Activation of the Pleiotropic Drug Resistance Pathway Can Promote Mitochondrial DNA Retention by Fusion-Defective Mitochondria in Saccharomyces cerevisiae

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ABSTRACT Genetic and microscopic approaches using Saccharomyces cerevisiae have identified many proteins that play a role in mitochondrial dynamics, but it is possible that other proteins and pathways that play a role in mitochondrial division and fusion remain to be discovered. Mutants lacking mitochondrial fusion are characterized by rapid loss of mitochondrial DNA. We took advantage of a petite-negative mutant that is unable to survive mitochondrial DNA loss to select for mutations that allow cells with fusion-deficient mitochondria to maintain the mitochondrial genome on fermentable medium. Next-generation sequencing revealed that all identified suppressor mutations not associated with known mitochondrial division components were localized to PDR1 or PDR3, which encode transcription factors promoting drug resistance. Further studies revealed that at least one, if not all, of these suppressor mutations dominantly increases resistance to known substrates of the pleiotropic drug resistance pathway. Interestingly, hyperactivation of this pathway did not significantly affect mitochondrial shape, suggesting that mitochondrial division was not greatly affected. Our results reveal an intriguing genetic connection between pleiotropic drug resistance and mitochondrial dynamics.

The abundance, shape, and size of mitochondria are determined by the rate of mitochondrial division and fusion. Both of these processes take place with the aid of GTPases that are conserved across the eukaryotic domain. The dynamin family member Dnm1p is key for mitochondrial division in Saccharomyces cerevisiae (Lackner and Nunnari 2009; Kageyama et al. 2011; Chan 2012; Westermann 2013). Dnm1p forms rings and spirals that are recruited to the sites of mitochondrial division by specific adaptor proteins (Ingerman et al. 2005; Naylor et al. 2006). These adaptor proteins differ among organisms but in S. cerevisiae consist of the soluble WD40-domain containing proteins Mdv1p and Caf4p and the membrane receptor Fis1p (Fekkes et al. 2000; Mozdy et al. 2000; Tieu and Nunnari 2000; Tieu et al. 2002; Cerveny and Jensen 2003; Griffin et al. 2005). Much evidence currently supports a model in which cooperative GTP hydrolysis by Dnm1p oligomers at the mitochondrial surface squeezes the mitochondrial tubule, thereby forcing mitochondrial scission (Mears et al. 2011).

Recent studies have illuminated numerous ways in which the process of mitochondrial division can be controlled by the cell. For example, phosphorylation of the human Dnm1p ortholog Drp1 by protein kinase A inhibits mitochondrial division and protects neurons under stress conditions (Merrill et al. 2011). In addition, Drp1 can be SUMOylated (Braschi et al. 2009; Zunino et al. 2009), S-nitrosylated (Cho et al. 2009), or ubiquitylated (Karbowski et al. 2007), all of which may bear upon the mitochondrial fission process. Moreover, association with another organelle system, the endoplasmic reticulum (ER), can determine the location of mitochondrial scission (Friedman et al. 2011). These exciting findings raise the possibility that additional genes and pathways that influence the rate of mitochondrial division might be identified and mechanistically studied using S. cerevisiae.

Like mitochondrial division, mitochondrial fusion is dependent upon dynamin-family GTPases. These fusion-mediating proteins reside...
at both the outer membrane (OM) and the inner membrane (IM) of mitochondria (Hopkins and Nunnari 2009; Chan 2012). In S. cerevisiae, Fzolp allows OM tethering and fusion (Meessen et al. 2004), and Mgm1p is thought to catalyze IM fusion (Meessen et al. 2006). Ugo1p connects the OM and IM fusion machineries (Wong et al. 2003; Sesaki and Jensen 2004). Mutations blocking mitochondrial fusion result in fragmentation of mitochondrial tubules and, for reasons that are not yet understood, mitochondrial DNA (mtDNA) loss (Hermann et al. 1998; Rapaport et al. 1998). However, this mitochondrial fragmentation is dependent upon the mitochondrial division machinery; cells lacking both the ability to fuse mitochondria and the capacity to divide mitochondria are able to maintain both tubular mitochondrial morphology and mtDNA (Bleazard et al. 1999; Sesaki and Jensen 1999). Selection for mutants that maintain mtDNA when mitochondrial fusion is blocked has successfully revealed mitochondrial division machinery components (Fekkes et al. 2000; Mozdy et al. 2000; Tieu and Nunnari 2000). However, selection for mtDNA retention was previously performed using nonfermentable medium, potentially excluding suppressors that block oxidative phosphorylation or otherwise inhibit proliferation under those specific culture conditions.

In this study, we applied a novel selection approach to search for new mutations that would allow cells unable to fuse mitochondria to maintain mtDNA. By sequencing the entire genomes of suppressor-containing isolates, we found that dominant mutations activating the pleiotropic drug resistance (PDR) pathway can allow cells lacking mitochondrial fusion components to keep the mitochondrial genome, providing additional evidence of a functional relationship between the PDR pathway and mitochondrial biogenesis.

MATERIALS AND METHODS

Yeast strains and culture conditions

Yeast media were prepared as described in Adams et al. (1997). Gene disruptions were performed as detailed in Sikorski and Hieter (1989) and Taxis and Knop (2006). Strains were cultured at 30°C except where otherwise indicated. The genotypes of strains used in the course of this study, along with construction details, are provided in Supporting Information, Table S1. Oligonucleotides used during this study are listed in Table S2. Ethidium bromide (Thermo-Fisher Scientific, Waltham, MA) was used at a concentration of 25 μg/mL to destroy mtDNA. Cycloheximide (CHX, Sigma-Aldrich, St. Louis, MO) was used at a concentration of 10 μg/mL for plasmid counterselection on yeast extract peptone dextrose broth (YEPD) medium, 3 μg/mL for plasmid counterselection on yeast extract peptone 3% glycerol+3% ethanol (YEPGE) medium, and at 0.2 μg/mL in YEPD medium to test for activation of the PDR pathway. Ketconazole (Tokyo Chemical Industry Co., Tokyo, Japan), another PDR substrate, was used at a concentration of 2 μg/mL in YEPD. To disrupt the actin cytoskeleton in logarithmic-phase cultures, latrunculin A (Santa Cruz Biotechnology, Dallas, TX) was used at a concentration of 10 μM in SD medium lacking leucine (SD-Leu), following the procedure of Hammermeister et al. (2010). Serial dilution assays were performed as in Garripler et al. (2014).

Plasmid construction and acquisition

To generate pFZO1-CYH2-TRP1 plasmid b19, the FZO1 open reading frame, along with 476 bp of upstream and 508 bp of downstream sequence, was amplified by polymerase chain reaction (PCR) with primers 1 and 2, digested with NolI, and ligated into NolI-linearized pKS1 (Ryan et al. 1998). For construction of pMG1-CYH2-TRP1 plasmid b86, the MGM1 coding region, together with 500 bp of upstream sequence and 108 bp of downstream sequence, was amplified by PCR with primers 42 and 43, digested with Xhol and NotI, and ligated into Xhol/NotI-cut pKS1. To construct plasmids containing PDR1 alleles, the PDR1 open reading frame, along with 556 bp of upstream and 284 bp of downstream sequence, was amplified by PCR with primers 459 and 460. This PCR product was digested using Xhol and NotI then ligated into pRS313 (Sikorski and Hieter 1989) subjected to cleavage with the same enzymes. Plasmids b60, b61, b62, b63, b64, and b65 contained PDR1 alleles amplified from strains CDD71, CDD91, CDD95, CDD98, CDD99, and CDD100, respectively. pHS12, a LEU2-containing plasmid expressing CoxI(1-21)-green fluorescent protein (GFP) (Sesaki and Jensen 1999) was a kind gift of Dr. Hiromi Sesaki, Johns Hopkins School of Medicine. pM390, an ARS/CEN-containing plasmid incorporating the LEU2 gene and expressing an Abf2-GFP fusion protein, was generously provided by Prof. Robert Jensen, Johns Hopkins School of Medicine.

Selection for suppressors of fzo1Δ aac2Δ synthetic lethality (sfa alleles)

Individual colonies of strain CDD71 were patched to YEPD medium and either left untreated (potential sfa isolates 1–22) or irradiated for 2 s at 312 nm with a Herolab UVT-20M, which provided a lethality rate of 74% (potential sfa isolates 23–265). We note that sfa is denoted in lowercase, because the dominance of characterized mutations was not established until after mutation identification. Patches were replica-plated to YEPD medium containing 10 μg/mL CHX then replica plated again to the same medium. A single cycloheximide-resistant (CHX8) clone was isolated from each individual patch. To test for a CHX8 phenotype caused by mutation of the plasmid borne CYH2 gene, isolates were replica-plated to synthetic dextrose medium lacking tryptophan (SD-Trp). Those isolates proliferating on SD-Trp medium were assumed to contain a mutated CYH2 allele within the b19 plasmid and discarded.

The remaining isolates were replica plated to fzo1Δ aac2Δ cyh2 cells of the opposite mating type that were deleted of DNMI (CDD72), MDV1 (CDD73), or FSI1 (CDD74) for the purpose of complementation testing. We note that fzo1Δ aac2Δ cells exhibit a “leaky” phenotype: some fzo1Δ aac2Δ mutant clones keep enough mtDNA to allow very slow proliferation on fermentable medium. Noncomplementation, during our triage of sfa alleles, was defined as the strong suppression of mtDNA loss from fzo1Δ aac2Δ cells at a level typical of mutations affecting known mitochondrial division components. Results from these crosses suggested that we had recovered 64 dnm1 alleles, 14 mdv1 alleles, and 11 fsi1 alleles. Twenty-nine sfa allele-containing isolates either provided inconsistent results among different complementation tests or, alternatively, demonstrated noncomplementation of the suppressor mutation by multiple deletions of division components. The isolation of mitochondrial division-blocking alleles that exhibit unlinked noncomplementation in combination with other division-hampering alleles has been described previously (Tieu and Nunnari 2000). Six other isolates failed to mate but were discarded after detecting the presence of FZO1 by PCR. Two other mutations were clearly and strongly dominant and were not studied further. We initially overlooked the at least partial dominance of the mutations that were later characterized in this study, because the strength of suppression in diploid cells generated during complementation testing appeared weak compared with the suppression conferred by homozygous deletion of known division components.

Twelve remaining fzo1Δ aac2Δ cyh2 sfa isolates were mated to cyh2 strain CDD51 transformed with plasmid b19 and lacking functional mtDNA following two days of culture in the presence of 25 μg/mL ethidium bromide (Goldring et al. 1970). All resulting diploids proliferated on YEPGE medium, suggesting that sfa mutations had allowed maintenance of mtDNA in the absence of FZO1 and, therefore, sfa alleles do not suppress the petite-negative phenotype of aac2Δ
cells. For many sfa alleles, including the PDR1-249 allele most prominently described in this work, absence of FZO1 coding sequence was also confirmed in the initial fzo1Δ aac2Δ sfa isolate by PCR before further characterization.

Next, fzo1Δ/FZO1 aac2Δ/AAC2 cyh2/cyh2 sfa/SFA pFZO1-CYH2-TRP1 strains generated by the aforementioned mating were sporulated, and haploid fzo1Δ aac2Δ cyh2 sfa progeny were tested for survival after loss of plasmid b19 on YEpd medium containing CHX. During our analysis comparing proliferation rates of haploid progeny after loss of FZO1, the suppressive phenotype of four presumptive sfa alleles did not appear to be mediated by a single allele segregating in a Mendelian fashion. Those four alleles were not studied further. However, later identification of one allele of PDR3 as a fzo1Δ aac2Δ suppressor raises the possibility that close linkage of PDR3 to AAC2 (Subik et al. 1986) may have confounded our analysis and that at least some of these four discarded sfa alleles could contain mutations at the PDR3 locus. The other eight alleles exhibited a segregation pattern characteristic of a single mutation existing on a nuclear chromosome.

**Bulk segregant analysis and next-generation sequencing**

To identify seven of the sfa mutations allowing mtDNA retention by cells lacking FZO1, bulk segregant analysis (Michelmore et al. 1991) was followed by next-generation sequencing. From fzo1Δ/FZO1 aac2Δ/AAC2 cyh2/cyh2 sfa/SFA diploids derived from strains CDD91, CDD95, CDD98, CDD99, CDD100, CDD104, and CDD105, suppressor-containing and suppressor-lacking meiotic products were combined into pools. At least three separate isolates were combined in each pool. Similarly, seven pools consisting of isolates lacking suppressors and derived from the same seven diploids were analyzed. For each genomic DNA preparation, equal volumes of saturated cultures of each fzo1Δ aac2Δ isolate either lacking or containing a sfa allele were mixed, and genomic DNA was extracted essentially as in Looke et al. (2011).

Paired-end library preparation was performed at the European Molecular Biology Laboratory (EMBL) Genomics Core Facility (Heidelberg, Germany) using the Illumina protocol for preparation of genomic DNA sequencing libraries. Library fragments averaging 300 bp (standard deviation of 25 bp) were sequenced on the Illumina HiSeq platform to a read length of 100 bp. Sequencing reads have been deposited into the Sequence Read Archive of the National Center for Biotechnology Information under accession number PRJNA229450.

**Bioinformatic analysis of genomic sequence**

Read mapping against the sacCer3 reference genome for S. cerevisiae was performed at EMBL and data resulting from the use of the Illumina ELAND algorithm were converted to BAM format. The sacCer3 reference genome was also used for all subsequent analysis. BAM files were sorted using samtools version 0.1.18 (Li et al. 2009). Next, the mpileup function of samtools was used to compare, for each suppressor mutation: the gDNA pool carrying the sfa allele, the gDNA pool not carrying the sfa allele, and, for subtraction of sequence differences between the S28C and W303 backgrounds, W303 control strain CDD101. VarScan version 2.3.5 (Koboldt et al. 2009) was used to analyze each mpileup output file to locate base pair changes that were private to gDNA pools containing sfa alleles. Minimum supporting reads necessary to identify a variant was set to 10, and minimum frequency for an allele to be considered homozygous was set to 0.90. VarScan output used to identify mutant bases associated with each characterized sfa allele is provided as File S1.

**Fluorescence microscopy**

Microscopy was accomplished using the equipment described in (Garipov and Dunn 2013). Mitotracker Green FM staining (Molecular Probes, Eugene, OR) was performed in culture medium for 20 min at a concentration of 500 nM before examination of mitochondrial morphology.

**Southern blotting**

Total genomic DNA was isolated essentially as in Looke et al. (2011). Ten micrograms of genomic DNA were digested by EcoRV, electrophoresed through a 0.8% agarose gel, then transferred to a nylon membrane and covalently bound to the membrane using a CL-508G UV cross-linker (UVitex Ltd., Cambridge, UK). A probe for quantification of nuclear DNA was synthesized by PCR using primers 537 and 538 and a genomic DNA template, thereby amplifying a 501 bp portion of the TDH1 open reading frame predicated to hybridize to a 3364 bp fragment of EcoRV-cut nuclear DNA. A probe for quantification of mtDNA was generated by PCR using primers 535 and 536 and a genomic DNA template to amplify a 563 bp portion of the COX3 open reading frame that was predicted to hybridize to a 8425 bp fragment of EcoRV-cut mtDNA. PCR probes were labeled with 32P using the Prime-a-Gene Labeling System (Promega, Madison, WI) and radiolabeled products were hybridizid to target sequences in PerfectHyb Plus buffer at 68°C using manufacturer’s instructions (Sigma-Aldrich, St. Louis, MO). A high stringency wash (0.5X saline-sodium citrate (SSC) buffer, 0.1% sodium dodecyl sulfate) was performed after hybridization with COX3 probe. Following phosphorimaging using a Perkin Elmer Cyclone (Waltham, MA), the same membrane was probed for TDH1 and a low stringency wash (2X SSC, 0.1% SDS) was carried out before signal detection.

**RESULTS**

A selection for mutations preventing mtDNA loss from cells lacking mitochondrial fusion machinery

Selection of mutations affecting mitochondrial division has previously depended upon the recovery of respiring cells from nonfermentable medium (Fekkes et al. 2000; Mozdy et al. 2000; Tieu and Nunnari 2000). With the goal of isolating new mitochondrial division machinery or proteins and pathways impinging upon the mitochondrial fission process, we similarly asked that fusion-incompetent cells maintain mtDNA, yet we performed our selection on glucose-containing medium. Our methodology took advantage of the petite-negative phenotype, or the inability of certain mutants to live in the absence of mtDNA, even upon fermentable medium (Chen and Clark-Walker 2000). Specifically, our selection scheme used a nuclear background lacking the major ATP/ADP antiporter of the mitochondrial IM, AAC2. Unlike wild-type S. cerevisiae cells, aac2Δ mutants cannot proliferate in the absence of mtDNA (Kovacova et al. 1968), yet are viable on nonfermentable medium (Kovacs et al. 1967; Beck et al. 1968).

In the background of an aac2Δ mutation, we removed the chromosomal copy of the FZO1 gene, required for OM fusion, yet provided a plasmid encoding both FZO1 and CYH2, allowing counter-selection by a high concentration of CHX in the presence of a chromosomal cyh2 mutation (Sikorski and Boeke 1991) (Figure 1). We then patched individual colonies from the resulting fzo1Δ aac2Δ cyh2 pFZO1-CYH2 strain (CDD71) to YEpd medium. Some patches were subjected to ultraviolet (UV) irradiation to promote the generation of new mutations. Patches were then replica-plated to medium containing 10 μg/mL CHX to select for the absence of the FZO1-containing plasmid and thereby ensure cessation of mitochondrial fusion. Although fzo1Δ aac2Δ cells proliferate very slowly, a single, rapidly growing colony
mutations might be found in the TED complementation tests to determine whether the newly isolated complementation testing suggested that we had isolated 64 mtDNA maintenance in the absence of mitochondrial fusion. Com-only three genes whose mutation has been demonstrated to allow were not further characterized. Some mdv1 tions, 14 mtDNA maintenance were called was isolated from each of more than 200 patches, making certain the independence of any suppressor mutations. Changes allowing mtDNA maintenance were called “sfa” alleles as an abbreviation for “suppressor of fzo1 Δ aac2 Δ synthetic lethality.”

After confirming loss of the FZO1-containing plasmid, we attempted complementation tests to determine whether the newly isolated sfa mutations might be found in the DNM1, MDV1, or FIS1 genes, the only three genes whose mutation has been demonstrated to allow mtDNA maintenance in the absence of mitochondrial fusion. Complementation testing suggested that we had isolated 64 dnm1 mutations, 14 mdv1 mutations, and 11 fis1 mutations, and those alleles were not further characterized. Some sfa alleles exhibited noncomplementation when combined with more than one division component, perhaps due to the reported unlinked noncomplementation of certain mutations affecting mitochondrial division (Tieu and Nunnari 2000) and also were not studied further. Other sfa alleles provided variable results during complementation testing, and these were similarly discarded. In addition, two clearly dominant sfa alleles were obtained, but were discarded with the expectation that they might be mutations in the DNM1 gene, in which such dominant-negative mutations have previously been isolated (Otsuga et al. 1998; Sesaki and Jensen 1999). Later, it became apparent that some sfa alleles originally classified as recessive suppressors were actually dominant in nature, yet provided weaker suppression than mutations of known division components. The dominant nature of several sfa alleles is further described below.

Twelve remaining fzo1 Δ aac2 Δ sfa isolates were crossed to a strain lacking mtDNA and carrying plasmid-borne FZO1. Resulting diploids were respiration competent, as expected for cells maintaining mtDNA, thereby demonstrating that sfa mutations do not suppress the petite-negative phenotype of aac2 Δ. Meiotic products of these diploids that lacked chromosomal FZO1 and AAC2 were tested for the ability to lose the FZO1-carrying plasmid. For eight of 12 isolates tested, Mendelian segregation of a single sfa allele appeared likely.

To identify alterations that promote mtDNA maintenance by cells lacking FZO1, we turned to bulk segregant analysis (Michelmore et al. 1991). From seven of eight fzo1 Δ/ΔAAC2 sfa/SFA diploids, those haploid segregants proliferating very slowly after loss of the FZO1 expressing plasmid were differentiated from those that were clearly suppressed and more rapidly proliferating. Suppressor-containing isolates were pooled, and suppressor-lacking isolates were placed into a separate pool. Next, we performed next-generation sequencing of genomic DNA and computationally identified mutations private only to the sfa allele-containing pools.

**Mutation of transcription factors of the PDR pathway promotes mtDNA maintenance of cells lacking both FZO1 and AAC2**

Surprisingly, all sfa mutations sequenced exhibited single amino acid changes to transcription factors Pdr1 or Pdr3, proteins responsible for activation of the PDR pathway and consequent resistance to a panoply
of proliferation by fzo1 mtDNA (Figure S3), suggesting that some fraction of allele was compared to the suppression mediated by deletion of a gene PDR3

Table 1 Alleles of drugs (Balzi et al. 1994; Katzmann et al. 1994; Prasad and Goffeau 2012). Six missense PDR1 mutations were identified, along with a single PDR3 mutation. Table 1 provides the location of each mutation within either Pdr1p or Pdr3p. Interestingly, two independently isolated L868S mutations within Pdr1p suppress the mtDNA loss of fzo1Δ aac2Δ cells. Therefore, we focused most of our subsequent efforts on the PDR1-249 allele, which carries this specific amino acid change.

Suppression of mtDNA loss from fzo1Δ aac2Δ cells by the PDR1-249 allele was compared to the suppression mediated by deletion of a gene encoding a known mitochondrial division component for S. cerevisiae, FIS1. In spot dilution assays, the PDR1-249 allele permitted a similar level of proliferation by fzo1Δ aac2Δ cells when compared with fzo1Δ aac2Δ fis1Δ cells (Figure 2A). Because counterselection of the FZO1-containing plasmid was based upon CHX treatment, and CHX is a known substrate of the PDR pathway (Saunders and Rank 1982), we verified that our isolation of PDR1 and PDR3 alleles as suppressors of fzo1Δ aac2Δ mtDNA loss was not an artifact of our selection scheme. Indeed, we found that the PDR1-249 allele does not cause resistance to the concentration of CHX used to counterselect for FZO1-containing plasmid, and PCR confirmed absence of FZO1 from fzo1Δ aac2Δ cells isolated following counterselection against the pFZO1-CYH2 plasmid (Figure S1). In addition, when CHX-based counterselection is applied to select for FZO1 loss, subsequent removal of CHX does not affect the further proliferation of fzo1Δ aac2Δ PDR1-249 cells, indicating that any CHX-driven modulation of the PDR pathway (Thakur et al. 2008) is not relevant to the genetic interaction that we have uncovered (Figure S2). Moreover, we were able to easily isolate fzo1Δ aac2Δ PDR1-249 CYH2 colonies that had lost the FZO1-containing plasmid without the use of counterselection agent CHX, and FZO1 loss from these isolates was additionally confirmed by PCR (C. D. Dunn, unpublished data), indicating that neither CHX nor a chromosomal cyh2 allele is required for the process of CHX used to counterselect for the pFZO1-CYH2 plasmid.

To further characterize the effects of PDR1 mutation, we tested the ability of the PDR1-249 allele to suppress mtDNA loss on nonfermentable medium from cells expressing AAC2 and deficient for mitochondrial fusion. fzo1Δ cyh2 pFZO1-CYH2 cells containing or lacking the PDR1-249 allele were allowed to lose the FZO1-encoding plasmid in YEPD medium then subjected to plasmid counterselection on YEPGE medium containing CHX. fzo1Δ cyh2 PDR1-249 cells, upon very extended incubation, did exhibit increased proliferation on YEPGE medium compared to fzo1Δ cyh2 cells (C. D. Dunn, unpublished data). However, analysis by PCR using primers amplifying FZO1 coding sequence (Figure S1) or a test of tryptophan prototrophy relying upon the TRP1 gene on the FZO1-containing plasmid (C. D. Dunn, unpublished data) demonstrated that FZO1 was still present. Therefore, in contrast with fzo1Δ aac2Δ PDR1-249 cells, fzo1Δ PDR1-249 cells tested upon YEPGE medium did not maintain mtDNA.

We performed additional experiments in YEPD medium testing the potential requirement for AAC2 deletion in the ability of fzo1Δ PDR1-249 mutants to maintain mtDNA. We transferred fzo1Δ fis1Δ strain CDD670 and fzo1Δ PDR1-249 strain CDD670, both strains containing plasmid-borne FZO1, expressing AAC2, and harboring a chromosomal cyh2 mutation, to YEPD liquid medium overnight. Overnight culture in YEPD medium allowed some cells to lose the FZO1-containing plasmid during cell division. Subsequently, we plated cells to YEPD medium containing 10 μg/mL CHX, upon which only cells lacking the FZO1-encoding plasmid would be expected to survive. Although 75% of individual fzo1Δ fis1Δ colonies (n = 394) had maintained mtDNA, as determined by replica-plating to solid YEPGE medium, not a single fzo1Δ PDR1-249 colony exhibited proliferation after replica-plating to YEPGE (n = 446), suggesting a very high rate of mtDNA loss. Matting to p strain CDD619, followed by replica-plating of resultant diploids to YEPGE medium confirmed the lack of mtDNA within these fzo1Δ PDR1-249 colonies. Failure of the PDR1-249 allele to allow fzo1Δ mutants expressing AAC2 to keep mtDNA on both fermentable and non-fermentable medium suggests that absence of AAC2 may be an important determinant of whether PDR1 and PDR3 mutations can allow fusion-defective mitochondria to keep mtDNA.

We asked whether the suppression of mtDNA loss by PDR1-249 was unique to cells lacking FZO1 or whether the loss of mtDNA prompted by other mutations blocking mitochondrial fusion similarly could be suppressed by PDR1 mutation in the background of an aac2Δ mutant. MGM1 is required for mitochondrial fusion (Sasaki et al. 2003; Wong et al. 2003), and mtDNA is, as in fzo1Δ cells, lost upon Mgm1p inactivation. We placed the PDR1-249 allele into the context of a mgm1Δ aac2Δ cell carrying a counterselectable plasmid encoding MGM1. Like fzo1Δ aac2Δ cells, mgm1Δ aac2Δ cells proliferate in the presence of the PDR1-249 allele (Figure 2B), demonstrating that the suppression mediated by PDR activation is not specific to the fzo1Δ mutant.

Disruption of the Mitochondrial-Endoplasmic Reticulum-Cortex Anchor Does not Suppress the defective proliferation of petite-negative cells lacking mitochondrial fusion

The Mitochondrial-Endoplasmic Reticulum-Cortex Anchor, or MECA, tethers mitochondria to the plasma membrane of the S. cerevisiae mother cell during cytokinesis (Lackner et al. 2013). Abrogation of the MECA leads to several phenotypes associated with a mitochondrial division defect, including a highly networked mitochondrial morphology (Cerveny et al. 2007; Hammermeister et al. 2010). The role of MECA in mitochondrial division is thought to be indirect (Lackner et al. 2013).
et al. 2013), resting upon its ability to tether mitochondria to the plasma membrane and to thereby provide tension to the mitochondrial tubule (Klecker et al. 2013). Deletion of MEGA component Num1p does not permit mtDNA maintenance by fzo1Δ cells on YEPGE (Cerveny et al. 2007). However, we considered that our selection scheme on fermentable medium might be a more sensitive scheme for uncovering mutants playing a role in mitochondrial division. Therefore, we also tested on YEPD medium whether deletion of the MEGA subunits Mdm36p or Num1p would allow proliferation of fzo1Δ aac2Δ cells. However, proliferation of neither fzo1Δ aac2Δ mdm36Δ nor fzo1Δ aac2Δ num1Δ mutants could surpass that of fzo1Δ aac2Δ cells (Figure 2C), suggesting that our selection scheme might not have isolated mutations only providing a partial loss of mitochondrial fission.

Suppression of mtDNA loss from cells lacking FZO1 and AAC2 is associated with dominant activation of the PDR pathway

Because the L868S mutation of Pdr1p was independently isolated twice during our initial selection, we inferred that our suppressor alleles may be dominant; if suppressor mutations in the nonessential PDR1 gene were recessive, loss-of-function alleles, it would be extremely unlikely to independently isolate the same amino acid change twice. To test whether PDR1-249 could dominantly suppress mtDNA loss from cells lacking FZO1 and AAC2, we generated diploid cells homozygous for fzo1Δ and aac2Δ mutations and either heterozygous for the PDR1-249 allele or homozygous for the WT PDR1 allele. Indeed, the presence of a single PDR1-249 allele allowed maintenance of mtDNA in fzo1Δfzo1Δ aac2Δ/aac2Δ cells (Figure 3A). Suggesting that PDR1-249 dominance does not result from haploinsufficiency, we found that deletion of PDR1 does not suppress mtDNA loss from fzo1Δ aac2Δ cells (Figure 3B). Furthermore, a plasmid carrying the PDR1-249 allele was able to dominantly suppress the loss of mtDNA from cells lacking both FZO1 and AAC2 (Figure 3C), and other isolated PDR1 alleles similarly provide dominant suppression of the fzo1Δ aac2Δ synthetic fitness defect (Figure 3D).

To further investigate whether PDR1-249 is a gain-of-function allele activating the PDR pathway, we plated PDR1-249 cells and isogenic WT cells to YEPD medium containing CHX (Saunders and Rank 1982) or ketoconazole, resistance to which is stimulated by PDR pathway activation (Saunders and Rank 1982; Carvajal et al. 1997). Note that the concentration of CHX used in this experiment assaying PDR activation is 20-fold lower than the CHX concentration used for plasmid counterselection purposes. Indeed, PDR1-249 cells were resistant to both drugs (Figure 3D), signaling activation of the PDR pathway. Cells lacking the mitochondrial division component Fis1p, however, were not resistant to these agents, and even exhibited increased sensitivity to CHX, demonstrating that not all mutations permitting mtDNA maintenance by fzo1Δ aac2Δ cells lead to PDR activation.

One previously isolated allele of PDR1 that stimulated drug resistance, PDR1-2, was reported to provide a proliferation defect on non-fermentable medium (Balzi et al. 1987). However, unlike the
PDR1-2 allele, the PDR1-249 mutation in an otherwise WT background causes no significant defect in cell proliferation on YEPD or YEPGE at any temperature (Figure S5).

Mitochondrial morphology is minimally affected by PDR activation

Because genetic evidence suggested that PDR activation might inhibit mitochondrial division, we examined the mitochondrial morphology of PDR1-249 cells by fluorescence microscopy after transformation with GFP targeted to mitochondria by the Cox4 presequence (Sesaki and Jensen 1999), but initial observation indicated that there was minimal, if any difference in mitochondrial morphology between WT and PDR1-249 cells. Disruption of the actin cytoskeleton causes fragmentation of all but the most highly networked mitochondrial tubules, better revealing fenestrated mitochondria that might result from a decrease in the rate of mitochondrial division (Bleazard et al. 1999). However, use of latrunculin A to disrupt the actin cytoskeleton and mitochondrial structure (Jensen et al. 2000; Hammermeister et al. 2010) did not reveal an apparent increase in mitochondrial fragmentation when comparing WT cells to PDR1-249 cells, while fis1Δ networks were readily seen both before and after treatment with latrunculin A (Figure 4A). Quantification of cells containing mitochondrial networks after the addition of latrunculin A also demonstrated that PDR activation does not increase the interconnectedness of mitochondria (Figure S6). Examination of PDR1-249 cells cultured in nonfermentable YEPGE medium by MitoTracker Green FM staining also did not uncover any mitochondrial morphology defect (Figure 4B).

We next examined the morphology of fis1Δ cells carrying the PDR1-249 allele to examine the effect of PDR activation when mitochondrial fusion is blocked. For cells expressing AAC2, we compared mutants lacking mtDNA so that fis1Δ cells with a wild-type PDR1 allele might be used as a control. However, this approach failed to uncover a difference between the mitochondrial morphology of fis1Δ ΔAAC2 and fis1Δ ΔAAC2 ΔPDR1-249 cells (Figure S7), although some fis1Δ ΔAAC2 ΔPDR1-249 cells did exhibit networked mitochondria. We also characterized the mitochondrial shape of fis1Δ ΔAAC2 ΔAAC2 ΔPDR1-249 ΔPDR1-249 cells and found that mitochondria were typically clustered at a single location and were not tubular. This mitochondrial morphology differed from that of fis1Δ ΔAAC2 ΔAAC2 ΔPDR1-249 ΔPDR1-249 cells, which exhibited a more elongated mitochondrial shape.

Altered mitochondrial genome distribution, increased mtDNA abundance, or overexpression of a mitochondrial protein import receptor is unlikely to allow maintenance of mtDNA upon a block to mitochondrial fusion

Potentially, an increase in the transport of mtDNA to the bud of dividing cells with an activated PDR pathway might promote mtDNA retention when mitochondrial fusion is perturbed. To visualize mtDNA in cells with a hyperactive PDR pathway, we expressed a fusion protein consisting of the nucleoid protein Abf2 linked to GFP (Okamoto et al. 1998). Upon examination of nucleoids in live WT and PDR1-249 cells using Abf2p-GFP, no obvious change in the distribution of nucleoids between mother and bud was apparent (Figure 5A), and similar results were obtained by DAPI staining of nucleoids in live cells (C. D. Dunn, unpublished data). Nucleoids did appear to be less well defined in PDR1-249 cells, with reduced delination between mitochondria (Figure S6).
mtDNA puncta; however, the etiology of any minor effect on inter-nucleoid distance induced by PDR activation remains to be determined. In any case, because mtDNA nucleoids did not seem to be increased in the bud of PDR1-249 cells compared with WT cells, more rapid transmission of mtDNA to the growing bud at the cost of maternal mtDNA retention is unlikely to be the mechanism by which fzo1Δ cells maintain mtDNA when PDR is activated.

Alternatively, a drastic increase in mtDNA abundance caused by PDR activation might counterbalance an increased rate of mtDNA loss from mitochondria unable to fuse. We examined mtDNA levels in PDR1-249 cells and in fzo1Δ aac2Δ PDR1-249 cells, but we found no increase in mtDNA compared to fzo1Δ aac2Δ fis1Δ cells, in which mtDNA is instead maintained due to a blockade of mitochondrial fission, or to fis1Δ cells (Figure 5B). Therefore, mtDNA amplification is also unlikely to be the mechanism by which PDR activation protects cells with defective mitochondrial fusion from mtDNA loss. Interestingly, we found a potential rearrangement of mtDNA in fzo1Δ aac2Δ PDR1-249 and fzo1Δ aac2Δ fis1Δ cells, as reported by a change in the mobility of an mtDNA restriction fragment containing COX3. In this regard, it is noteworthy that mtDNA instability is a well-known consequence of mutating the ANT1 ATP/ADP antiporter in mammals (Esposito et al. 1999; Kaukonen et al. 2000). In any case, we have confirmed that the suppressive effect of PDR activation on mtDNA loss from mitochondrial fusion mutants is not dependent upon any specific mtDNA structure found in the strain used for our initial selection; we regenerated a fzo1Δ aac2Δ PDR1-249 strain whose parental mtDNA is inherited from a wild-type strain of the same background (BMA64-1A) and then recapitulated our results demonstrating that PDR activation suppresses mtDNA disappearance from aac2Δ cells after loss of FZO1 (C. D. Dunn, unpublished data).

DISCUSSION
We performed a genetic selection, taking advantage of the petite-negative phenotype, the goal of which was to identify new genes and pathways involved in mitochondrial dynamics. After next-generation sequencing of suppressor-containing genomes, we found that activation
degeneration sequencing of segregant pools greatly facilitated the rapid Num1p or Mdm36p (Dimmer mitochondrial division, such as removal of MECA complex members changes to mitochondrial morphology by indirectly reducing mito-
diferentiation and, together, these mutations lead to a block to mitochondrial function. Supporting this possibility, removal of AAC2 affects mitochondrial morphology (Altmann and Westermann 2005), potentially signaling an uncharacterized role for the Aac2 protein in mitochondrial dynamics that is worthy of future study.

Pursuing other possible ways in which PDR activation might allow mtDNA to be maintained by mitochondrial fusion mutants, we also investigated whether PDR activation greatly altered mtDNA distribution or abundance. However, nucleoid distribution between mother and bud appeared normal in PDR1-249 mutants, and mtDNA levels were not elevated when compared to cells lacking Fis1p, a protein required for mitochondrial fission, suggesting that amplification of mtDNA or more rapid transfer of mtDNA into the bud does not promote mtDNA retention by the fzo1Δ mutant. Moreover, overexpression of a PDR-activated protein import receptor, Tom71p, seems irrelevant to the apparent effects of PDR activation on mitochondrial dynamics. It is possible that increased sphingolipid biosynthesis could be relevant to suppression of mtDNA loss from fzo1Δ mutants by PDR activation, as several enzymes playing a role in sphingolipid biosynthesis are up-regulated by the PDR pathway (Hallstrom 2001; Ferrari et al. 2004). We sought to perturb sphingolipid biosynthesis by deleting SUR4, which plays a role in synthesis of the major sphingolipid forms of S. cerevisiae (Oh et al. 1997; Dickson 2008). However, we found that sur4Δ mutants proliferated poorly following germination of haploid spores from a heterozygous diploid (Figure S9), and fzo1Δ aac2Δ sur4Δ mutants expressing plasmidborne FZO1 also exhibited severe proliferation defects on YEPO (C. D. Dunn, unpublished data), confounding immediate analysis of any role for up-regulated sphingolipid biosynthesis downstream of PDR activation.

It has been suggested that screens and selections for new components playing a direct role in mitochondrial division might be saturated (Lackner and Nunnari 2009). Have, in fact, all of the proteins directly taking part in the mitochondrial division reaction in S. cerevisiae been identified? Before initiating this study, we noted that numerous proteins involved in membrane curvature and endocytosis

![Figure 5](https://academic.oup.com/g3journal/article/4/7/1247/6025936)
at the plasma membrane, such as the BAR domain containing proteins Yvs161 and Yvs167, are required for proliferation on non-fermentable media in at least one yeast background (Ren et al. 2006), suggesting potential functionality at mitochondria. Our selection design would likely have discovered any substantial role for such endocytosis regulators in mitochondrial scission, yet no mutations in genes encoding endocytosis components were identified.

We had also hoped that our selection might uncover machinery used to divide the mitochondrial IM, as no proteins have yet been identified that demonstrably play a direct role in the mitochondrial division reaction at this location (Westermann 2010). In fact, exciting new data indicate that some proteins can participate in both oxidative phosphorylation and mitochondrial protein import (Gebert et al. 2011), permitting speculation that other IM proteins might also play a dual role, functioning in both mitochondrial ATP generation and mitochondrial division. Our selection scheme did not require that mutations affecting mitochondrial division respire, and so we could have conceivably identified such dual-use proteins. However, we did not isolate mutations in any gene playing a direct role in oxidative phosphorylation. Perhaps a partially functional, yet still petite-negative allele of AAC2 would have been more suitable for this purpose, since complete AAC2 deletion is synthetically lethal with an absolute block to either the electron transport chain or to the ATP synthase complex (Smith and Thorsness 2008). Specific allele selection may be particularly important in this context: as described above, the full deletion of AAC2 may have facilitated recovery of mutations impinging upon mtDNA loss from fusion defective mitochondria.

To summarize, our selection scheme uncovered regulatory proteins that genetically interface with mitochondrial dynamics in an intriguing way. Further studies should elucidate the mechanism by which PDR pathway transcriptional targets allows mtDNA maintenance when mitochondrial fusion is lacking. We note that, although rapid mtDNA loss from a mitochondrial fusion mutant seems related to generation of small, fragmented mitochondria, it is not clear why mtDNA is lost from these fragments. PDR activation may prevent mtDNA loss by addressing the most proximal cause of mtDNA loss from fusion-deficient mitochondria rather than impinging in any way upon mitochondrial dynamics. Finally, our work suggests that alternative methodologies not taking advantage of direct selection are likely to be required for identification of new S. cerevisiae proteins that play a direct role in mitochondrial division.

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