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# Using Genetic Reporters to Assess Stability and Mutation of the Yeast Mitochondrial Genome

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## 1. Introduction

The mitochondrion has been an identified subcellular organelle since the late 1800s, though its function and importance remained relatively cryptic until almost a century later. With the molecular renaissance of the 1950s and 1960s came the tools and perspectives, including peptide sequencing, microscopy, and the chemiosmotic hypothesis, with which to formulate and test crucial questions about what mitochondria do and how they function. In addition, shortly after the structure of DNA was elucidated and its role as the genetic material of the cell established, the presence of extranuclear DNA was revealed by electron microscopy (Nass and Nass, 1963) and by density gradient fractionation (Haslbrunner et al., 1964), identifying this DNA as part of highly purified mitochondria.

The goal of targeted genetic manipulation within the mitochondrial genome is rapidly driving a second renaissance in mitochondrial biology. Current approaches to the mitochondrial uptake of DNA have recently been reviewed in detail (Mileshina et al., 2011a). Several model organisms are currently amenable to this technique although no vertebrate species are among them. In our view, successful mitochondrial transformation is hindered by our limited knowledge of the fundamental processes of mitochondrial DNA maintenance and repair. We are hopeful that a greater understanding of the replication, repair, and maintenance of the mitochondrial genome, afforded by the tools currently in existence and presented here, will soon allow the construction of mammalian mitochondrial disease models (Dunn et al., 2011), or lead directly to gene therapy for the treatment of mitochondrial diseases in patients (Schon and Gilkerson, 2010). In this review, we will discuss the current state of mitochondrial genetic reporters and their application toward understanding and manipulating mitochondrial genome maintenance.

In cells, mitochondrial DNA is organized into protein-associated structures called nucleoids. Quantitative PCR coupled with immunofluorescence microscopy revealed an estimated 2-8 mitochondrial genomes per nucleoid in human immortalized cell culture (Legros et al., 2004); higher-resolution microscopy identified more nucleoids per cell, bringing this estimate down to only 1-2 copies per nucleoid (Kukat et al., 2011). A growing number of the proteins associated with the mitochondrial nucleoid have been identified from eukaryotic model systems (Bogenhagen et al., 2003; Garrido et al., 2003; Kienhöfer et al., 2009). The conserved and abundant HMG protein, Abf2p in yeast, and mtTFA (or TFAM) in

vertebrates, is thought to organize and compact the mitochondrial genome, and is proposed to play a histone-like role in mitochondrial DNA organization (Kaufman et al., 2007; Pohjoismaki et al., 2006). In addition to these properties, TFAM has recently been reported to contribute to mitochondrial DNA replication (Pohjoismaki et al., 2006) and repair (Canugovi et al., 2010).

The mitochondrial genome is vitally important to the organelle's proper function. Its encoded proteins are almost all subunits of the mitochondrial respiratory complexes; mutations to these genes can disrupt bioenergetic function and control. There is also growing evidence that proper maintenance of the mitochondrial genome is tightly associated with normal mitochondrial behavior, including fusion, fission, and intracellular migration (Baker and Haynes, 2011; Chen et al., 2010; Gilkerson, 2009). The reasons for this are currently unknown, but we can speculate that nucleoids may help to define a minimal mitochondrial "unit" and that disruption of mitochondrial DNA resolution affects mitochondrial dynamics and transmission in dividing cells (Margineantu et al., 2002). Alternately, nucleoids may help to catalyze organization of cristae, respiratory complexes, or other submitochondrial structures, as has been suggested for the Complex V ATP synthase (Strauss et al., 2008), and faulty mitochondrial DNA maintenance disrupts this patterning.

Fission and fusion, coupled with autophagic degradation of mitochondrial material ("mitophagy"), are increasingly appreciated as a primary means of mitochondrial quality control. Instability of the mitochondrial genome is therefore not just a problem affecting its encoded products, but potentially the structural integrity of the entire organelle. Recent proposals suggest that the cellular dysfunction underlying neurodegeneration in Alzheimer's and Parkinson's diseases is caused or at least exacerbated by defective mitophagy and increased mitochondrial DNA instability (Chang, 2000; Corral-Debrinski et al., 1994; Coskun et al., 2011; Narendra et al., 2010; Narendra et al., 2008; Sasaki et al., 1998; Suen et al., 2010).

### 1.1 Yeast gene nomenclature

A comprehensive guide to yeast gene nomenclature is both published (*Trends in Genetics, Volume 14, Issue 11, Supplement 1, 1998, Pages S.10-S.11*) and available online (<http://www.yeastgenome.org/help/yeastGeneNomenclature.shtml>). We will summarize here the points that are most relevant to the following sections.

There are two nomenclature systems, reflecting the advent of genomic analysis. The ORF naming system, instituted in the post-genomic era, gives each predicted open reading frame a systematic designation reflecting its chromosomal position and strand orientation. This system will not be discussed here. We will focus on gene symbol nomenclature, which applies only to ORFs that express a known gene product and is in common use for most proteins.

In gene symbol nomenclature, yeast genes are given a three-letter "name" followed by a number. Gene names are italicized, with dominant (usually wild-type) alleles in capital and recessive (usually mutant) alleles in lowercase type. For example, *ABC1* denotes a wild-type, dominant gene; *abc1* a generic mutant. The names themselves correspond to characterized features, either phenotypic, biochemical, enzymatic, or genetic. Mutant alleles can carry a second number to specify a particular mutation, e.g., *abc1-1*, or a descriptive name. For

example, *abc1-G43V* describes a point mutant that bears a glycine-to-valine amino acid substitution at position 43. Gene knockouts are denoted with a delta symbol, e.g., *abc1-Δ*. Gene disruptions by insertion of another gene are denoted with a double colon, e.g., *abc1::URA3*. If a gene is fully replaced with the insertion, this is indicated as *abc1Δ::URA3*. In yeast, gene products may also carry the gene name, e.g., Abc1p or *abc1p-G43V*. Gene product names are interchangeable with a functional name, for example, Arg8p = acetylmornithone transaminase.

Mitochondrial dysfunction can occur through single gene mutations in both nuclear and mitochondrial genomes, forming two classes of mutants. *Pet* mutants are those with nuclear DNA mutations, while *mit* mutants are those with mitochondrial DNA mutations. Additionally, mitochondrial genome status is also categorized. Respiring yeast strains carrying wild-type mitochondrial DNA are referred to as  $\rho^+$ . When yeast cells are grown on a fermentable carbon source such as glucose, spontaneous variants arise that cannot respire and form smaller colonies, termed “*petite*” mutants. The vast majority of spontaneous *petite* strains contain large-scale mitochondrial DNA deletions and rearrangements. Often these strains carry only a small fraction of their original mitochondrial DNA; retention of only 1% of the genome is commonly observed. The total mitochondrial DNA content of  $\rho^-$  and  $\rho^+$  strains are equivalent, however, as the remaining fragments in  $\rho^-$  strains are amplified accordingly (Dujon, 1981).

Generation of mitochondrial DNA-free derivatives of any strain is achieved by culturing cells with ethidium bromide, which blocks mitochondrial DNA replication without affecting nuclear DNA (Meyer and Simpson, 1969). These  $\rho^0$  strains can be studied directly or used as a tool for mitochondrial DNA manipulation.

## 1.2 The presence of mitochondrial DNA repair mechanisms

The assertion that mitochondrial DNA did not undergo repair was made as late as 1990 (Singh and Maniccia-Bozzo, 1990), in agreement with early reports (Clayton et al., 1974). Part of the appeal of this idea was the observation that mitochondrial DNA depletion can be induced by various insults, including oxidative stress (Shokolenko et al., 2009), ethanol (Ibeas and Jimenez, 1997) and zidovudine (AZT) (Arnaudo et al., 1991) treatment. This was interpreted as evidence that, when damaged, mitochondrial DNA was simply eliminated rather than repaired. However, in 1992, the first evidence for photolyase repair of UV-induced mitochondrial DNA damage in yeast was provided (Yasui et al., 1992), followed quickly by work from the Bohr and Campbell groups demonstrating uncharacterized repair activities and homologous recombination, respectively, in two mammalian mitochondrial systems (LeDoux et al., 1992; Thyagarajan et al., 1996). The fifteen years since have revealed an extensive set of mitochondrial DNA repair pathways, including base excision repair (BER), homologous recombination (HR), and non-homologous end joining (NHEJ). A recent review summarizes our current understanding of these pathways in mitochondria and other organellar genomes (Boesch et al., 2010).

## 1.3 Nuclear and mitochondrial DNA repair pathways share protein components

Many of the known mitochondrial DNA repair pathway proteins are mitochondrially-localized proteins initially characterized in nuclear repair. The first mitochondrial DNA repair protein identified, photolyase, was demonstrated to be one such dual-localized protein (Green and MacQuillan, 1980). Subsequent studies indicated localization of base

excision repair proteins to both subcellular compartments, including the yeast glycosylases Ntg1p (You et al., 1999), Ung1p (Chatterjee and Singh, 2001) and Ogg1p (Singh et al., 2001), the mammalian glycosylases UNG1 (Nilsen et al., 1997), MTH1 (Kang et al., 1995), OGG1 (Nishioka et al., 1999), and MYH (De Souza-Pinto et al., 2009; Nakabeppu et al., 2006), and the yeast AP endonuclease Apn1p (Ramotar et al., 1993). In human lymphoblasts, BER proteins were associated with the mitochondrial inner membrane fraction, where mitochondrial nucleoids are also found (Stuart and Brown, 2006). These findings illustrate the high evolutionary conservation of mitochondrial BER. Factors that regulate the subcellular localization of these proteins are not well understood; however, changes to localization in response to stress has recently been demonstrated (Griffiths et al., 2009; Swartzlander et al., 2010). This apparent recruitment of DNA repair proteins to the mitochondria may represent a DNA-specific communication pathway between the intramitochondrial and extramitochondrial environments.

Other DNA repair proteins have been shown to affect mitochondrial DNA maintenance in mammalian cells, including the BER flap endonuclease FEN1 (Liu et al., 2008), DNA double-strand break repair proteins, Rad51p (Sage et al., 2010), Mre11 (Dmitrieva et al., 2011) and Ku80 (Coffey et al., 1999), and the nucleotide excision repair protein CSA (Kamenisch et al., 2010). In addition, in yeast, DNA damage tolerance pathways that utilize the translesion polymerase complexes encoded by Rev1p, Rev3p, and Rev7p also impact mitochondrial mutagenesis (Kalifa and Sia, 2007; Zhang et al., 2006).

## 2. Manipulation of the yeast mitochondrial genome

### 2.1 Basic features of the mitochondrial genome

The yeast mitochondrial genome consists of 75-85 kb of double-stranded DNA, encoding seven protein products (Cox I, II, III, Atpase 6, 9, cyt b, Var1), 2 rRNAs and 24 tRNAs, while the human mitochondrial genome is a much smaller 16.5 kb and encodes 13 protein products (cox I, II, III, ND1-6, 4L, Atpase 6, 8, cyt b), 2 rRNAs and 22 tRNAs. Aside from the presence of complex I (NADH:ubiquinone oxidoreductase) subunit genes in the human mitochondrial genome and not yeast, and the presence of non-coding regions in the yeast genome, the two are remarkably similar in structure and encoded products, giving yeast mitochondrial genome manipulation great power to inform our understanding of mammalian mitochondrial DNA defects.

Mitochondrial and nuclear DNA in yeast are compositionally different; mitochondrial DNA is relatively AT-rich and highly repetitive, with G and C bases further segregated in coding regions. This repetition made initial sequencing of the entire yeast mitochondrial genome difficult (Foury et al., 1998) and is a continued challenge in targeted gene manipulation, particularly in the intergenic AT-rich regions. Yeast mitochondrial DNA has multiple regions of non-coding DNA, which are the primary contributors to the 83% AT bias of the genome. The size difference between human and yeast mitochondrial DNA is almost entirely due to the absence of these intergenic regions in the human mitochondrial genome.

### 2.2 Organisms with tractable mitochondrial genomes

To date, the number of organisms that have successfully undergone mitochondrial transformation remains small and is restricted to unicellular eukaryotes. The most widely used model remains the budding yeast *Saccharomyces cerevisiae* (Johnston et al., 1988), which

presents multiple biological advantages beyond the accessibility of its mitochondrial genome. Budding yeast are facultative anaerobes. This organism can survive by fermentation when oxygen is unavailable or when respiratory mechanisms are disrupted, as occurs during biolistic transformation (Section 2.3). Moreover, our comprehensive understanding and molecular tool base for the nuclear genome enables efficient analysis and powerful interpretation of mitochondrial phenotypes. Finally, homologous recombination in yeast is highly efficient in both nuclear and mitochondrial genomes, facilitating the introduction of targeted sequences to either genome.

The mitochondria of hyphal yeast *Candida glabrata* have also recently been reported to take up biolistically delivered DNA. Unlike wild-type *S. cerevisiae*, *C. glabrata* can maintain relatively stable heteroplasmy, maintaining mixed populations of transformed and endogenous DNA, which the authors propose as an ideal characteristic for studying the regulation of mitochondrial DNA transmission. Under selective pressure the exogenous genotype can be fixed to homoplasmy (Zhou et al., 2010). Heteroplasmy is the state of harboring multiple different mitochondrial genomes, while in homoplasmy only one type is present.

An important requirement of successful mitochondrial manipulation is the ability to select and purify a rare transformation event; in *S. cerevisiae*, the efficiency of transformation using microprojectile bombardment is on the order of one in  $10^7$  (Bonney and Fox, 2007). As described in Section 2.3, this is the sole method currently known for successful mitochondrial incorporation of exogenous DNA into whole cells.

The only known algal species to date that can be biolistically transformed is the green alga *Chlamydomonas reinhardtii*. This species can incorporate exogenous DNA into both mitochondrial and chloroplast genomes, providing a unique model for studying the genetic and functional interactions of these two organelles (Randolph-Anderson et al., 1993). Here, the development of a selectable mutant facilitated the initial isolation of mitochondrial transformants. *C. reinhardtii* are normally able to grow in the dark if supplemented with acetate as a carbon source; due to a deletion disruption of the mitochondrial CYB gene encoding apocytochrome b, the *dum-1* mutant cannot. Transforming *dum-1* cells with mitochondrial DNA isolated from a *DUM-1* strain and growing cells in darkness plus acetate selects for restoration of a growth phenotype, indicating mitochondrial uptake of the wild-type CYB gene.

Biolistic transformation in multicellular organisms is hampered by an inability to select and amplify individual transformants. A promising recent attempt to transform mitochondria in a mouse embryonic fibroblast line with a “universal” neomycin marker via a bacterial conjugation-like mechanism was not successful in generating mitochondrial transformants (Yoon and Koob, 2011). However, mitochondria isolated from both mammalian and plant sources have been successfully transformed *in vitro* (Mileshina et al., 2011b; Yoon, 2005). Multiple methods, including DNA targeting with a protein localization signal, electroporation, and spontaneous mitochondrial uptake of linear DNA have all given rise to mitochondrial uptake of exogenous DNA, but these constructs could not be propagated in the mitochondrial genome of a viable and dividing cell (Mileshina et al., 2011a).

### 2.3 Biolistic transformation of yeast and selection of transformed clones

Microprojectile bombardment of DNA on a carrier is an effective method for delivering DNA past the plasma membrane and two mitochondrial membranes into the mitochondrial

matrix. This method was pioneered in plants by John Sanford (Sanford et al., 1987), and first demonstrated in yeast by Sanford, Butow and colleagues (Johnston et al., 1988). Described below is the general transformation procedure used for *S. cerevisiae* (Bonney and Fox, 2007).

The linear or circular plasmid DNA to be transformed is alcohol-precipitated onto a carrier substrate, usually tungsten or gold particles <1 $\mu$ m. The bombardment itself occurs in a biolistic gun chamber (Sanford, 1988), where rising vacuum pressure ruptures a pressure sensitive disk holding the DNA-precipitated particles, driving the particles onto a freshly plated lawn of haploid yeast cells. The plate medium then selects for uptake of either the plasmid of interest or of a co-transformed marker if the target plasmid does not confer a selectable phenotype.

The target cells for mitochondrial transformation are typically  $\rho^0$  (non-mitochondrial DNA-containing) derivatives of a chosen strain, to ensure that the only mitochondrial DNA present is transformation-derived. After selection for the co-transformed nuclear marker, transformants must be screened for the presence of the transforming mitochondrial DNA. Following mitochondrial uptake of the desired DNA, positive haploid clones are mated to a strain containing wild-type mitochondrial genomes, allowing mixing of the transformed DNA with the target mitochondrial DNA. Generally, the desired outcome is integration of the synthetic DNA construct into the mitochondrial genome, although mitochondrial plasmid maintenance can also occur. The specific example of *ARG8<sup>m</sup>* integration is provided below in Section 3.1.

### 3. The *ARG8<sup>m</sup>* auxotrophic mitochondrial reporter gene

#### 3.1 Building an auxotrophic mitochondrial reporter: the *ARG8<sup>m</sup>* gene

The phenotypic output of a genetic reporter system determines its strengths and weaknesses as an analytic tool. In yeast, multiple auxotrophic (factor-requiring) mutants have historically been used with great success as both selective markers and phenotypic reporters. The defined requirements of yeast grown in culture allow for synthetic reconstitution of growth media lacking a specific amino acid. Commonly used auxotrophic markers in yeast include growth status on media lacking uracil, histidine, leucine, arginine, methionine, and lysine. Many laboratory strains, including our wild-type strain, DFS188 and its derivatives, lack the ability to make these amino acids due to specific nuclear mutations. These strains are typically maintained in rich media, allowing unrestricted growth. Withdrawal of the amino acid in question results in cell death. Rescue of a growth phenotype occurs when the gene that complements the nuclear mutation for that amino acid's synthesis is supplied on a plasmid or as part of a conditional reversion construct, allowing cells to regain prototrophic (factor-independent) growth.

While an ideal system to assess mechanisms associated with nuclear gene expression, the mitochondrial genome has long been inaccessible to auxotrophic reporter manipulation because it does not encode any amino acid biosynthetic enzymes. Direct insertion of a nuclear gene is impossible, as the codon usage of the mitochondrial genome differs from the nuclear genome both in the preferred codon frequency and in some codon products. Multiple nuclear leucine codons encode threonine in mitochondria, and a nuclear UGA stop codon encodes tryptophan in the mitochondria of yeast (Bonitz et al., 1980). Generating a

mitochondrial auxotrophic reporter thus requires mutating a nuclear gene to enable its expression from the mitochondrial genome. Once constructed, this gene must be introduced into the mitochondrial genome with the appropriate transcriptional and translational cues. To date, the only such auxotrophic marker gene to be engineered in this way is the synthetic *ARG8<sup>m</sup>* gene, made by Tom Fox and colleagues (Steele et al., 1996).

The nuclear *ARG8* gene encodes acetylornithine transaminase, which catalyzes an early step in the biosynthesis of ornithine, a precursor to both arginine and proline. The Arg8 protein is normally localized to the mitochondrial matrix and yields the active mitochondrial transaminase following cleavage of its N-terminal targeting sequence (Steele et al., 1996). It is therefore an ideal candidate for reconfiguration as a mitochondrial gene, as its product functions within the matrix and does not require mitochondrial export for phenotypic expression.

Fox and group began by synthetically generating a 1.3 kilobase fragment encoding the entire 423 amino acid acetylornithine transaminase enzyme. Substitutions were made at 12 CUN codons (n: Leu; mt: Thr) and 6 AUA codons (n: Ile; mt: Met) to maintain the Leu and Ile residues. In addition, each of the two Trp codons was changed to UGA (n: STOP; mt: Trp) ensuring *ARG8<sup>m</sup>* expression from the mitochondrial genome only. This construct was introduced into a plasmid containing mitochondrial DNA sequence flanking the *COX3* gene, providing sequences for recombination-dependent integration and ensuring the presence of correct transcriptional and translational processing signals.

Several steps were then required to generate the desired end product of the *cox3::ARG8<sup>m</sup>* sequence incorporated into the mitochondrial genome while maintaining isogenicity of both mitochondrial and nuclear genomes. These steps are a “shell game” of genetic manipulation, designed to shield various DNA pools from one another until the desired product is achieved (Fig. 1).

The plasmid containing *cox3::ARG8<sup>m</sup>* was biolistically transformed into a  $\rho^0$  haploid yeast strain, ensuring that plasmid DNA was the only DNA present in the mitochondrial compartment (Fig. 1). In addition, these yeast cells lack the nuclear *ARG8* gene and carry a mutation in the *KAR1* gene that prevents nuclear fusion during mating. A second plasmid carrying a functional *LEU2* allele was co-transformed to allow selection of successful DNA uptake by a Leu<sup>+</sup> phenotype. Note that the mitochondrial DNA targeted construct, though present, is inactive; screening for its presence therefore requires selection of a DNA-dependent effect. Transformants were screened for the presence of the *ARG8<sup>m</sup>* gene in the mitochondria by mating with a strain carrying a mitochondrial genome deletion in the 5' untranslated region upstream of the *COX3* gene. Only Leu<sup>+</sup> transformants that also carry the mitochondrial plasmid with the wild-type *COX3* 5' sequence will complement the deletion to give rise to respiring recombinants. Positive mitochondrial transformants identified by this test mating were purified and used to generate the integrated reporters.

To allow mixing of the mitochondrial plasmid DNA with intact mitochondrial genomes, the biolistically transformed cells were mated with a second haploid strain bearing normal mitochondrial DNA (Fig. 1, Fig. 2A). Since one strain is karyogamy-deficient, the nuclear envelopes do not fuse. Cell division gives rise to haploid cells, and one haploid genome can be selected in subsequent divisions. The mitochondria, however, undergo rapid fusion, allowing interaction between plasmid and mitochondrial DNA. This process is known as

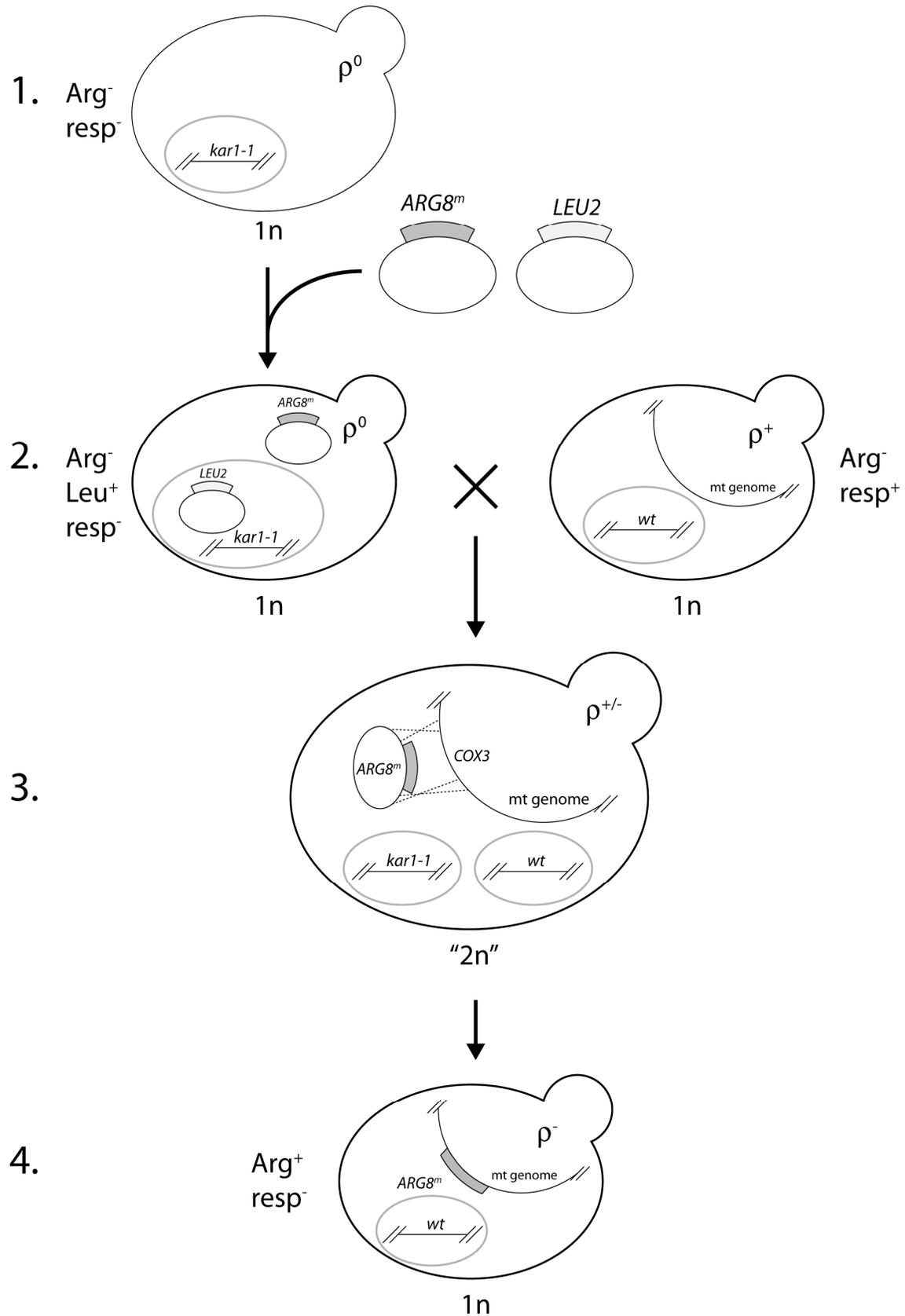


Fig. 1. Diagrammatic representation of biolistic transformation and integration of exogenous DNA into the yeast mitochondrial genome. Mitochondrial genome status is given in  $\rho$

nomenclature. Slash marks indicate endogenous genomic DNA; ovals with bars for relevant genes represent plasmid DNA. Gray line denotes the nucleus. Selectable phenotypes of the genotype shown are written outside the cell. Ploidy is written below the cell. **Step 1.** A haploid,  $\rho^0$ , nuclear fusion-deficient mutant (*kar1-1*) is biolistically transformed with two plasmids, one bearing the *LEU2* allele and one the *ARG8<sup>m</sup>* allele with *COX3* flanking sequence. **Step 2.** Transformants are selected by *Leu<sup>+</sup>* growth, reflecting nuclear uptake of the *LEU2*-bearing plasmid (and possible uptake of the *ARG8<sup>m</sup>*-bearing plasmid; confirmation of uptake is described in Sections 2.3 and 3.1). *Leu<sup>+</sup>* transformants are crossed with wild-type cells. **Step 3.** Mixing of the *ARG8<sup>m</sup>* plasmid with endogenous mitochondrial genomes; homologous regions of *COX3* non-coding sequence flanking *ARG8<sup>m</sup>* mediate recombination at the mitochondrial *COX3* locus. **Step 4.** Replacement of endogenous *COX3* with *ARG8<sup>m</sup>* in the mitochondrial genome, maintained by *Arg<sup>+</sup>* selection. Loss of *COX3* confers respiration failure.

cytoduction. Homologous recombination between the *COX3* flanking sequences mediated replacement of the endogenous *COX3* gene with plasmid-derived *cox3::ARG8<sup>m</sup>* sequence. By simultaneously selecting for both mitochondrial (*Arg<sup>+</sup>*) and nuclear markers, a haploid strain of the desired nuclear and mitochondrial backgrounds results that is phenotypically *Arg<sup>+</sup>* and respiration-deficient, requiring a fermentable carbon source.

Mitochondrial genome incorporation of *cox3::ARG8<sup>m</sup>* was confirmed by Southern blotting, while its requirement for the *Arg<sup>+</sup>* phenotype was shown by curing yeast of their mitochondrial DNA and observing a reversion to an *Arg<sup>-</sup>* phenotype. The *Arg<sup>+</sup>* phenotype was also dependent on the *COX3* translational activation complex, as deletion of the genes encoding the complex components result in *Arg<sup>-</sup>* cells. Finally, immunoblot analysis demonstrated that the protein product of the *ARG8<sup>m</sup>* gene is identical in size to that of the nuclear *ARG8*, suggesting correct N-terminal processing. These controls elegantly demonstrate the correct location, expression and control of the *cox3::ARG8<sup>m</sup>* reporter gene (Steele et al., 1996).

The *ARG8<sup>m</sup>* reporter was initially used by Fox and group to examine mechanisms of mitochondrial translation (Bonney and Fox, 2000; Dunstan et al., 1997), but its utility as a reporter extends to any assay in which reporter expression can be made a meaningful indicator of the function of interest. Fox and colleagues have also used the *ARG8<sup>m</sup>* and other reporters (including a recoded mitochondrial GFP (Cohen and Fox, 2001)) to measure peptide import and export from the mitochondria (He and Fox, 1997; Torello et al., 1997), and to generate mutations in respiratory complex subunits (Ding et al., 2008). In addition, we and others have used *ARG8<sup>m</sup>* expression to measure various aspects of mitochondrial DNA instability. The following sections will describe the construction, insertion, and use of these reporters in detail.

### 3.2 *ARG8<sup>m</sup>* as a reporter of mitochondrial translation, DNA repair, recombination, and heteroplasmy

Prior to the construction of the *ARG8<sup>m</sup>* mitochondrial reporter and the advent of qPCR and high-throughput sequencing, only two methods were available to easily assess mitochondrial DNA stability. First,  $\rho^-$  yeast cells with grossly defective mitochondrial DNA are respiration deficient and display slower growth on fermentable media, forming “petite”

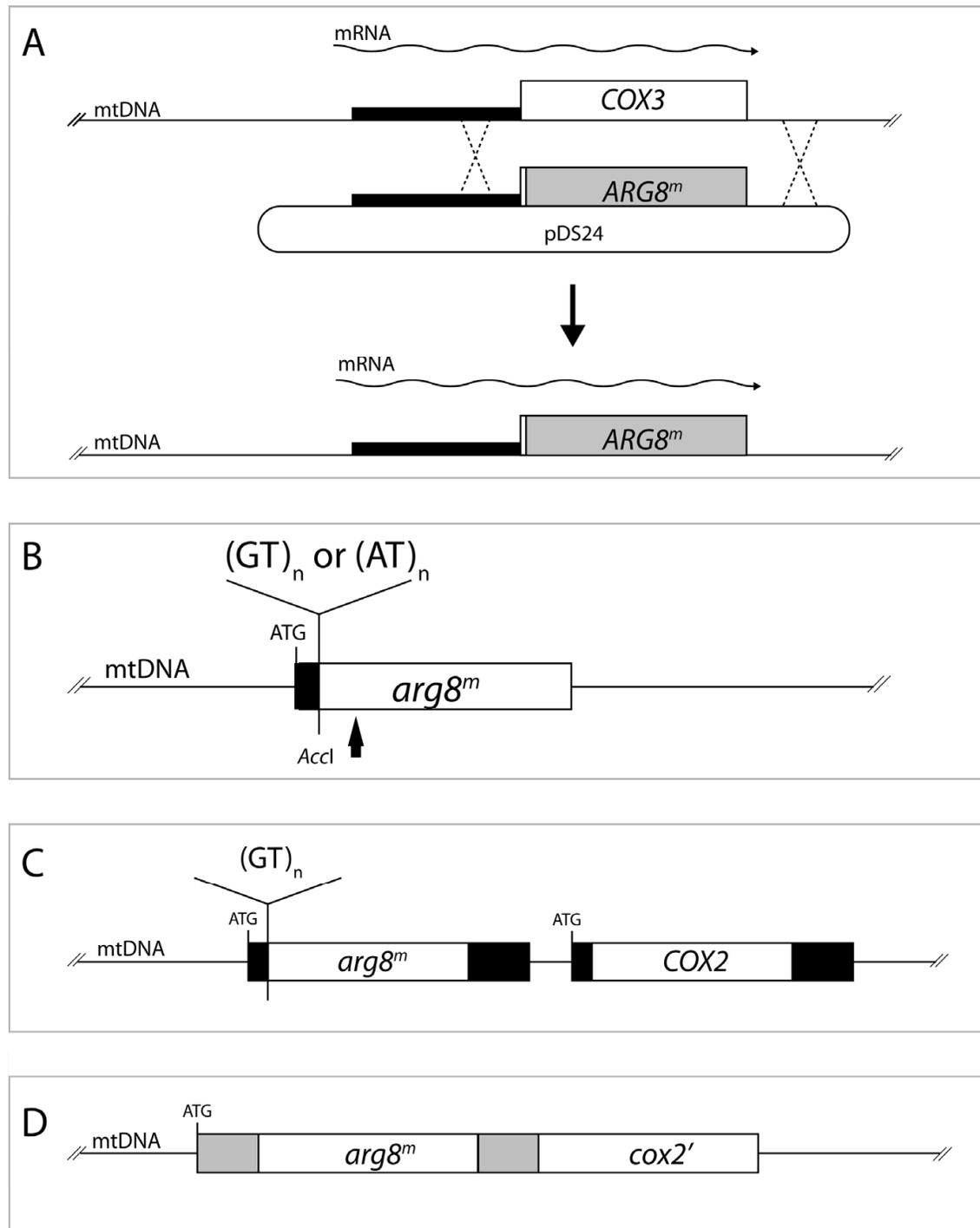


Fig. 2. *ARG8<sup>m</sup>* construct diagrams. Slash marks indicate endogenous genomic DNA; ovals with bars for relevant genes represent plasmid DNA. **A.** Endogenous *COX3* locus (top) and replacement by *ARG8<sup>m</sup>* borne on plasmid pDS24 to generate the mitochondrial genome-integrated *ARG8<sup>m</sup>* allele (bottom). Thin black bar represents non-translated sequence; labeled white and gray bars denote the *COX3* and *ARG8<sup>m</sup>* ORFs, respectively. Adapted from (Steele et al., 1996). **B.** Insertion of GT or AT dinucleotide repeats into a *cox3::ARG8<sup>m</sup>* reporter fusion using an internal *AccI* restriction endonuclease site. Black bar represents *COX3* coding sequence, ATG denotes *COX3* translational start, arrow represents post-translational cleavage site to yield functional Arg8p. Adapted from (Sia et al., 2000). **C.** Respiring

microsatellite reporter insertion upstream of the endogenous *COX2* locus, flanked by *COX2* non-coding sequence (black bars) including the translational start (ATG). Adapted from (Mookerjee and Sia, 2006). **D.** *Rep96::arg8<sup>m</sup>::cox2'* direct repeat-mediated deletion (DRMD) reporter. Gray bars represent the first 96 base pairs of *COX2* translated sequence, either containing (ATG, left of *arg8<sup>m</sup>* locus) or missing (right of *arg8<sup>m</sup>* locus) the ATG translational start codon. Adapted from (Phadnis et al., 2005).

colonies. Though petite formation is a somewhat useful indicator of gross mitochondrial DNA abnormality, its pleiotropic nature (the petite phenotype may result from nuclear *pet* as well as mitochondrial *mit* mutations), its origin via multiple types of mitochondrial DNA mutation, and the lack of a mechanistic explanation for the generation of  $\rho^-$  genomes all limit its utility. Second, because mitochondria retain many prokaryotic properties, they are selectively sensitive to antibiotic drugs, including erythromycin. Erythromycin binds to the 21S mitochondrial ribosomal RNA, disrupting mitochondrial protein synthesis. Specific point mutations to the mitochondrial 21S rRNA gene prevent erythromycin from binding to the gene product, conferring an erythromycin resistant (Ery<sup>R</sup>) phenotype (Cui and Mason, 1989; Kalifa and Sia, 2007). In this way, the appearance of Ery<sup>R</sup> colonies can be used to estimate mitochondrial DNA point mutation accumulation rates using various calculation methods (Lea and Coulson, 1949; Luria and Delbruck, 1943). Ery<sup>R</sup> acquisition is useful because it is largely restricted to one mutation type. However, the spectrum of mutations that can be obtained is biased in that mutants must maintain mitochondrial ribosome function to preserve respiration competence. Since erythromycin does not affect yeast viability, only respiration, yeast strains are resistant to erythromycin in fermentable media. This makes it impossible to select point mutations under non-respiring conditions using Ery<sup>R</sup>.

A spontaneous *cox3::arg8<sup>m</sup>* mutant, isolated by Fox and group and reported by Strand and Copeland, is an Arg<sup>-</sup> revertant that was shown to contain two nucleotide substitutions and a +1 frameshift insertion mutation (Strand and Copeland, 2002). Deletion of a single nucleotide restores the proper reading frame and allows reversion to an Arg<sup>+</sup> phenotype (Zhang et al., 2006). This mutant was proposed as a replacement for the Ery<sup>R</sup> mutation assay, which measures true point mutations. While this reporter has some advantages over Ery<sup>R</sup> acquisition, this approach fails to distinguish between different types of mitochondrial DNA mutation and is therefore less helpful at elucidating the mechanisms responsible for their generation. Our subsequent work has demonstrated that the proteins that impact mitochondrial nucleotide substitutions and mitochondrial insertion/deletion mutations are not always the same, allowing the dissection of pathways that differentially affect point and frameshift mutation as described below.

#### 4. Measuring mitochondrial microsatellite instability

Short, repetitive sequences consisting of di- and tri-nucleotide repeats are abundant in the nuclear genome, in both coding and non-coding regions. Their appearance and inherent instability in the coding regions of several proteins is the underlying cause of the polyglutamine diseases, including Huntington's disease and multiple types of spinocerebellar ataxia. These microsatellites are an important source of mutation in nuclear DNA through the internal repetition that facilitates repeat length changes by polymerase slippage, and through the ability of separate repeated regions to undergo homologous recombination in *trans*.

Yeast mitochondrial DNA is highly microsatellite-enriched, partially due to the abundance (83%) of A and T. Mammalian mitochondrial DNA lacks most of the non-coding AT-rich DNA (~44% A/T) that gives rise to this bias, but still contains AT-rich repetitive regions (Anderson et al., 1980). If this repetition confers higher mitochondrial DNA instability, similar to its effects on nuclear DNA (Wierdl et al., 1997), it could play an important role in mitochondrial dysfunction as it relates to aging and aging-related diseases. These findings provided the impetus for making a mitochondrial microsatellite reporter system.

In 2000, Petes, Fox, and colleagues published an analysis of mitochondrial DNA microsatellite instability using the *ARG8<sup>m</sup>* reporter as a marker of frameshift mutation within a microsatellite repeat (Sia et al., 2000). This approach built on a previous reporter of yeast nuclear microsatellite instability (Henderson and Petes, 1992). They generated *cox3Δ::arg8<sup>m</sup>*-bearing plasmids that contained poly-GT and poly-AT tracts 15-17 repeats in length 5' to the *ARG8<sup>m</sup>* sequence, shifting the reading frame of the gene either 1 or 2 nucleotides out of frame (Fig 2B). Once incorporated and expressed, these *cox3Δ::arg8<sup>m</sup>(G/A-T)* constructs express a nonsense transcript. However, a frameshift mutation in the microsatellite tract that restores the correct reading frame restores a functional gene product, conferring an Arg<sup>+</sup> phenotype. As a control, plasmids were also made bearing microsatellite inserts that did not result in an *ARG8<sup>m</sup>* frameshift, conferring a constitutive Arg<sup>+</sup> phenotype, confirming that insertion of the repetitive sequence did not disrupt *ARG8<sup>m</sup>* function.

The experiments carried out with these strains were among the first to demonstrate fundamental differences between nuclear and mitochondrial DNA processing and maintenance. Unlike nuclear DNA, in which poly(AT) and poly(GT) tracts have similar levels of instability, with repeat addition favored, mitochondrial poly(AT) tracts are much more stable than poly(GT) tracts, and repeat deletion predominates. These and other differences suggest that assumptions of mitochondrial DNA behavior based on nuclear DNA may be inherently flawed, preventing a clear understanding of how to predictably manipulate mitochondrial DNA.

To allow microsatellite instability measurement in respiring cells, a state that imposes a respiration requirement on mitochondrial DNA maintenance, we developed and characterized a version of the microsatellite reporter that is respiration competent (Kalifa and Sia, 2007; Mookerjee and Sia, 2006). This ensures that the measured microsatellite instability occurs in an otherwise functional background. This new reporter serves two useful purposes, allowing us to determine the effect of an active respiratory chain on mitochondrial mutagenesis, and to assess microsatellite instability in mutant strains that only maintain mitochondrial DNA under constant respiratory selection. For this reporter, instead of replacing *COX3*, *ARG8<sup>m</sup>* was inserted with *COX2* flanking sequence upstream of the endogenous *COX2* locus (Fig. 2C). These untranslated flanking sequences ensure correct expression while avoiding disruption of cytochrome oxidase subunits. In this new genetic and functional context, poly(GT)<sub>16</sub> repeats in the +1 frame were approximately 10-fold more unstable than the original *cox3Δ::arg8<sup>m</sup>(G/T)<sub>16</sub>* (Kalifa and Sia, 2007). Further experiments will be required to determine whether this is due to the altered flanking sequence or respiration status of the cell.

## 5. Measuring direct repeat-mediated deletions

Accumulation of mitochondrial deletions is associated with multiple pathologies and with aging. These deletions are commonly flanked by direct repeats, raising speculation that they

are recombination-mediated. A detailed understanding of mitochondrial DNA recombination has lagged far behind equivalent nuclear recombination processes, which in turn limits our efforts to use recombination both as a molecular tool for genome manipulation and as a biological correlate of mitochondrial dysfunction. By manipulating the sequence context of the *ARG8<sup>m</sup>* gene, we developed reporters to measure and characterize mitochondrial recombination in yeast. These studies have revealed detailed information about the requirements for and mechanisms of recombination in the yeast mitochondrial genome.

### 5.1 DFS188 *Rep96::ARG8<sup>m</sup>::cox2'* reporter and variants

We generated a synthetic deletion substrate with the *ARG8<sup>m</sup>* gene fused in frame to the first 99 bp of the mitochondrial *COX2* gene. This construct was followed by the entire *COX2* coding sequence, lacking the 5' start codon, giving rise to *ARG8<sup>m</sup>* flanked by 96 bp of directly repeated *COX2* sequence (Fig. 2D). This deletion substrate expresses functional acetylornithine transaminase and confers an Arg<sup>+</sup> phenotype. *COX2* is not expressed, as translation terminates with Arg8p. These cells are therefore non-respiring. However, deletion between the flanking 96-bp repeats excises *ARG8<sup>m</sup>* and restores a functional *COX2* sequence with the appropriate initiation and termination signals. Cells that have undergone either sufficient recombination events, or homoplasmic fixation of one or a few recombination events, display a phenotypic shift to respiration competence and arginine auxotrophy. The work that followed development of the *Rep96::ARG8<sup>m</sup>::cox2'* reporter used either this form or modified versions with changes to the flanking repeats.

Yeast bearing the *Rep96::ARG8<sup>m</sup>::cox2'* reporter can be assayed by imposing different nutrient conditions that select for either the original construct or the deleted one. Cells are initially grown on medium lacking arginine to maintain the original reporter. Individual colonies are then separately diluted and plated on glycerol medium (YPG) to select for deletion events, which are scored after 3 days. The median number of colonies appearing on YPG plates is used to estimate the mutation rate using the method of the median (Lea and Coulson, 1949), which incorporates statistical assumptions about the number of mutational events vs. colony number.

### 5.2 Characterizing direct-repeat mediated deletion (DRMD) in yeast

Work by Phadnis *et al.* (2005) demonstrates the utility of this reporter in characterizing mitochondrial recombination. First, generation of deletion reporters containing different repeat lengths revealed that the rate of *ARG8<sup>m</sup>* deletion is linearly dependent on repeat lengths between 33 and 96 bp; a 21-bp repeat did not facilitate a significant deletion rate. This establishes the minimal efficient processed sequence (MEPS) between 21 and 33 bp long. This MEPS is longer than the direct repeats flanking mitochondrial DNA deletions in mammalian systems, including the 13 bp flanking the 5-kb "common deletion" (Schon *et al.*, 1989) and a 7-bp recombination-associated sequence (Myers *et al.*, 2008). It should be noted that in humans, direct repeats mediate only some of the total detectable mitochondrial DNA deletions (Guo *et al.*, 2010; Srivastava and Moraes, 2005).

Second, the effects of heterology on deletion efficiency were tested by introducing silent mutations into either the leading or following repeat (relative to the direction of

transcription), giving rise to ~2% heterology between the repeat sequences. This design was meant to allow comparison to similar work in the yeast nuclear genome, where a 3% heterology between 205 bp repeats decreased the rate of deletion formation 6-fold (Sugawara et al., 2004). These experiments revealed similar behavior in mitochondrial DNA, where a 3- to 4-fold reduction in deletion formation rate was observed. Interestingly, this effect was dependent on mutation placement in the leading repeat; the same mutations in the following repeat had no effect on deletion rate. One explanation for this is that the heteroduplex rejection mechanisms may act more stringently on particular mispair orientations. In successful deletion events, the final sequence was almost always that of the repeat closest to the remaining *COX2* sequence. These findings are likely to be related to the specific types of mechanisms that mediate DRMD, but at present are unexplained.

The possible mechanisms mediating repeat-dependent deletion can be partially distinguished based on the DNA products they generate, namely, whether detectable products are reciprocal or non-reciprocal. Unlike qPCR detection methods that are commonly used to measure mitochondrial DNA deletions *in vivo*, we used Southern blot analysis to examine the products of direct repeat dependent deletion. In theory, the reciprocal products, either a circular molecule or tandem duplication on another mitochondrial genome would both be detectable. We did not observe such species using this technique. However, we detected reciprocal products through PCR amplification and electrophoresis, indicating that reciprocal events do occur. This method cannot distinguish between circular “pop-outs” or tandem duplications. This analysis revealed that the majority of deletion events were non-reciprocal, suggesting a replication- or single strand annealing-based mechanism.

Third, genes believed to be involved in repeat-mediated deletion were tested to determine their effects on the rate of mutation. Mutations to the proof-reading domain of the mitochondrial DNA polymerase were shown to affect *Rep96::ARG8<sup>m</sup>::cox2'* deletion. The *mip1-D347A* exonuclease proofreading mutation, which confers a approximately 500-fold increase in point mutation rates as measured by Ery<sup>R</sup>, actually decreases direct repeat-mediated deletion nearly 5-fold (Phadnis et al., 2005). Interestingly, several years later Vermulst *et al.* (2008) determined that the analogous Pol $\gamma$  mutation shifts deletion formation from a repeat-mediated to a non-repeat-mediated mechanism (Vermulst et al., 2008). That the analogous mutation appears to limit DRMD in both yeast and vertebrates suggests that at least some of the mechanisms for mitochondrial deletion may be conserved between these organisms.

### 5.3 Assaying heteroplasmy in yeast

The *Rep96::ARG8<sup>m</sup>::cox2'* reporter, by virtue of its construction, can also be used as a phenotypic marker of mitochondrial DNA heteroplasmy. Unlike mammalian cells, yeast containing multiple different mitochondrial DNA types will purify this population to fix one type within 6-10 budding cycles (Dujon, 1981). Several factors are thought to be involved in this process of homoplasmic sorting. First, compaction of multiple, possibly clonal genome copies into nucleoids reduces the number of heritable units. Second, a limited number of nucleoids are transmitted to daughter cells, and may undergo regulated sorting, further restricting mitochondrial DNA inheritance (Spelbrink, 2009). In mammalian systems, a third factor may be that nucleoids do not appear to readily mix, even when given the opportunity to do so by cytoduction (Schon and Gilkerson, 2010).

As stated earlier, the *Rep96::ARG8<sup>m</sup>::cox2'* reporter confers respiration incompetence and prototrophy. This phenotype is mutually exclusive with that of the deleted reporter, which confers respiration competence and arginine auxotrophy. Therefore, growing yeast under conditions that select for both respiration competence and arginine prototrophy, representing one phenotype of each possible state of the reporter, should select for yeast that maintain heteroplasmy. This was shown successfully in an analysis of point mutants of *MSH1* a yeast homolog of the MutS mismatch repair initiation protein (Mookerjee and Sia, 2006). In contrast to wild-type yeast, in which heteroplasmic maintenance occurred at a frequency on the order of  $10^{-5}$ , *msh1* alleles permitted frequencies of heteroplasmy up to 0.25, 4 to 5 orders of magnitude greater. The role of Msh1p in homoplasmic sorting remains undetermined.

## 6. Elucidation of mitochondrial DNA repair pathways

With specific reporters of microsatellite instability, point mutation, and direct repeat instability, coupled with direct sequencing and Southern blotting, the pathways of mitochondrial DNA repair become more readily accessible to quantitative analysis. This section will discuss some of the research findings resulting from use of the mitochondrial reporters described above.

### 6.1 Mismatch recognition combines with recombination and base excision repair pathways

Use of the *Rep96::ARG8<sup>m</sup>::cox2'* reporter allowed the initial characterization of mitochondrial DNA recombination requirements, including repeat size, degree of sequence identity, and directional/positional repair bias as described in Section 5.2 and in Phadnis et al. (2005). We have further applied the reporters to determining the total complement of mitochondrial DNA repair mechanisms and their interactions

Mismatch repair has been a predicted pathway of mitochondrial DNA repair since the identification of the mitochondrially-localized MutS homolog, Msh1p. However, there is currently no direct evidence in yeast for mismatch repair activity. Human mitochondria do have a putative mismatch repair mechanism (De Souza-Pinto et al., 2009), but do not possess a MutS homolog. Point mutation accumulation rates increase with Msh1p disruption, but evidence from multiple groups suggests that this is due to base excision repair (BER), rather than mismatch repair (MMR), defects. Further, no other characterized mismatch repair proteins are known to localize to the mitochondria.

Haploid yeast strains with deletions of the *MSH1* gene cannot maintain wild-type mitochondrial DNA, generating  $\rho^-$  petites at a high frequency even in the presence of selection on a non-fermentable carbon source (Chi and Kolodner, 1994). To explore this problem in detail, we characterized the mutagenic consequences of three point mutations to *MSH1*. These mutations are analogous to well-studied mutations in *E. coli* MutS and yeast nuclear MutS homologs and were chosen based on the biochemical functions of the mutant proteins and their ability to maintain mitochondrial function when under selection. The *msh1-F105A* substitution lies in the conserved DNA binding domain, and is predicted by its homology to MutS and Msh6p to impair DNA binding and mismatch recognition (Bowers et al., 1999; Schofield et al., 2001). The *msh1-G776D* and *msh1-R813W* substitutions both lie

within the highly conserved ATPase domain, although they are predicted to have different phenotypic and biochemical consequences. The yeast *msh6p-G987D*, analogous to our *msh1p-G776D* mutant, is significantly impaired in ATP-binding and displays an ability to bind mismatches, but is defective in further processing. The *msh2p-R730W* mutation, analogous to our *msh1p-R813W*, is able to bind ATP, but is defective in hydrolysis. While complexes containing this mutant form of Msh2p cannot perform mismatch repair, they remain at least partially functional for promoting deletions at directly repeated sequences (Kijas et al., 2003; Studamire et al., 1998).

By comparison with the known mutations, all three *msh1* mutations were predicted to result in loss of any mismatch repair activity. Consistent with this hypothesis, all three *msh1* mutants displayed increased point mutation rates. However, this increase was insufficient to explain the catastrophic loss of wild-type mitochondrial DNA and respiratory function (Mookerjee et al., 2005; Mookerjee and Sia, 2006), as mutations in the proof-reading domain of the mitochondrial replicative polymerase display more than ten-fold higher rates of point mutation, but can maintain  $\rho^+$  DNA (Foury and Vanderstraeten, 1992).

We then characterized the effects of *MSH1* mutation on microsatellite instability, hypothesizing that since nuclear mismatch repair disruption greatly increases nuclear microsatellite instability, a similar observation in mitochondria would support a bona fide mismatch repair function. Examination of *msh1* alleles with disruptions in the DNA-binding (*msh1-F105A*) and ATPase (*msh1-R813W*) domains revealed no significant changes in GT microsatellite instability, suggesting that Msh1p initiates non-mismatch repair mechanisms. It is formally possible that mitochondrial mismatch repair is not equivalent with respect to microsatellites between mitochondria and the nucleus due to other differences between the two compartments. Still, this finding prompted us to search for other repair activities involving Msh1p.

Mismatch repair proteins have been shown to function in other DNA repair pathways, including BER, nucleotide excision repair (NER), and homologous recombination (Goldfarb and Alani, 2005; Polosina and Cupples, 2010). Using the mitochondrial reporters, we were able to examine the genetic interaction of *MSH1* with putative recombination (Mookerjee and Sia, 2006) and BER components (Pogorzala et al., 2009).

Though widely accepted as a functional mechanism in mitochondrial DNA, the proteins that carry out recombination, and the specific mechanisms themselves, are largely unknown. Due to differences in the available proteins, in the substrate, or in the presumably constant availability of a homologous template, mitochondria may combine existing repair components in ways not seen in the nucleus (Masuda et al., 2009). We speculated that Msh1p, like its nuclear homologs, may play a role in the generation of deletions at directly-repeated sequences, and therefore would be predicted to result in reduced DRMD. Unexpectedly, we found that all three *msh1* mutations increase deletion rate approximately 100-fold, revealing a novel role for Msh1p function in mitochondrial recombination suppression that is not mirrored by any of its nuclear homologs.

Previously, examination of the deletion junctions of  $\rho^-$  genomes in spontaneous *petite* strains had suggested that recombination utilizing repeated sequences may be the initiating event in their generation (Dujon, 1981). If so, all three of the *msh1* mutants would be predicted to give rise to similar, high levels of non-respiring cells. However, while the *msh1*-

*F105A*, *msh1-G776D*, and the *msh1-R813W* mutant strains all display high rates of point mutation and DRMD, the *msh1-R813W* mutant strain displays significantly lower rates of respiration loss than strains expressing the other two *MSH1* mutant alleles. This result calls into question several assumptions about how cells become  $\rho$ -*petite*. In these cells, respiration capability is lost with deletion of the majority of the mitochondrial genome, but it is clear that gene disruptions leading to increased rates of both point mutation accumulation and repeat-mediated deletion do not necessarily lead to loss of  $\rho$ + mitochondrial DNA.

If Msh1p mutation allows it to bind but not release mismatched DNA, it is tempting to speculate that DNA molecules might become aberrantly linked. While not direct confirmation of this, we did find that the *msh1-G776D* and *msh1-R813W* alleles, and to a lesser extent, the *msh1-F105A* allele, conferred increases in the frequency of heteroplasmic maintenance of at least three or more orders of magnitude.

In addition to suppressing DRMD, Msh1p is also an important component of mitochondrial base excision repair. Through conventional epistasis analysis of *MSH1* and the BER genes *OGG1*, *NTG1*, and *APN1*, we found that Msh1p defects gave rise to different mutator phenotypes depending on the form of BER, and proposed that mismatch or lesion recognition contributes to short-patch BER, while DNA binding plays a stabilizing role during long-patch BER (Pogorzala et al., 2009).

## 6.2 Translesion polymerases facilitate frameshifts but suppress mitochondrial point mutation

The high fidelity of replicative DNA polymerases arises through extremely stringent requirements for nucleotide binding in the active site. While normally desirable, the inflexibility of these domains to accept alternate substrates causes replication fork stalling or collapse in the presence of cyclobutane-pyrimidine dimers, a common UV-induced product, and other damage resulting in large adducts.

To remedy this, a second class of polymerases exists that have much less stringent binding requirements for nucleotide incorporation. These translesion polymerases sacrifice fidelity for greater flexibility in template usage, favoring processivity but leading to mutagenic DNA synthesis that introduces both point and frameshift mutations. Consequently, their disruption gives rise to higher sensitivity to DNA damaging agents (e.g., UV) but lower nuclear DNA mutation rates in surviving cells.

Poly is the only known mitochondrial replicative polymerase and, until recently, the only polymerase with known mitochondrial DNA activity. In yeast, *REV3*, encoding the catalytic subunit of the Pol $\zeta$  translesion polymerase, was previously implicated in mitochondrial DNA maintenance (Smolińska, 1987). Rev1p and Rev7p interact with Rev3p and are required for Rev3p-dependent synthesis opposite damaged templates (Lawrence et al., 2000). Subsequently, the Singh group observed that Rev1p, Rev3p, and Rev7p have N-terminal mitochondrial localization signals and demonstrated the ability of these N-terminal sequences to deliver GFP to mitochondria. They used the *cox3::arg8<sup>m</sup>* variant isolated by T. Fox to analyze Arg<sup>+</sup> reversion as a measure of mutation, and found that single deletion of Rev1p, Rev3p, and Rev7p all result in a decrease in mutation frequency (Zhang et al., 2006). This reporter reverts to Arg<sup>+</sup> via loss of a single base pair, indicating

that Pol $\zeta$  is important in the generation of mitochondrial insertion/deletion (indel) mutations.

Using the respiring (GT<sub>16</sub>+1) and (GT<sub>16</sub>+2) reporters (Fig 2C), (Mookerjee and Sia, 2006), we found that single deletions of *REV1*, *REV3*, and *REV7* all led to decreases in both spontaneous and UV-induced frameshift consistent with previously published work and supporting a role for all three gene products in the generation of mitochondrial indels. However, unlike nuclear DNA, which displays decreases in both frameshifts and point mutations, the *rev3*- $\Delta$  and *rev7*- $\Delta$  strains also showed unexpected increases in spontaneous mitochondrial DNA point mutation rate, and all three deletions gave rise to increases in UV-induced point mutation rates. Sequence analysis of these mutants revealed a UV-dependent shift favoring A to T transversion in the absence of Rev1p, consistent with repair of a thymine dimer by a mechanism biased towards adenine insertion (Kalifa and Sia, 2007).

These observations emphasize the importance of examining the effect of disrupting repair or damage tolerance pathways on multiple types of mutations, as they are often generated and repaired via distinct mechanisms. The employment of multiple mutagenesis reporters allows the proper dissection of these pathways.

## 7. Conclusion

Increasingly, proteins previously considered to be nuclear DNA repair factors are found to also display mitochondrial localization (Section 1.3), suggesting significant overlap between nuclear and mitochondrial DNA repair pathways with respect to their protein components. However, our analysis of the phenotypic consequences of mutating the relevant genes reveals significant differences in the contribution of these proteins to mitochondrial mutagenesis and repair (Sections 5 and 6). These differences likely result from compositional DNA differences, the different packaging and DNA-binding proteins, the different regulatory control of mitochondrial DNA replication and transmission, the exposure to certain kinds of damage, and the availability of other repair proteins, between nuclear and mitochondrial DNA. We should not expect that studies of nuclear DNA repair can simply be extrapolated to generate correct mitochondrial models.

Careful analysis of these pathways within the mitochondrion will require tools like those we have described here. These reporters provide us with the ability to differentiate between point substitutions, frameshift mutations, and deletion events and will be critical to elucidating specific pathways. While *Saccharomyces cerevisiae* is currently the only model system available for these studies, the conservation of DNA repair pathways among eukaryotes supports the hypothesis that some, if not most of these repair proteins will have conserved mitochondrial roles. Finally, understanding mitochondrial DNA repair serves two practical purposes. First, it allows us to understand an important genetic system whose failure is heavily implicated in aging. Second, understanding the mechanisms of repair may facilitate their exploitation for mitochondrial genome manipulation in other systems.

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