., 2008; Chatre and Ricchetti, 2011). However, to date there has not been any link between mitochondrial DNA and replication control in *S. pombe*.

It is accepted that successful progression through the cell cycle requires coordination between the nuclear and mitochondrial genomes (Chatre and Ricchetti, 2013; Chu et al., 2007; Crider et al., 2012; Lebedeva and Gerald S. Shadel, 2007; Mandal et al., 2005; Mitra et al., 2009). For instance, in *S. cerevisiae*, the G1 to S phase cell cycle checkpoint is regulated by mitochondrial DNA (Mandal et al., 2005; Mitra et al., 2009). Specifically, in the absence of mtDNA, the Rad53 DNA damage response checkpoint is activated and the G1 to S phase cell cycle transition is inhibited (Crider et al., 2012). However, the mechanism by which this regulation occurs remains to be determined.

S. pombe is a paradigm for cell cycle research sharing many features with higher eukaryotes (Chiron et al., 2007; Coudreuse and Nurse, 2010; Fantes and Nurse, 1978; Nurse et al., 1976), including a dependence upon respiration for survival (Schäfer, 2003; Weir and Yaffe, 2004). *S. pombe* cells have a small nuclear genome and can be synchronised, making it an excellent choice for studying mt-nDNA interactions. Here we characterize the relationship between mt-nDNA interactions and cellular function over the course of the cell cycle in *S. pombe*. We identify specific mt-nDNA interactions with high efficiency, early firing origins of replication in the G1 phase of the cell cycle. Furthermore, we detect mt-nDNA interactions with genes required for nucleotide synthesis and ribosome biogenesis during mitotic anaphase. Our results suggest that the formation of specific mt-nDNA interactions contribute to the regulation of cell cycle progression in *S. pombe* by promoting nuclear DNA replication and protein synthesis following exit from mitotic anaphase.

2. Materials and Methods

2.1 Strains, growth conditions and synchronization

Schizosaccharomyces pombe strains (Supplementary Table S1) were recovered from -80°C on YES (Sabatinosa and Forsburga, 2010) (2% agar) plates (26°C , 4 days). YES medium (12 ml) starter cultures were inoculated and incubated (26°C , 200 rpm) until the OD_{595} measured ~ 0.8 (~ 24 h). Synchronization cultures (125 ml EMM2 (Sabatinosa and Forsburga, 2010), in baffled flasks) were inoculated with starter culture to an $\text{OD}_{595} = \sim 0.05$ and incubated (26°C , 120 rpm). Cultures were grown for four generations ($\text{OD}_{595} \sim 0.8$) before synchronization was induced by the addition of pre-warmed EMM2 medium (125 ml, 46°C), instantly raising the culture temperature to a restrictive 36°C . Cultures were incubated (36°C , 140 rpm, for 4 h) to complete synchronization.

2.2 Synchronization efficiency

Synchronization efficiency was determined using cells harvested (1 ml, 4,000 rpm, 2 min), and snap frozen (dry ice/ethanol (100%) bath), from cultures before induction and following synchronization. Cells collected during synchronization were thawed, washed once with ice-cold 1% PBS (500 μl , 4,000 rpm, 2 min) and suspended in PBS (100 μl). Pre- and post-synchronization cells were stained with calcofluor white (1 g/L with 10% Potassium Hydroxide) and DAPI (25 mg/ml) and photographed using a fluorescence microscope (ZEISS, HBO 100 Axiostart plus). The numbers of visible septa were counted in at least 200 cells (total) from 10 fields of view. Cell cycle phase synchronization was calculated for the G1 and G2 phases by comparing the proportion of cells that had a visible septum in the pre-synchronized and synchronized cell populations (Supplementary Fig. S1 and Table S2). The estimation of $>80\%$ synchronization for M phase cells was based on the observation of traits characteristic of synchronized *nuc2* cultures (Hirano et al., 1988) including: 1) an increased septation index (*i.e.* increased from $\sim 16\%$ to $\sim 50\%$); 2) highly condensed chromosomes; and 3) the presence of enucleate cells, following DAPI staining.

2.3 Chromatin isolation for Genome Conformation Capture (GCC)

Chromatin isolation and GCC were performed as previously described (Rodley et al. 2009, Cagliero et al. 2013) with minor modifications. Following synchronization, cultures (200 ml) were cross-linked, washed, and suspended in FA-lysis buffer. Aliquots containing $\sim 9.5 \times 10^8$ cells were made up to a volume of 330 μl with FA-lysis buffer and the cell walls were digested with T20 Zymolyase (70 μl at 75 mg/ml; 35°C , 40 min with periodic inversion) before heat inactivation (60°C , 5 min). Acid washed glass beads (500 μl) were added to each sample before disruption in a Geno/Grinder (-20°C ; 1,750 rpm, 2×30 sec on 60 sec off; SPEX sample prep 2010). Chromatin was isolated by centrifugation through a pin hole into a clean tube (2,000 rpm, 1 min) before being pelleted (13,000 rpm, 15 min, 4°C), washed with FA-lysis buffer, suspended in chromatin digestion buffer and stored (-80°C).

Each chromatin sample was split into ten aliquots, each containing the equivalent of chromatin from 9.5×10^7 cells. Samples were digested with AseI (100U, New England Biolabs, 37°C , 2 h). A ligation control (Supplementary Table S3) was added to the AseI digested chromatin before samples were diluted (~ 20 -fold) and ligated with T4 DNA ligase

(20U, Invitrogen). Following ligation, cross-links, protein and RNA were removed. pUC19 plasmid (27.4pg/2ml) was added, as a control for the ligation step in the sequencing library preparation, before phenol: chloroform (1:1) extraction and column purification. GCC libraries (3 µg of each) were sent for paired-end sequencing (50bp BGI China).

2.4 Network Assembly

GCC networks were constructed from 50bp paired-end Illumina Genome Analyser sequence reads using the Topography suite v1.19 (Rodley et al., 2009). Topography used the SOAP algorithm (Li et al., 2008) to position PE tags and single end reads, which contained a *AseI* restriction enzyme site, onto the reference genome. The reference genome consisted of the *Schizosaccharomyces pombe* (ASM294v2), pUC19 (SYNPUC19CV), *E. coli*, pRS426 and Lambda phage ligation control sequences. No mismatches or unassigned bases (N) were allowed during positioning.

Unless explicitly stated, all analyses were performed on significant, uniquely mapped, non-adjacent (only interactions between restriction fragments that were not adjacent to each other in the linear sequence) interaction data using in house Perl and Python scripts. Significant interactions were defined as those that occurred at levels above the significance cut-off value (see section 2.5). Except where indicated, statistical analyses were performed using R and Venn-diagrams were drawn using the Vennerable package (R Development Core Team, 2008).

2.5 Significance cut-off calculations

Random ligation events can occur during the two ligation steps in the GCC protocol: 1) the ligation of the cross-linked fragments; and 2) linker addition during preparation for sequencing. External ligation controls (Supplementary Table S3) were produced according to Cagliero et al. 2013 and incorporated into the samples prior to the GCC ligation step to enable the estimation of the frequency of inter-molecular ligation events. We only detected a single ligation event between the *E. coli* ligation control, which was included in the G1 phase GCC preparations, and the mitochondrial genome in one of the G1 phase biological replicates. Therefore, interactions that occurred with the mitochondrial genome were deemed significant if they were detected at a frequency ≥ 2 in both biological replicates.

2.6 Generating heat maps

GCC interaction networks were displayed as heat maps using 75 Kbp segments (Gehlen et al., 2012). Interaction frequencies were represented as the percentage total interactions for the uniquely mapped interactions. This allows cell cycle phase comparisons of interaction frequency (Fig. 1).

2.7 Loop lengths of interactions within the mitochondrial genome

The loop length (bp) between interacting fragments on the mitochondrial genome was calculated for significant uniquely mapped intra-mitochondrial interactions detected at each cell cycle phase. Where the two interacting mitochondrial fragments were defined by coordinates $F_{n_{start}} - F_{n_{end}}$. The loop length (L) was defined as:

$$L=|F1_{\text{end}}-F2_{\text{start}}|$$

Where interacting fragments were ordered so that $F1_{\text{end}} < F2_{\text{Start}}$.

Loop lengths were plotted as a histogram (100bp bins).

2.8 Extracting nuclear genes associated with mt-nDNA interactions for GO and transcription analyses

Nuclear encoded genes associated with mt-nDNA interactions were extracted and used for gene ontology (GO) analysis. Genes that were internal to, overlapped with or were the nearest up and down stream genes to the nuclear restriction fragments, involved in the mt-nDNA interactions, were extracted. Gene sets associated with specific mt-nDNA interactions (*e.g.* G1 or M phase specific) were used for GO enrichment analysis using AmiGO (see section 2.13) and their presence in transcription data sets was determined (see section 2.12).

2.9 Determining whether nuclear restriction fragments involved in mt-nDNA interactions overlap with nuclear encoded mitochondrial genes and origin of replication sites

We determined if the nuclear restriction fragments involved in the mt-nDNA interactions overlapped nuclear encoded mitochondrial genes more than expected at random. Genomic coordinates of nuclear encoded mitochondrial genes were obtained from PomBase using the advance search filter: GO_ID; GO:0005739 - mitochondrion. The proportion of mt-nDNA interactions that overlapped nuclear encoded mitochondrial genes (gene co-ordinates and +/- 500 bp) was calculated for specific interaction sets.

We generated sets of random genomic regions containing the same number of identically sized nuclear fragments by randomly selecting a start coordinate for each region and then adding the length (bp) of the restriction fragment to obtain the end coordinate. Two separate populations of random sets were generated: 1) the conserved random [CR] sets. CR sets conserved the number of genomic regions per chromosome and ensured that significant results were not due to the linear distribution of the regions on a particular chromosome(s); and 2) the random [R] sets where regions were randomly selected across the entire genome, with chromosome selection determined at a frequency that was relative to the chromosome lengths. One thousand random data sets were generated for the CR and R methods. The frequency with which the restriction fragments involved in the mt-nDNA interactions overlapped nuclear encoded mitochondrial genes within the actual data was higher or lower than the random data sets was used to estimate significance.

The frequency with which restriction fragments involved in mt-nDNA interactions associated with known origins of DNA replication (henceforth called origins) was compared to sets of random genomic regions to determine if the association was non-random. Chromosomal coordinates for the strongest 401 origins and their efficiency and replication timing were obtained from Heichinger C et al. 2006. Origin regions were defined as the centre coordinate for each origin (Heichinger et al., 2006) +/-125 bp or +/-5,000 bp. The frequency with

which mt-nDNA interactions overlapped the origins was calculated for specific interaction sets and compared to sets of random genomic regions as above. When there was a significant association with origins, we performed a Kolmogorov-Smirnov (K-S) test to determine if the contacted sites had a non-random linear chromosomal distribution. Origins were further divided into low (<18; 100), medial (18–40; 204), and high (>40; 97) efficiency; and early (<80 min; 360) and late (41) firing origins. The proportion of contacts with each origin category in the real and random sets was compared and used to determine significance.

2.10 RNA extraction

For RNA extraction, cells were harvested (4,000 rpm, 2 min, RT) from each synchronized cell culture (12 ml) prior to cross-linking, washed (5 ml) and suspended in AE buffer (400 µl; 50 mM Sodium Acetate, 10 mM EDTA, pH 5.3). Cell suspensions were transferred to tubes containing an equal volume of phenol/chloroform/isoamyl alcohol (24/24/1 Ambion) and acid washed glass beads (400 µl; Invitrogen). Cells were mechanically lysed (1,750 rpm, 8 × 30 sec on 60 sec off) in aGeno/Grinder (SPEX sample prep 2010; block pre-chilled to –20°C). Lysis was completed by freeze (–80°C, ~15 min), thaw before centrifugation (14,500 rpm, 5 min, 4°C). The aqueous phase was extracted three times with an equal volume of phenol/chloroform/isoamyl alcohol. RNA was isolated by precipitation with 1/10th volume ammonium acetate (5 M) and 2 volumes of 100% ethanol at –80°C (>30 min) before centrifugation (14,500 rpm, 10 min, 4°C). RNA was washed with 70% (v/v) ethanol (350 µl; 14,500 rpm, 5 min, 4°C) and air dried (37°C, ~15 min). RNA pellets were suspended in RNA Secure (80 µl; Ambion) and dissolved by heating (60°C, 10 min). RNA concentration was determined by Nano-Drop (ACT Gene ASP-3700) and each sample (2 µg) was visually inspected following electrophoresis through a 1% (w/v) agarose gel. RNA was stored (–80°C) before sequencing (BGI China, 90 bp paired-end RNA sequencing analysis).

2.11 Transcriptome analysis

RNA sequences (90 bp) were quality assessed using FastQC (<http://www.bioinformatics.babraham.ac.uk/projects/fastqc/>). To maximize read quality, 10 bp was trimmed off either end of the sequences using fastx_trimmer (http://hannonlab.cshl.edu/fastx_toolkit/index.html).

Differentially expressed genes were identified using cufflinks (Trapnell et al., 2012) to analyse the trimmed RNA sequences as a time course. This enabled the isolation of effects due to the temperature shift, thus maximizing the chances of identifying genes that are differentially regulated during each cell cycle transition. Briefly, trimmed RNA-seq reads were aligned to the *S. pombe* reference genome (ASM294v2) using Tophat (<http://tophat.cbcb.umd.edu/>) without providing the *S. pombe* GTF file. Aligned reads were assembled for differential expression analysis using cufflinks (<http://cufflinks.cbcb.umd.edu/>) and merged using cuffmerge (<http://cufflinks.cbcb.umd.edu/manual.html#cuffmerge>) with an “assemblies” file containing the transcripts.gtf output files from cufflinks for the two biological replicates of each cell cycle phase in the order G1 – G2 – M – G1. Finally, differential expression analysis was performed using the merged.gtf

output file from cuffmerge, the -T option, and the accepted_hits.bam output files from tophat in the time series order G1 – G2 – M – G1.

Raw transcript levels for genes in individual biological replicates were highly correlated ($R^2 > 0.91$). For downstream analyses, transcription data sets were divided into: 1) genes that were in the top and bottom 5% of transcript levels in each cell cycle phase (Supplementary Table S4); and 2) genes whose transcript levels were differentially regulated during the cell cycle transitions (G1 – G2, G2 – M, and M – G1) (Supplementary Table S4). Except where indicated, statistical analyses were performed in R (R Development Core Team, 2008). Venn-diagrams were drawn using the Vennr package available for R.

2.12 Identification of an association between nuclear encoded gene sets associated with mt-nDNA interactions and specific transcription data sets

The transcript levels of nuclear encoded genes associated with mt-nDNA interactions and the likelihood that these genes were found in specific transcription data sets (*i.e.* high, low, or differentially regulated during cell cycle transitions) were compared to random gene sets. Briefly, we generated one thousand random sets containing equivalent numbers of genes and used the number of times the random gene sets had a higher or lower proportion of genes associated with the transcription gene sets to estimate significance. If there was a significantly high proportion of mt-nDNA associated genes detected in a transcription data set, the shared genes were used for GO Term Enrichment analysis (see section 2.13).

2.13 Gene Ontology (GO) analysis

The AmiGO Term Enrichment online resource (http://amigo.geneontology.org/cgi-bin/amigo/term_enrichment) was used to determine if particular gene sets were enriched in GO terms within Pombase. We used a maximum p-value of 0.05 and required a minimum of two gene products for enrichment.

3. Results

3.1 Mt-nDNA interactions vary throughout the *S. pombe* cell cycle

Mt-nDNA interactions are dynamic in the Budding yeast (*Saccharomyces cerevisiae*) and change if the yeast is respiring or fermenting (Rodley et al., 2012, 2009). We used Genome Conformation Capture (GCC) to identify connections within the mitochondrial genome(s) and between the mitochondrial genome and the nuclear chromosomes (mt-nDNA interactions) in the G1, G2, and M phases of the fission yeast cell cycle (See Supplementary Spreadsheets). Significant interactions within the individual biological replicates were highly correlated and replicates were combined for further analysis (Supplementary Fig. S2A–C).

The number and strength of interactions, both within the mitochondrial genome and between the mitochondrial genome and nuclear chromosomes, varied in the different phases of the cell cycle (Fig. 1, Supplementary Fig. S2D–F and S3, and Supplementary Table S5). The relative ratio of mt-nDNA: nDNA-nDNA interactions was 1:8, 1:28, and 1:10 in the G1, G2 and M phases, respectively. The majority of mt-nDNA interactions are specific to each

phase of the cell cycle (Supplementary Fig. S3B). Despite this, the linear distribution of the nuclear interaction partners along the *S. pombe* nuclear chromosomes was not different from random (K–S test), although there were obvious differences in the distributions between cell cycle phases (Fig. 1A). The variability and specificity of the interactions supports the conclusion that the mt-nDNA interactions are not arte facts of the GCC technique.

The mitochondrial DNA fragments that directly participate in interactions with nuclear chromosomes are distributed across the mitochondrial genome (Fig. 1B). However, three mitochondrial fragments account for the majority of the interactions that occur in the G1, G2 and M phases of the *S. pombe* cell cycle: 1) 21S-15SrRNA A (2,021-3,809bp); 2) Cox1-SPMIT.02 (5,243-6,621bp); and 3) Cox3-Cob1-SPMIT.06 (9,433-11,427bp)(Fig. 1B). Critically, there is cell cycle phase variation in both the frequency with which each of these fragments is involved in mt-nDNA interactions and the number of nuclear loci that they contact. For example, the 21S-15SrRNA fragment accounts for: 36% of the total interaction frequency and contacts 82 nuclear loci in G1 phase; 41% and 26 loci in G2 phase; and 21% and 45 loci in M phase. Thus the frequency of interactions and number of nuclear loci that interact with a particular mtDNA fragment changes throughout the cell cycle.

The number and contact frequency of interactions between all restriction fragments within the mitochondrial genome also varied between the cell cycle phases (Supplementary Fig. S3 and 4). Of note, there are a large number of within mitochondrial genome interactions that form specifically during mitotic anaphase and are shared with the G1 phase of the cell cycle (Supplementary Fig. S3C). However, there was no obvious bias in the lengths of the DNA loops that formed between the interacting fragments within the mitochondrial genome (Supplementary Fig. S4B–D). Collectively, these results suggest that there is not one particular mitochondrial region that preferentially directs mitochondrial genome structure. Moreover, the connectivity between and within the mitochondrial genome(s) increases during mitotic anaphase, in a manner reminiscent of the increased condensation seen for the nuclear chromosomes.

3.2 Mt-nDNA interactions are not enriched for nuclear encoded mitochondrial genes

The majority of genes required for mitochondrial function are encoded in the nuclear chromosomes (Timmis et al., 2004). Thus the transcription of nuclear genes required for mitochondrial function must be precisely coordinated in response to mitochondrial demand (Butow and Avadhani, 2004; Liu and Butow, 2006). We determined whether mt-nDNA interactions preferentially formed with nuclear encoded mitochondrial genes. A subset of the mt-nDNA interactions involved nuclear encoded mitochondrial genes in all data sets (Supplementary Fig. S5A). However, in general, the genes involved in mt-nDNA interactions were depleted for nuclear encoded mitochondrial genes when compared to random sets (Supplementary Fig. S5A).

Previously, specific mt-nDNA interactions have been shown to influence the transcript levels of nuclear encoded mitochondrial genes (Rodley et al., 2012). We examined whether the formation of mt-nDNA interactions with nuclear encoded mitochondrial genes correlated with their transcript levels. Nuclear encoded mitochondrial genes were not often found in the high, or low transcription data sets (Supplementary Fig. S5B and C). However of note,

mt-nDNA interactions that formed specifically during the G2 phase were highly associated with nuclear encoded mitochondrial genes that were subsequently down regulated during the G2-M phase cell cycle transition (Supplementary Fig. S5C(ii)).

3.3 Mt-nDNA interactions formed during mitotic anaphase occur with genes required for cell growth and DNA synthesis

Mitochondria have a central role in cellular metabolism. Therefore, we hypothesized that this would be reflected in connections to genes that affect metabolic processes central to cell viability. We determined if mt-nDNA interactions that were specific to or shared by the different cell cycle phases involved specific nuclear gene sets. Only mt-nDNA interactions that specifically formed during mitotic anaphase were enriched for a gene set (Fig. 2A). Interestingly, the cellular functions of these genes were associated with molecular binding activity and related to energy availability and growth (Fig. 2B).

Mt-nDNA interactions that specifically formed during mitotic anaphase were associated with nuclear genes that had high transcript levels in that phase of the cell cycle (Fig. 3A(i)). Furthermore, the nuclear gene set involved in the mitotic anaphase specific interactions was enriched for genes involved in ribosome structure and function (Fig. 3B).

By contrast, mt-nDNA interactions were not enriched with nuclear genes that had low transcript levels (Fig. 3A(ii)) or that were differentially regulated during the G1–G2 or M–G1 phase cell cycle transitions (Supplementary Fig. S6). However, a high proportion of mt-nDNA interactions that were present in both the G2 and M phases of the cell cycle, and those formed specifically during M phase, associated with genes whose transcript levels increased significantly ($+>1.5$ -fold) during the G2-M phase cell cycle transition (Supplementary Fig. S6A). Despite this, the up regulated genes associated with these mt-nDNA interactions were not enriched for any specific functions.

3.4 G1 phase mt-nDNA interactions are associated with high efficiency, early firing origins of DNA replication

NUMTs and the cell's mitochondrial DNA concentration have been implicated in promoting nuclear DNA replication in *S. cerevisiae* (Blank et al., 2008; Chatre and Ricchetti, 2011). We investigated if cell cycle specific mt-nDNA interactions preferentially formed with known *S. pombe* origins of replication (henceforth called origins) (Heichinger et al., 2006). A significantly high proportion of interactions detected during the G1 phase of the cell cycle occurred with origins (Fig. 4A). By contrast, mt-nDNA interactions that formed in the G2 and M phases of the cell cycle were not enriched for origins (Fig. 4A). The nuclear origins that were involved in these interactions were randomly distributed across the linear sequence of all three nuclear chromosomes (Fig. 4B). However, some 50 Kbp regions on chromosomes I and III contain 2–3 origins that were involved in mt-nDNA interactions. Moreover, the nuclear origins involved in the G1 phase mt-nDNA interactions were enriched for high efficiency and early firing origins (Fig. 4C). Finally, the 21S-15SrRNA, Cox1-SPMIT.02 and Cox3-Cob1-SPMIT.06 mitochondrial fragments participated in the majority of the origin interactions during the G1 phase (Supplementary Fig. 7).

4. Discussion

In this study, we detected variations in the number and frequency of interactions between the mitochondrial and nuclear chromosomes during the G1, G2, and M phases of the fission yeast cell cycle. This supports our earlier observations where mt-nDNA interactions were captured in *S. cerevisiae*, with their numbers and positions being dependent upon carbon source and metabolism (Rodley et al., 2012, 2009). GCC is probabilistic and does not enable us to estimate the numbers of cells within the population in which the interactions are occurring, either singly or in combination (O'Sullivan et al., 2013), however these results support earlier observations that there is continual transfer of specific mitochondrial DNA regions to the nucleus and this transfer varies according to the cell's energy state and cell cycle phase (Ricchetti et al., 2004, 1999; Thorsness and Fox, 1993, 1990). Moreover, the specificity of cell cycle phase mt-nDNA interactions suggests a regulatory role for these connections in cell function and growth.

Mitochondrial genomes exist as a population of nucleoids that are individually comprised of protein complexes and one or more copies of the mitochondrial chromosome (Chen et al., 2005; Kucej and Butow, 2007). These mitochondrial nucleoids must be segregated during cell division. In the Budding yeast, nucleoids have been shown to be selectively sorted during cell division, however, the molecular mechanism(s) of how this occurs is unknown (Kucej and Butow, 2007; Okamoto et al., 1998). The intra-mitochondrial genome interactions that were maintained in all cell cycle phases: G1, G2, and M phase, may reflect the presence of a "stable" nucleoid organization that is maintained throughout the fission yeast cell cycle. Interestingly, a large number of intra-mitochondrial interactions formed specifically during mitotic anaphase and were shared with the G1 phase of the cell cycle, consistent with the hypothesis that condensation of a single, or aggregation of multiple copies of the mitochondrial chromosome occurs analogous to, and simultaneously with, nuclear chromosome condensation. Thus, the apparent condensation of mitochondrial genomes during mitotic anaphase may reflect mechanisms involved in the faithful segregation of nucleoids during cell division.

The formation of functional mitochondria requires the coordination of gene expression from both mitochondrial and nuclear encoded genes (Butow and Avadhani, 2004; Liu and Butow, 2006). We have previously identified specific contacts between the mitochondrial genome and nuclear encoded genes in *S. cerevisiae* (Rodley et al., 2012). Moreover, we demonstrated that these interactions occur within a sufficiently large part of the population such that interfering with their formation resulted in a population level increase in the transcript levels of the nuclear genes (Rodley et al., 2012). Thus, the formation of a high proportion of interactions between mtDNA fragments and nuclear encoded mitochondrial genes during the G2-M phase cell cycle transition (Supplementary Fig. S5C(ii)) is consistent with our earlier observations of a repressive role for these connections (Rodley et al., 2012). However, the bulk of the mt-nDNA interactions we detected in *S. pombe* did not associate specifically with nuclear encoded mitochondrial genes.

We hypothesize that interactions between mtDNA and other subsets of nuclear genes have positive effects on transcript levels, either by promoting/inhibiting transcription or changing

the stability of the transcripts through an as yet undetermined mechanism. This hypothesis is supported by observations that mt-nDNA interactions that specifically formed during mitotic anaphase, contacted genes that were enriched in various molecular binding functions (including anion, ATP, purine and nucleoside binding), and genes that had high transcript levels required for ribosome structure and function. While not specifically annotated as being related to mitochondrial function, these genes are essential for viable cell growth and the synthesis of DNA during S-phase. Considering that the majority of transcription is thought to be suppressed during metaphase, it is possible to speculate that these mt-nDNA interactions facilitate/stimulate transcription activation following mitotic anaphase exit. This hypothesis gains further support from our earlier work, in *S. cerevisiae*, where we demonstrated that suppression of mt-nDNA interactions correlated with changes in the nuclear genes transcript levels (Rodley et al., 2012).

In the Budding yeast, NUMTs are rich in ARS consensus motifs and promote nuclear DNA replication (Blank et al., 2008; Chatre and Ricchetti, 2011). Similarly, NUMTs are also enriched at or near origins of replication in the fission yeast (Lenglez et al., 2010). Interestingly, merely increasing the amount of mitochondrial DNA has been shown to promote nuclear DNA replication in *S. cerevisiae* by preventing the Sir2 mediated deacetylation of ARS sites (Blank et al., 2008). We determined that a high proportion of mt-nDNA interactions formed during the G1 phase of the cell cycle were associated with previously identified strong, high efficiency, early firing origins in *S. pombe* (Heichinger et al., 2006). Due to the known insertion of NUMTs near origins in the fission yeast (Lenglez et al., 2010), the analyses were also performed on data that had interactions with NUMTs removed and the same results were obtained with only two interactions between the mtDNA and NUMTs captured in the G1 and M phases of the cell cycle (Supplementary Spreadsheet S1). We interpret these results as supporting a role for mt-nDNA interactions in the regulation of origins of replication. Specifically, our results support those of Blank et al. (Blank et al., 2008) who demonstrated that the “level of Sir2p bound at the rDNA ARS elements is inversely related to the amount of mtDNA in the cell”. The formation of mt-nDNA interactions at high efficiency early firing origins raises the possibility that the presence of mtDNA directly or indirectly prevents the recruitment of the Sir2 protein thus promoting activation of the sites. Further experiments are required to clarify whether the interactions are associated with origin of replication activity or inactivity. However, this hypothesis gains further support from observations that mitochondria regulate the G1-S phase cell cycle transition in a number of organisms (Crider et al., 2012; Mandal et al., 2005; Mitra et al., 2009).

Three mitochondrial fragments (*i.e.* 21S-15SrRNA, Cox1-SPMIT.02, Cox3-Cob1-SPMIT.06) were involved in the majority of interactions between the mitochondrial and nuclear chromosomes, including the mt-nDNA interactions formed with origins in the G1 phase. Two of these fragments (*i.e.* Cox1-SPMIT.02, Cox3-Cob1-SPMIT.06) overlapped group II introns (Lang et al., 1984). The finding that group II intron containing regions were heavily involved in mt-nDNA interactions in *S. pombe* fits with our earlier observations in *S. cerevisiae* where the group I and II introns within the Cox1 gene were demonstrated to have a role in interaction formation or maintenance (Rodley et al., 2012).

The finding that three mitochondrial regions (*i.e.* 21S-15SrRNA, Cox1-SPMIT.02, Cox3-Cob1-SPMIT.06) participated in the majority of interactions with different nuclear regions that were captured in the different stages of the cell cycle can be interpreted as indicating that: 1) the mt-nDNA interactions simply occur with 'open' regions (*i.e.* actively transcribed genes and origins) within the nuclear genome; or 2) that there is a level of regulation of this process which is dependent upon other factors (*i.e.* cell cycle dependent proteins or RNA). These two interpretations are not mutually exclusive, nor do they automatically mean that the interactions are functional or non-functional. However, observations that some fragments in the mitochondrial genome (*e.g.* the mitochondrial region from SPMIT.08 to atp9) predominantly participated in within mitochondrial interactions tends to support interaction formation and capture being dependent upon a non-random process. Moreover, the observed variation in the frequencies and numbers of partners for particular mitochondrial regions again indicates that the formation and capture of these interactions is non-random and dependent upon other nuclear factors (*e.g.* proteins).

It is clear that the transfer of mtDNA into nuclei is an ongoing process. Yet the precise mechanism(s) remain(s) unknown and potentially include combinations of direct or indirect transfer (Campbell and Thorsness, 1998; Shafer et al., 1999; Thorsness and Fox, 1993, 1990). Both active and passive transport mechanisms are capable of transferring mitochondrial nucleic acid into the nucleus. If, as these findings and our earlier data (Rodley et al., 2012) suggest, the mt-nDNA interactions have regulatory functions then it is likely they reflect mitochondrial function. Thus, one can hypothesize that if the interactions reflect a damaged/dying population of mitochondria and are predominantly negative regulators of nuclear functions (*e.g.* transcription and replication) then it is likely the transfer mechanism involves the release of either DNA or RNA into the cytoplasm from damaged mitochondria. This cytoplasmic DNA or RNA would subsequently be taken up by the nucleus. Alternatively, if the interactions reflect a very active mitochondrial population and are predominantly positive, or both positive and negative, then specific transport of DNA or RNA may occur. In this scenario, mitochondrial RNA would reflect activity and mitochondrial DNA would reflect the status of mitochondrial replication and genome stability. These models are not mutually exclusive.

In conclusion, we have identified correlations that suggest functional roles for mitochondrial DNA fragments that are participating in cell cycle dependent interactions within the *S. pombe* nucleus. Specifically, we identified the formation of mt-nDNA interactions with nuclear genes required for DNA replication, energy availability, and protein synthesis. Moreover, mt-nDNA interactions detected during the G1 phase of the cell cycle are enriched for high efficiency and early firing origins of DNA replication. Collectively, these results suggest that mt-nDNA interactions play a role in the regulation of cell growth and the G1-S phase cell cycle checkpoint. We propose that aberrant mitochondrial to nuclear DNA transfer may contribute to the pleomorphic effects manifest by mitochondrial dysfunction in complex diseases (Koopman et al., 2013; Wallace, 2012).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors would like to thank Lynsey Cree and Julia Horsfield for comments on this manuscript. This work was supported by The Marsden fund (JMOS) and a Massey University Doctoral Scholarship (RSG).

References

- Aves SJ, Durkacz BW, Carr A, Nurse P. Cloning, sequencing and transcriptional control of the *Schizosaccharomyces pombe* cdc10 “start” gene. *EMBO J.* 1985; 4:457–463. [PubMed: 4018034]
- Blank HM, Li C, Mueller JE, Bogomolnaya LM, Bryk M, Polymenis M. An increase in mitochondrial DNA promotes nuclear DNA replication in yeast. *PLoS Genet.* 2008; 4:e1000047. [PubMed: 18404213]
- Butow RA, Avadhani NG. Mitochondrial signaling: The retrograde response. *Mol Cell.* 2004; 14:1–15. [PubMed: 15068799]
- Cagliero C, Grand RS, Jones MB, Jin DJ, O’Sullivan JM. Genome conformation capture reveals that the *Escherichia coli* chromosome is organized by replication and transcription. *Nucleic Acids Res.* 2013; 41:6058–6071. [PubMed: 23632166]
- Campbell CL, Thorsness PE. Escape of mitochondrial DNA to the nucleus in yme1 yeast is mediated by vacuolar-dependent turnover of abnormal mitochondrial compartments. *J Cell Sci.* 1998; 111:2455–2464. [PubMed: 9683639]
- Chatre L, Ricchetti M. Nuclear mitochondrial DNA activates replication in *Saccharomyces cerevisiae*. *PLoS One.* 2011; 6:e17235. [PubMed: 21408151]
- Chatre L, Ricchetti M. Prevalent coordination of mitochondrial DNA transcription and initiation of replication with the cell cycle. *Nucleic Acids Res.* 2013; 41:3068–78. [PubMed: 23345615]
- Chen XJ, Butow Ra, Xin JC. The organization and inheritance of the mitochondrial genome. *Nat Rev Genet.* 2005; 6:815–825. [PubMed: 16304597]
- Chiron S, Gaisne M, Guillou E, Belenguer P, Clark-Walker GD, Bonnefoy N. Studying mitochondria in an attractive model: *Schizosaccharomyces pombe*. *Methods Mol Biol.* 2007; 372:91–105. [PubMed: 18314720]
- Chu Z, Li J, Eshaghi M, Karuturi RKM, Lin K, Liu J. Adaptive expression responses in the Pol-gamma null strain of *S. pombe* depleted of mitochondrial genome. *BMC Genomics.* 2007; 8
- Coudreuse D, Nurse P. Driving the cell cycle with a minimal CDK control network. *Nature.* 2010; 468:1074–9. [PubMed: 21179163]
- Crider DG, García-Rodríguez LJ, Srivastava P, Peraza-Reyes L, Upadhyaya K, Boldogh IR, Pon La. Rad53 is essential for a mitochondrial DNA inheritance checkpoint regulating G1 to S progression. *J Cell Biol.* 2012; 198:793–8. [PubMed: 22927468]
- Fantes PA, Nurse P. Control of the timing of cell division in the fission yeast. 1978; 115:317–329.
- Gehlen LR, Gruenert G, Jones MB, Rodley CD, Langowski J, O’Sullivan JM. Chromosome positioning and the clustering of functionally related loci in yeast is driven by chromosomal interactions. *Nucl (United States).* 2012; 3:13.
- Heichinger C, Penkett CJ, Bahler J, Nurse P. Genome-wide characterization of fission yeast DNA replication origins. *EMBO J.* 2006; 25:5171–5179. [PubMed: 17053780]
- Hirano T, Hiraoka Y, Yanagida M. A temperature-sensitive mutation of the *Schizosaccharomyces pombe* gene nuc2+ that encodes a nuclear scaffold-like protein blocks spindle elongation in mitotic anaphase. *J Cell Biol.* 1988; 106:1171–1183. [PubMed: 3283148]
- Koopman WJH, Distelmaier F, Smeitink JAM, Willems PHGM. OXPHOS mutations and neurodegeneration. *EMBO J.* 2013; 32:9–29. [PubMed: 23149385]
- Kucej M, Butow RA. Evolutionary tinkering with mitochondrial nucleoids. *Trends Cell Biol.* 2007; 17:586–592. [PubMed: 17981466]
- Lang BF, Ia W, Munchen D, Davies CRW, The S. The mitochondrial genome of the fission yeast *Schizosaccharomyces pombe*: highly homologous introns are inserted at the same position of the otherwise less conserved cox1 genes in *Schizosaccharomyces pombe* and *Aspergillus nidulans*. *EMBO J.* 1984; 3:2129–2136. [PubMed: 6092057]

- Lebedeva MA, Shadel Gerald S. Cell Cycle- and Ribonucleotide Reductase-Driven Changes in mtDNA Copy Number Influence mtDNA Inheritance Without Compromising Mitochondrial Gene Expression. *Cell Cycle*. 2007; 6:2048–2057. [PubMed: 17721079]
- Lenglez S, Hermand D, Decottignies A. Genome-wide mapping of nuclear mitochondrial DNA sequences links DNA replication origins to chromosomal double-strand break formation in *Schizosaccharomyces pombe*. *Genome Res*. 2010; 20:1250–61. [PubMed: 20688779]
- Li R, Li Y, Kristiansen K, Wang J. SOAP: short oligonucleotide alignment program. *Bioinform Appl Note*. 2008; 24:713–714.
- Liu Z, Butow RA. Mitochondrial retrograde signaling. *Annu Rev Genet*. 2006; 40:159–185. [PubMed: 16771627]
- Mandal S, Guptan P, Owusu-Ansah E, Banerjee U. Mitochondrial regulation of cell cycle progression during development as revealed by the tenured mutation in *Drosophila*. *Dev Cell*. 2005; 9:843–54. [PubMed: 16326395]
- Mitra K, Wunder C, Roysam B, Lin G, Lippincott-Schwartz J. A hyperfused mitochondrial state achieved at G1-S regulates cyclin E buildup and entry into S phase. *Proc Natl Acad Sci U S A*. 2009; 106:11960–5. [PubMed: 19617534]
- Nurse P, Thuriaux P, Nasmyth K. Genetic control of the cell division cycle in the fission yeast *Schizosaccharomyces pombe*. *Mol Gen Genet*. 1976; 146:167–178. [PubMed: 958201]
- O'Sullivan JM, Hendy MD, Pichugina T, Wake GC, Langowski J. The statistical-mechanics of chromosome conformation capture. *Nucl (United States)*. 2013; 4
- Okamoto K, Perlman PS, Butow Ra. The sorting of mitochondrial DNA and mitochondrial proteins in zygotes: preferential transmission of mitochondrial DNA to the medial bud. *J Cell Biol*. 1998; 142:613–23. [PubMed: 9700153]
- R Development Core Team. R Foundation for Statistical Computing; Vienna, Austria: 2008. R: A language and environment for statistical computing. [WWW Document] URL <http://www.R-project.org>
- Ricchetti M, Fairhead C, Dujon B. Mitochondrial DNA repairs double-strand breaks in yeast chromosomes. *Nature*. 1999; 402:96–100. [PubMed: 10573425]
- Ricchetti M, Tekaia F, Dujon B. Continued colonization of the human genome by mitochondrial DNA. *PLoS Biol*. 2004; 2
- Rodley CDM, Bertels F, Jones B, O'Sullivan JM. Global identification of yeast chromosome interactions using Genome conformation capture. *Fungal Genet Biol*. 2009; 46:879–886. [PubMed: 19628047]
- Rodley CDM, Grand RS, Gehlen LR, Greyling G, Jones MB, O'Sullivan JM. Mitochondrial-nuclear DNA interactions contribute to the regulation of nuclear transcript levels as part of the inter-organellar communication system. *PLoS One*. 2012; 7:e30943. [PubMed: 22292080]
- Sabatina SA, Forsburg SL. Molecular Genetics of *Schizosaccharomyces pombe*. *Methods in Enzymology*. 2010; 759–795. [PubMed: 20946835]
- Schäfer B. Genetic conservation versus variability in mitochondria: The architecture of the mitochondrial genome in the petite-negative yeast *Schizosaccharomyces pombe*. *Curr Genet*. 2003; 43:311–326. [PubMed: 12739049]
- Shafer KS, Hanekamp T, White KH, Thorsness PE. Mechanisms of mitochondrial DNA escape to the nucleus in the yeast *Saccharomyces cerevisiae*. *Curr Genet*. 1999; 36:183–194. [PubMed: 10541855]
- Thorsness PE, Fox TD. Escape of DNA from mitochondria to the nucleus in *Saccharomyces cerevisiae*. *Nature*. 1990; 346:376–379. [PubMed: 2165219]
- Thorsness PE, Fox TD. Nuclear mutations in *Saccharomyces cerevisiae* that affect the escape of DNA from mitochondria to the nucleus. *Genetics*. 1993; 134:21–28. [PubMed: 8514129]
- Timmis JN, Ayliff MA, Huang CY, Martin W. Endosymbiotic gene transfer: Organelle genomes forge eukaryotic chromosomes. *Nat Rev Genet*. 2004; 5:123–135. [PubMed: 14735123]
- Trapnell C, Roberts A, Goff L, Pertea G, Kim D, Kelley DR, Pimentel H, Salzberg SL, Rinn JL, Pachter L. Differential gene and transcript expression analysis of RNA-seq experiments with TopHat and Cufflinks. *Nat Protoc*. 2012; 7:562–78. [PubMed: 22383036]
- Wallace DC. Mitochondria and cancer. *Nat Rev Cancer*. 2012; 12:685–98. [PubMed: 23001348]

Weir BA, Yaffe MP. Mmd1p, a Novel, Conserved Protein Essential for Normal Mitochondrial Morphology and Distribution in the Fission Yeast *Schizosaccharomyces pombe*. *Mol Biol Cell*. 2004; 15:1656–1665. [PubMed: 14767070]

Fig. 1.

Mt-nDNA interactions are cell cycle dependent. (A) There were differences in the identity, number and contact frequencies of mt-nDNA interactions detected at the G1, G2 and M phases of the fission yeast cell cycle. Mt-nDNA interactions (% total mt-nDNA interactions) are plotted as heat maps for 75 Kbp nuclear fragments. The linear chromosomal distribution of the nuclear interacting partners was not different from random (K-S test). (B) Three mitochondrial DNA fragments participate in the majority of the mt-nDNA interactions: 1) 2,021–3,809, 2) 5,243–6,621, and 3) 9,433–11,427; at each phase of the cell cycle (G1 (67%), G2 (62%) and M phase (66%)). However, there is substantial variation in the distribution of the contact frequency and number of nuclear partners among these three fragments. The proportion of the total mt-nDNA contact frequency that each restriction fragment is involved in (black) and number of nuclear partners (red) of the individual mitochondrial fragments was plotted for the three cell cycle phases (G1, G2, and M). The mitochondrial genes and AseI restriction enzyme sites are illustrated. Columns are not proportional to restriction fragment length to enable side by side comparisons of interaction frequency and number of nuclear partners.

Fig. 2. Nuclear genes associated with mt-nDNA interactions formed specifically during mitotic anaphase are enriched in biological functions (A) related to genomic DNA synthesis and energy regulation (B). Genes that were associated with nuclear DNA restriction fragments within specific mt-nDNA interaction sets were extracted and used for gene ontology term enrichment analysis. Ontological terms related to genomic DNA synthesis and the regulation of energy availability were enriched within the M phase interactions.

Fig. 3. Mt-nDNA interactions that formed specifically during mitotic anaphase were associated with highly transcribed nuclear genes required for ribosome function. (A(i)) A high proportion of the nuclear genes that were associated with M phase specific mt-nDNA interactions had high transcript levels compared to random gene sets. By contrast, mt-nDNA interactions specific to and shared by the other cell cycle phases were not enriched for genes that had high transcript levels. (A(ii)) Nuclear genes associated with mt-nDNA interactions specifically formed during the G1 phase were less likely than random to involve genes with low transcript levels. Moreover, interactions that were shared by consecutive cell cycle phases did not involve nuclear genes whose transcript levels were within the lowest 5% in each condition. (B) The high transcript level genes that were associated with the M phase specific mt-nDNA interactions were enriched in GO terms related to ribosome structure and function.

Fig. 4.

G1 phase mt-nDNA interactions are enriched for high efficiency, early firing origins of replication. (A) mt-nDNA interactions detected during the G1 phase of the cell cycle are enriched for known nuclear origins. The proportion of mt-nDNA interactions for which the nuclear partner overlapped a nuclear origin (± 500 bp) (Heichinger et al., 2006) was compared to the overlap at an equivalent number of randomly selected nuclear loci. (B) The distribution, within the linear sequences of the nuclear chromosomes, of the origins that were associated with the G1 phase mt-nDNA interactions was not different from random (K-S test). (C) There was a significant enrichment for high efficiency and early firing origins while interactions with low efficiency origins were depleted within the nuclear loci associated with the G1 phase mt-nDNA interactions.