

Fis1 deficiency selects for compensatory mutations responsible for cell death and growth control defects

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Genetic mutations affecting mitochondrial fission and fusion proteins cause human neurological disorders, but are assumed to be well tolerated in yeast. The conserved mitochondrial fission protein Dnm1/Drp1 is required for normal mitochondrial division, but also promotes cell death in mammals and yeast. Fis1, an outer mitochondrial membrane-anchored receptor for Dnm1/Drp1, also can promote cell death in mammals, but appears to have prosurvival activity in yeast. Here we report that deletion of the *FIS1* gene in yeast consistently results in acquisition of a secondary mutation that confers sensitivity to cell death. In several independently derived *FIS1* knockouts, tiling arrays and genomic sequencing identified the secondary mutation as a premature termination in the same stress-response gene, *WHI2*. The *WHI2* mutation rescues the mitochondrial respiratory defect (petite formation) caused by *FIS1* deficiency, but also causes a failure to suppress cell growth during amino-acid deprivation. Thus, loss of Fis1 drives the selection for specific compensatory mutations that confer defective growth control and cell death regulation, characteristic of human tumor cells. The important long-term survival function of Fis1 that is compensated by *WHI2* mutation appears to be independent of fission factor Dnm1/Drp1 and its adaptor Mdv1, but may be mediated through a second adaptor Caf4, as *WHI2* is also mutated in a *CAF4* knockout.

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Mitochondria are dynamic organelles that continuously undergo fission and fusion for organelle maintenance.¹ Mitochondrial fission and fusion events are mediated by distinct molecular complexes that are characterized best in the budding yeast *Saccharomyces cerevisiae*.² Deletion or depletion of the small, membrane-anchored fission protein Fis1 from yeast or mammalian cells results in a more connected mitochondrial network. Yeast Fis1 forms a complex with dynamin-related 1 (Dnm1), a dynamin-like GTPase conserved in animals (Drp1/DLP1) and plants (ADL2b) through either of two WD40-repeat adaptor proteins Mdv1 or its paralog Caf4.^{2–4} Mammalian Drp1 facilitates normal synaptic activity in neurons, and a mutation in human Drp1 is linked to severe neurological deficits.^{5–7} In addition to its critical role in healthy neurons, mammalian Drp1 also interacts with Bcl-2 family proteins and promotes the rapid mitochondrial fragmentation that is characteristic of apoptotic cells.^{6,8,9} Furthermore, Drp1/Dnm1 was shown to actively promote cell death in yeast, worms and flies, and a small molecule inhibitor of Drp1/Dnm1 inhibits cytochrome *c* release and delays mammalian cell death.^{10–13} However, the pro-death function of Drp1/Dnm1 may be separable from its fission function.

Consistent with the model that mitochondrial fission factors have an evolutionarily conserved pro-death function,

mammalian Fis1 has been reported to promote mammalian cell apoptosis based on depletion of Fis1 by RNAi^{14,15} and overexpression of Fis1 by transfection.^{16–19} Thus, it was initially unexpected that deletion of *FIS1* in yeast increases their sensitivity to multiple death stimuli, including acetic acid, H₂O₂, heat shock, ethanol and viruses.^{10,20,21} However, a protective function for Fis1 is supported by overexpression studies where yeast and human Fis1 rescue yeast *FIS1*-knockout strains from cell death, though Fis1 does not improve survival beyond that of wild-type.¹⁰ Furthermore, depletion of human Fis1 results in mammalian cell senescence.^{22,37}

Here we report that yeast Fis1 has a long-term survival function, as *de novo* deletion of the *FIS1* gene in *S. cerevisiae* selects for chromosome abnormalities and mitochondrial deficits, but does not immediately result in sensitivity to cell death. Rather, the acute sensitivity to cell death is due to secondary mutations in whiskey 2 (*WHI2*), a stress-response gene,²³ that specifically arose in *FIS1* knockouts but not in the mitochondrial fission-defective knockouts of *DNM1* or *MDV1*. This process in yeast may share similarities to human tumorigenesis, where one mutation selects for additional mutations that confer loss of growth control despite the accompanying sensitivity to cell death induced by toxic chemicals such as anticancer agents.

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Abbreviations: *CAF4*, CCR4 associated factor; CSH, Cold Spring Harbor Laboratory Press; G418^R, G418 resistant; ME, *Methods in Enzymology*; SCD, synthetic complete dextrose medium; *WHI2*, whiskey 2; YKO, yeast knockout

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Results

FIS1-knockout strains fail to suppress growth during amino-acid deprivation. Yeast deletion mutants lacking either *FIS1* (*fis1Δ*), *MDV1* (*mdv1Δ*) or *DNM1* (*dnm1Δ*) all have netted mitochondrial morphology and, as a consequence, a mild mitochondrial segregation defect during sporulation, but lack obvious growth defects.²⁴ However, *FIS1* knockouts are uniquely sensitive to a variety of cell death stimuli, unlike *DNM1* and *MDV1* knockouts that are death resistant.^{10,20} The haploid *fis1Δ* strains in the yeast knockout (YKO) collection,²⁵ designated here as *fis1Δd₁* and *fis1Δd₂* (Supplemental Table S1), were compared to their wild-type background strains WT₁ (BY4741) and WT₂ (BY4742), respectively. As previously reported,¹⁰ both of these haploid *FIS1*-knockout strains are sensitive to cell death induced by acetic acid, a mimic of overgrowth conditions (Figure 1a). This cell death phenotype was not caused by a general defect in cell health, because these strains grow normally on rich medium (yeast extract, peptone, dextrose, YPD) and on synthetic complete medium (SCD_{CSH}, Cold Spring Harbor²⁶).

Serendipitously, we found that *FIS1*-knockout strains grow robustly compared to controls if the synthetic complete medium was prepared by a different recipe (SCD_{ME}, *Methods in Enzymology*²⁷) (Figure 1b and c). Thus, the reduced growth of wild-type strains on SCD_{ME} medium is the default response to a signal that is apparently ignored by the *FIS1*-knockout strains, as *fis1Δd₁* and *fis1Δd₂* continue to grow robustly. Growth of *DNM1* knockouts was similar to wild-type, suggesting that defective mitochondrial division is not the cause of the overgrowth phenotype. To identify which media components reveal the overgrowth control defect of *FIS1* knockouts, two additional media were prepared to evaluate all media component differences. Reducing the amino-acid content of SCD_{CSH} medium to match that of SCD_{ME}

recapitulated the large growth differences between wild-type and *FIS1*-knockout strains (Figure 1d, right panels). In contrast, adjusting the four non-amino-acid ingredients, PIUA (PABA (*para*-aminobenzoic acid used in folate/vitamin B₉ biosynthesis), inositol (incorporated into phospholipids), uracil and adenine), in the SCD_{CSH} medium to the levels found in SCD_{ME} had no effect (Figure 1d, left panels). Therefore, we conclude that the wild-type control strains sense the reduced amino-acid supply in SCD_{ME} and deliberately repress their growth, whereas the *FIS1*-knockout strains *fis1Δd₁* and *fis1Δd₂* have a growth control defect allowing them to ignore these low-nutrient signals.

FIS1-knockout strains carry a previously unknown second mutation. To definitively determine whether *FIS1* deficiency is responsible for the cell death and growth control defects, genetic linkage between these phenotypes and the *FIS1* gene locus was assessed by tetrad analysis.²⁷ *FIS1*-knockout strains *fis1Δd₁* and *fis1Δd₂* were mated to wild-type strains. The resulting heterozygous diploids were sporulated and the four haploid spores (a–d) in tetrads produced by each mating pair were analyzed for the segregation of genetic markers. As expected, *FIS1* gene deletion (Δ , G418^R) co-segregated with netted (N) mitochondrial morphology (Figure 2a and b). If *FIS1* deletion is directly responsible for the cell death and growth control defects, these phenotypes will co-segregate with *FIS1*-deletion (Δ , G418^R). However, this was not the case. Instead, *FIS1* deletion segregated independently, indicating that *FIS1* deletion is not directly responsible for these phenotypes (Figure 2a). However, in all tetrads analyzed, the cell death and growth control phenotypes perfectly co-segregated with each other (2:2 segregation), suggesting that *FIS1*-knockout strains *fis1Δd₁* and *fis1Δd₂* each have a second previously unknown gene mutation,

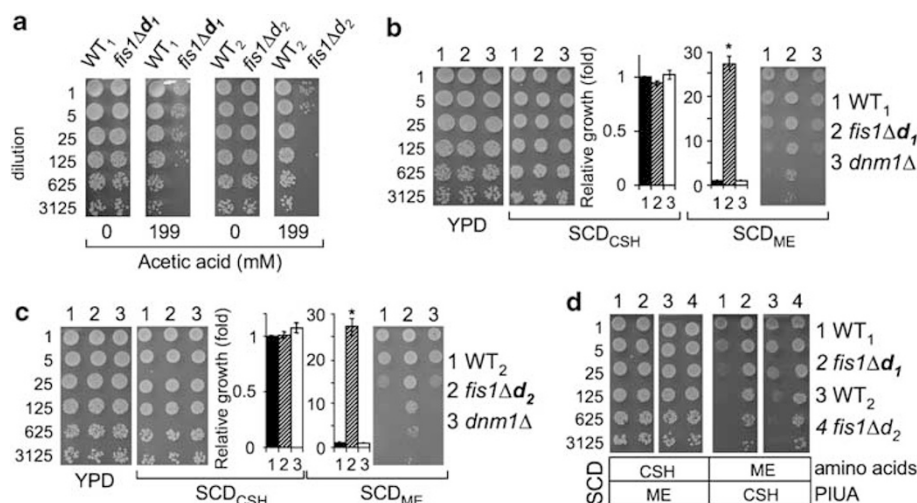


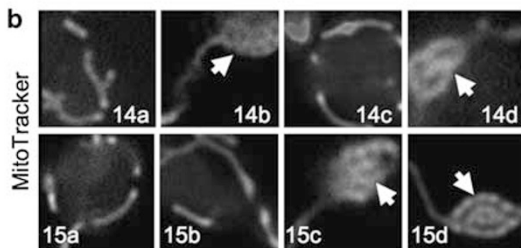
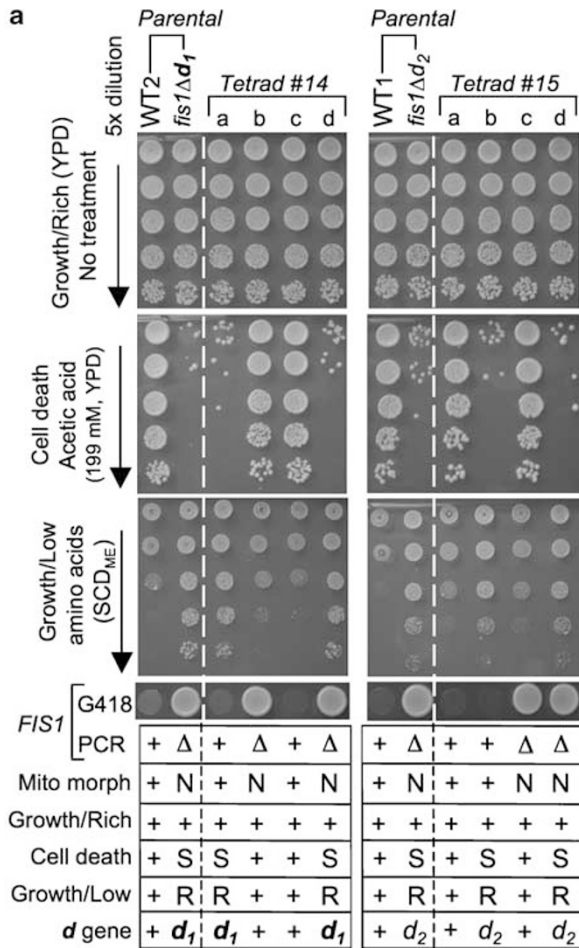
Figure 1 Fission 1 (*FIS1*) knockouts exhibit cell death sensitivity and growth control defects. (a) Growth of the indicated haploid yeast strains plated at the indicated dilutions on rich medium (YPD) with or without pretreatment with acetic acid. (b, c) Growth properties of two independent *FIS1*-knockout strains are compared to wild-type and a *DNM1* knockout on the three indicated media. Quantified results are from 4 to 6 independent experiments presented as mean \pm S.E.M. Growth of *fis1Δ* strains is \sim 27-fold over WT, $*P < 10^{-5}$ for *fis1Δd₁*; $*P < 0.0005$ for *fis1Δd₂*. (d) Low amino-acid levels in SCD_{ME} explain reduced growth by wild-type strains (see text). Representative images from four independent experiments are shown

designated *d* (death), that is responsible for both the cell death and growth control defects (Figure 2c).

***d*₁ and *d*₂ are recessive mutations in the same gene.** To determine if the secondary mutations *d*₁ and *d*₂ in the two *FIS1*-knockout strains are in the same or different genes, a complementation test was performed. However, this genetic

test can only be applied if the mutations are recessive. To determine if *d*₁ and *d*₂ are recessive or dominant, we used *FIS1*⁺ strains containing only *d*₁ or *d*₂ (e.g. spores 14a and 15b, Figure 2a). These *d*₁ and *d*₂ haploid strains were mated to wild-type strains to create heterozygous diploids and tested for cell death sensitivity. The *d* mutations were complemented by the wild-type gene, as the heterozygous diploids were indistinguishable from the control diploid (WT × WT) when treated with acetic acid, indicating that the *d* mutations are recessive (Figure 3a, middle). Therefore, to determine if the *d* mutations are in the same complementation group, single mutants for *d*₁ and *d*₂ were mated together to generate double *d*₁ × *d*₂ mutants. Because *d*₁ (*d*₂) failed to rescue the cell death phenotype of *d*₂ (*d*₁), based on similar cell death sensitivities for both heterozygous (*d*₁ × *d*₂) and homozygous diploids (*d*₁ × *d*₁ and *d*₂ × *d*₂), *d*₁ and *d*₂ are in the same cell death complementation group (Figure 3b, middle). Although *d*₁ × *d*₂ also failed to rescue the overgrowth phenotype on low amino-acid medium SCD_{ME} (Figure 3b, lower), both heterozygous diploids *WT* × *d*₁ and *WT* × *d*₂ had an intermediate phenotype indicating haploinsufficiency, compromising interpretation of the complementation test for this phenotype (Figure 3a). Nevertheless, because the cell death phenotype genetically co-segregates with the overgrowth phenotype, we conclude that *d*₁ and *d*₂ mutations are likely to be in the same gene.

Secondary mutations in *WHI2* explain death sensitivity and growth control defects. To identify the gene responsible for both the cell death and growth control defects, a resequencing technique employing the tiling microarray was used to detect genome-wide sequence polymorphisms at single-nucleotide resolution.^{28,29} The tiling microarray predicted a mutation in the coding sequence of *WHI2*, a stress-response gene,²³ in both *d*₁ and *d*₂ strains (*FIS1*⁺). Direct sequencing of genomic DNA (~1600 nt) surrounding the polymorphism confirmed the



c Summary of Tetrad analysis

Linkage	WT × <i>fis1</i> Δ <i>d</i> ₁	WT × <i>fis1</i> Δ <i>d</i> ₂
Cell death vs. Growth on low amino acids (SCD _{ME})	100%	100%
Cell death vs. <i>fis1</i> Δ	4% (<i>p</i> >0.25)	10% (<i>p</i> >0.5)

Figure 2 Tetrad analysis reveals a second mutation responsible for cell death and growth control defects. (a) Haploid parental strains and the four spores/strains of a representative tetrad derived from their cross was analyzed for growth on rich medium (YPD, top), tubular (+/normal) or netted (N) mitochondrial morphology, sensitivity (S) to acetic acid-induced cell death, resistance (R) to growth inhibition due to low amino acids (synthetic complete dextrose medium, SCD_{ME}, *Methods in Enzymology*), and G418 resistance (indicating *FIS1* gene replacement). Data are summarized for three or more independent experiments per condition in the table below; + indicates wild-type phenotype. (b) Mitochondrial morphology of each spore in tetrads no. 14 and no. 15 from (a) were visualized by MitoTracker staining.¹⁰ Arrows mark netted mitochondrial morphology. (c) Summary of genetic linkage determined by tetrad analyses as illustrated in (a). *FIS1* deletion is unlinked to the cell death *d* gene, as the ratios of PD (parental ditype) : NP (nonparental ditype) : TT (tetratype) within tetrads were 1 : 6 : 17 for *fis1*Δ*d*₁ (24 tetrads for cell death and/or overgrowth on SCD_{ME}) and 2 : 3 : 15 for *fis1*Δ*d*₂ (20 tetrads), which are close to random segregation (1 : 1 : 4, or 17% for PD (linked) versus 83% for NP + TT (unlinked)). In contrast, cell death sensitivity was 100% linked to resistance to growth inhibition on low amino acids (SCD_{ME}), with ratios of 8 : 0 : 0 (*fis1*Δ*d*₁) and 20 : 0 : 0 (*fis1*Δ*d*₂). χ^2 goodness-of-fit test was used to determine whether *FIS1*-deletion (*fis1*Δ) segregated independently from mutation *d* (death) at the expected ratio of 1 : 1 : 4 for PD : NP : T. As none of the *p* values rejected the null hypothesis, *FIS1*-deletion segregated independently from mutation *d*

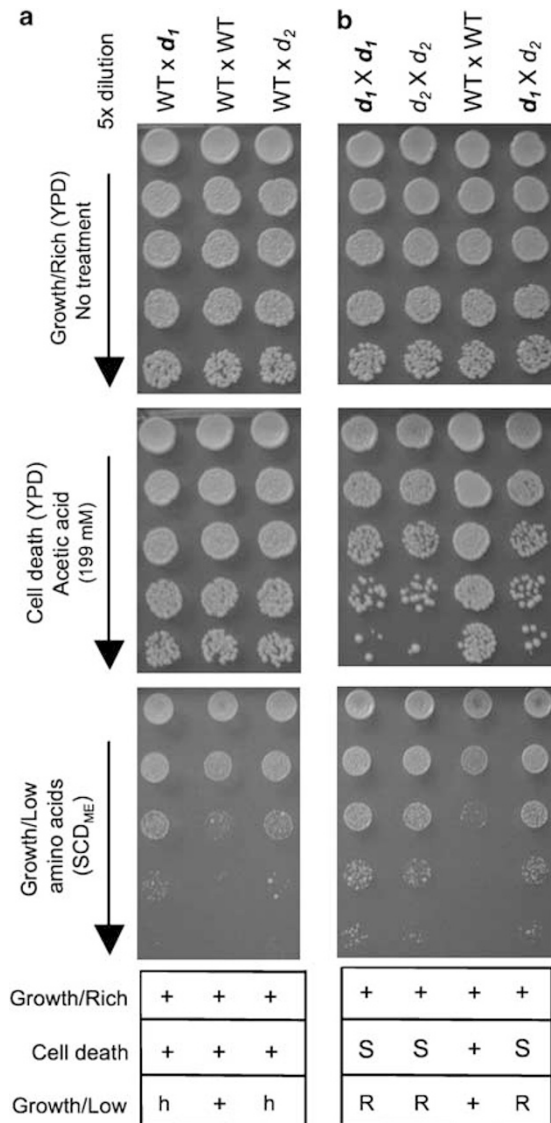


Figure 3 Mutations d_1 and d_2 are in the same complementation group. (a) d_1 is a recessive mutation. Diploid strains heterozygous for d_1 ($WT \times d_1$) or d_2 ($WT \times d_2$) and a wild-type control diploid ($WT \times WT$) were assayed for death sensitivity and the synthetic complete dextrose medium (SCD_{ME} , *Methods in Enzymology*) overgrowth phenotypes. (b) Complementation test. Growth of the indicated homozygous and heterozygous diploids generated from the indicated crosses was treated as in (a). Data for four independent experiments for each assay are summarized below representative images; + (WT-like), h (haploinsufficiency), S (sensitive), R (resistant)

predicted single nucleotide changes on chromosome 15 (G to T at 411326 (d_1), T to G at 411075 (d_2)) that introduce premature termination codons, implying loss-of-function (Figure 4a). The same mutations were confirmed in three d_1 and two d_2 mutant strains (including spores 14a, 14d, 15b and 15d, Figure 2a), and in their respective parental strains ($fis1\Delta d_1$ and $fis1\Delta d_2$) (Figure 4b and data not shown). Importantly, *WHI2* mutations were not found in WT_1 , WT_2 or four $fis1\Delta$ -only spores with no cell death or growth control phenotypes (14b, 14c, 15a and 15c, Figure 2a). Furthermore, the homozygous *FIS1* knockout in the YKO collection

(derived by mating the haploid knockouts) was heterozygous for both point mutations, indicating that these secondary mutations arose independently before mating (Supplemental Figure S1).

Acquisition of *WHI2* mutations in both *FIS1*-knockout strains raised the concern that this phenomenon could be restricted to the YKO collection. To address this point, we acquired a third independently constructed *FIS1* knockout on the Winston background ($fis1\Delta d_3$, RJ1365) in which the *FIS1* ORF had been replaced with the *HIS3* selection marker.³⁰ This $fis1\Delta d_3$ strain also has both the cell death¹⁰ and growth control phenotypes, and tetrad analysis verified that both phenotypes are caused by a secondary mutation, designated d_3 (Figure 4c; W-C Cheng and JM Hardwick, unpublished data). Furthermore, sequence analysis of d_3 revealed a premature stop codon after residue 27 in *WHI2* that is distinct from d_1 and d_2 (Figure 4a and b). Remarkably, three independently derived *FIS1*-knockout strains all acquired a loss-of-function mutation in the same gene, *WHI2*.

To further verify that loss-of-function mutations in *WHI2* are responsible for the observed death sensitivity and growth control defects of *FIS1* knockouts ($fis1\Delta d_{1-3}$), we reconstituted *WHI2* into strains carrying only mutation d_1 , d_2 or d_3 (*FIS1*+). As expected, expression of *WHI2* by its endogenous promoter on a low copy plasmid rescued both phenotypes, thereby restoring cell survival following a death stimulus and limiting cell growth during amino-acid deprivation (Figure 4c). Exogenous *WHI2* also rescued both phenotypes in the parental strain $fis1\Delta d_1$ as expected (Supplemental Figure S2).

***FIS1* deficiency decreases respiratory competency independently of fission.**

To gain insight into the critical function of *FIS1* that drives selection for a secondary mutation, we sequenced the *WHI2* gene in the *DNM1* and *MDV1* knockouts from the YKO collection, which have netted mitochondrial morphology due to defective fission similar to *FIS1* knockouts.¹ No *WHI2* mutations were present, consistent with their cell death (resistance) and normal growth control phenotypes (Figures 1b, c and 5a and data not shown). However, a screen of the yeast knockout collection for strains that exhibit overgrowth phenotypes in low amino acids identified *CAF4*, which encodes an Mdv1-like protein that also binds Fis1, though the knockout has nearly normal mitochondrial morphology.³¹ Direct sequencing revealed a frameshift (T deletion) following amino acid 75 of *Whi2*, resulting in a termination codon 10 residues later (Figure 4b, right). These findings suggest that a specific function of the Fis1 protein that is independent of Dnm1 and Mdv1, but potentially dependent on Caf4, is compensated by loss of *Whi2* function.

Loss or mutation of mitochondrial DNA abolishes oxidative phosphorylation in yeast causing a petite (slow growth) phenotype on rich medium containing glucose (YPD) and a failure to grow on glucose-free medium containing glycerol as the carbon source (yeast extract, peptone, glycerol, YPG), where mitochondrial respiration is important for survival. Therefore, mitochondrial respiratory deficiency was assessed by replica plating strains derived from sets of tetrads (similar to those in Figure 2) from YPD onto YPG plates.²⁴

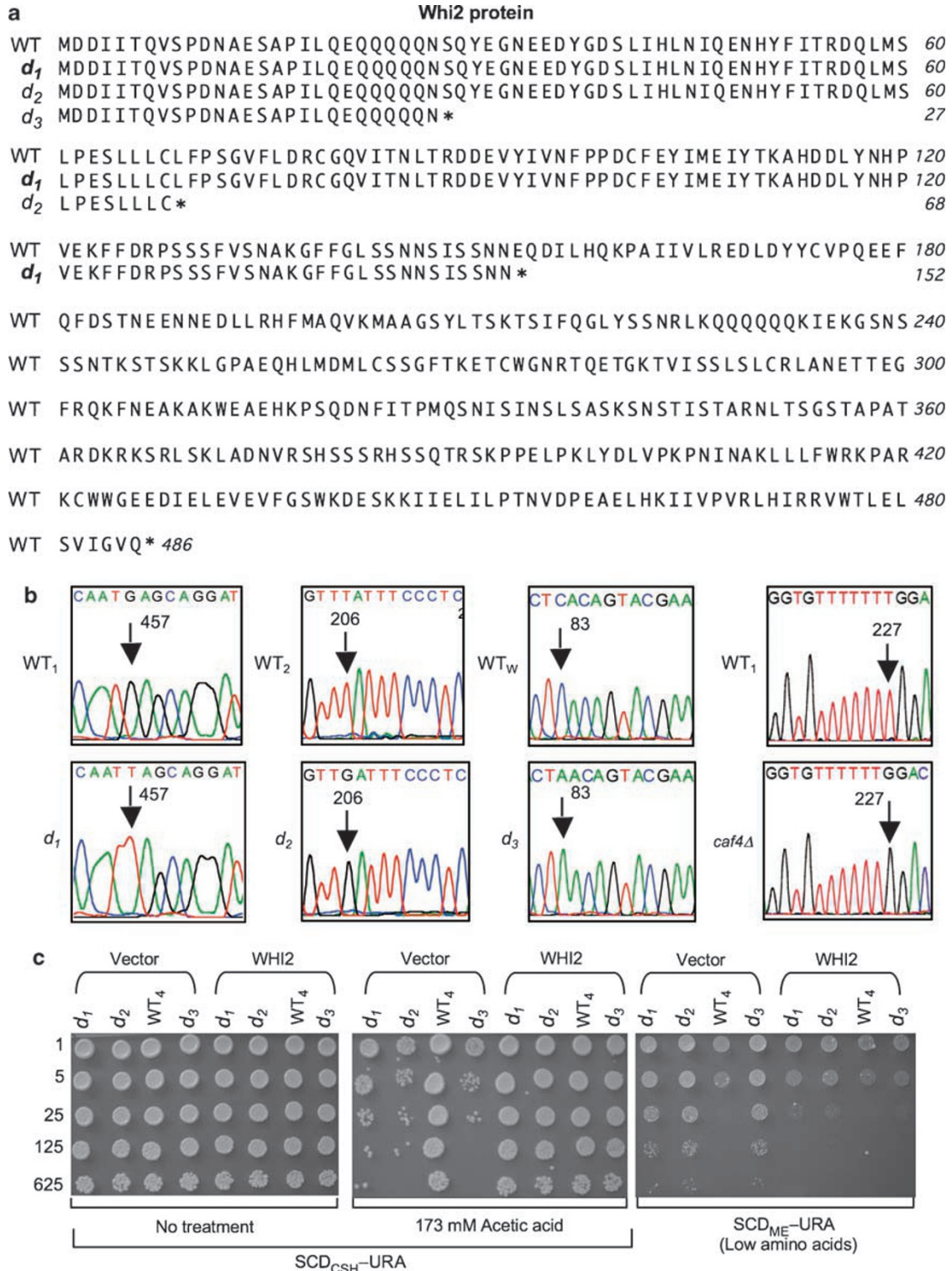


Figure 4 Secondary mutations in whiskey 2 (*WHI2*) are responsible for the cell death and overgrowth phenotypes. (a) Genomic sequencing identified premature terminations (*) in *Whi2* from three independently derived *FIS1*-knockout strains. (b) DNA sequence chromatograms of *WHI2* mutations in mutant strains *d*₁, *d*₂, *d*₃, *caf4Δ* and their respective wild-type strains. Nucleotide position numbers in the *WHI2* ORF mark the mutations (arrows). (c) A low-copy plasmid (CEN-URA) expressing *WHI2* (by its own endogenous promoter) in *d*₁, *d*₂ and *d*₃ strains, but not the empty plasmid vector, restores cell survival and reinstates the ability to reduce growth on low amino-acid medium. Note, a slightly lower dose of acetic acid is required when plating on synthetic medium. A representative of three independent experiments per condition is shown

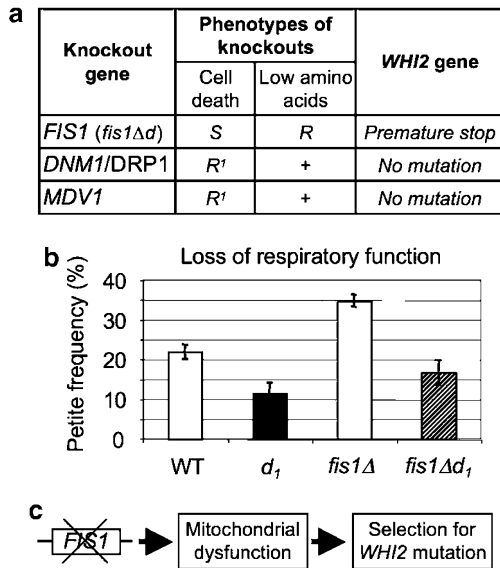


Figure 5 *FIS1* deletion increases mitochondrial respiratory defects. (a) Results showing that whiskey 2 (*WHI2*) mutations do not arise in other knockout strains (dynamin-related 1, *DNM1*, or mitochondrial division 1, *MDV1*) that are defective for mitochondrial fission, or in another strain where secondary mutations are responsible for cell death sensitivity and overgrowth during amino-acid deprivation. (b) Loss of respiratory function measured as petite frequency (comparing strains with the indicated genotypes derived from eight spores of four tetrads) is caused by *FIS1* deletion and is alleviated by *WHI2* mutations. Means \pm S.E. are presented for four independent experiments (9–10 determinations per genotype). $P=0.02$ for WT versus d_1 ; $P=0.004$ for WT versus *fis1Δ*, $P=0.006$ for *fis1Δ* versus *fis1Δd₁*. (c) Model summarizes data presented, indicating that loss of the *FIS1* gene compromises mitochondrial function, which is compensated by selection of *WHI2* loss-of-function mutations

FIS1-knockout strains with no *WHI2* mutations had noticeably more petite colonies, consistent with an increased petite frequency when replica plated onto YPG (Figure 5b). Importantly, this mitochondrial defect in the *FIS1* knockout was rescued by the *d* mutation in *WHI2* (*fis1Δd₁*), indicating that the *WHI2* loss-of-function mutation protects the *FIS1* knockout from mitochondrial damage. Taken together, these data suggest a model where deletion of *FIS1* causes a mitochondrial defect that is compensated by a loss-of-function mutation in *WHI2* (Figure 5c).

Gene duplication upon *de novo* deletion of *FIS1*. To further evaluate the importance of *FIS1* to cell survival, we generated new *FIS1*-knockout strains by homologous recombination with a targeting cassette containing the auxotroph selection marker *URA3* plus 200bp arms of *FIS1* ORF flanking sequences (Figure 6a). This targeting cassette was introduced into a wild-type strain (WT₁) and transformants were selected on uracil-minus medium. Successful recombination at both 5' and 3' ends of the selection marker at the *FIS1* locus of the resulting clones was confirmed by PCR using recommended primer pairs³² and by verifying netted mitochondrial morphology (Figure 6b, data not shown). These new *FIS1*-deleted strains lack a cell death phenotype (Supplemental Figure S3), and do not have *WHI2* mutations (data not shown), confirming that *WHI2* mutations do not develop immediately following deletion of *FIS1*.

In contrast to the recombination strategy above, attempts to delete *FIS1* were unsuccessful with the strategy used for the YKO collection with only 40bp homologous arms.²⁵ All 14

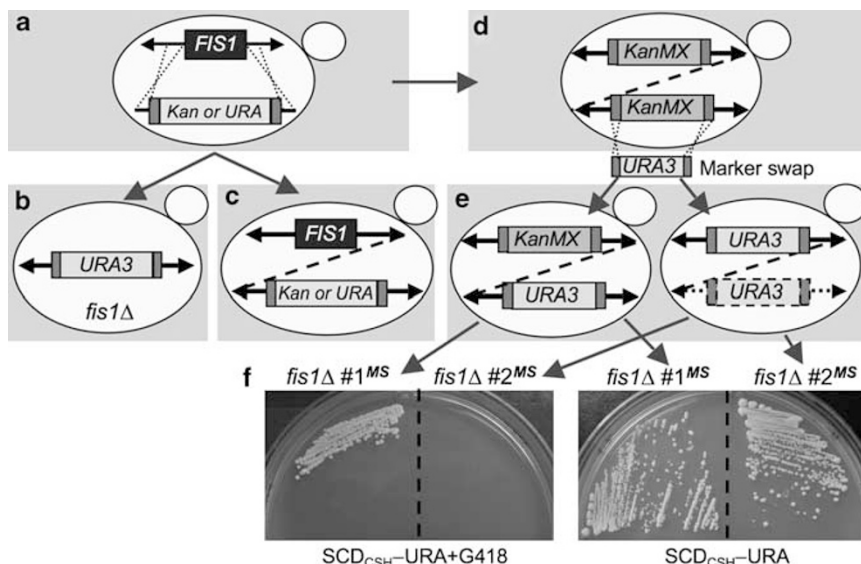


Figure 6 *FIS1* deletion induces gene duplication. (a) Gene replacement strategy for generating knockouts of the *FIS1* ORF (blue box) with targeting cassettes consisting of the *KanMX4* or *URA3* selection marker flanked by *TEF* sequences for selection marker expression (red boxes). (b, c) Results of successful gene replacements (b) and unsuccessful attempts (c) resulting in strains with both *FIS1* and the *URA3/KanMX4* selection markers inserted in *cis* (dashed line) or in *trans* (solid arrows). (d) Presumed rare event of gene (dashed line) or segmental/chromosome duplication upon replacement of *FIS1* with *KanMX4* for G418^R in the YKO collection, resulting in two copies of *KanMX4* cassettes per cell. (e) Results obtained by marker swap (MS) of the *KanMX4* cassette in the YKO collection for a *URA3* cassette resulting in 0–1 copies of *KanMX4* and 1–2 copies of *URA3*. (f) Examples of growth on synthetic complete dextrose medium (SCD_{CSH}, Cold Spring Harbor) minus uracil plates, with or without G418, confirm PCR genotype analysis from strains as shown in (e). A colour version of this figure is available online

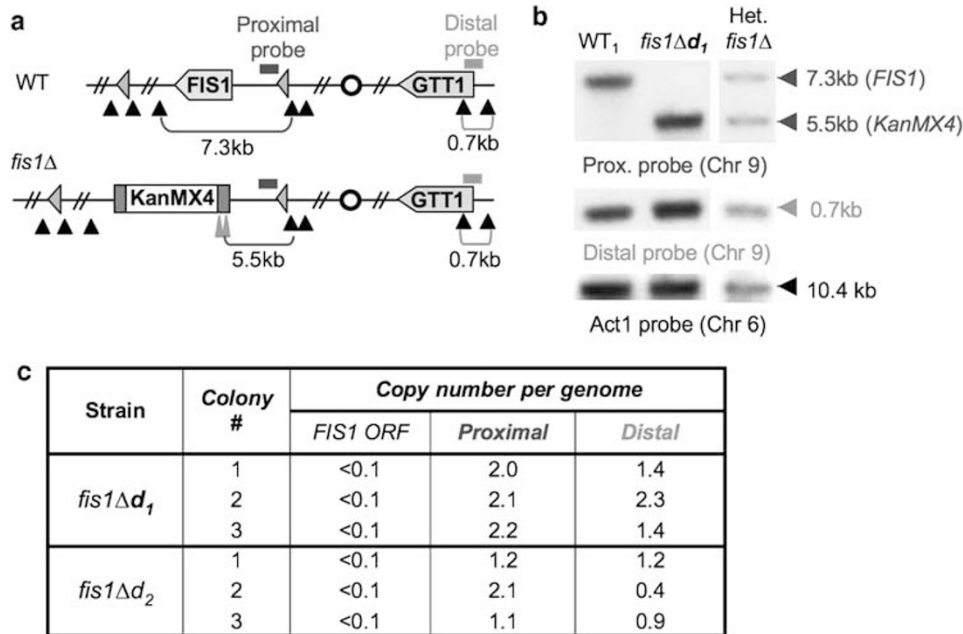


Figure 7 Evidence for segmental and chromosome duplication in *FIS1* knockouts from the YKO collection. (a) Chromosome 9 for parental and *FIS1*-deleted strains shows approximate locations of the intergenic probe proximal to the *FIS1* gene (blue bar), distal probe near end of opposite arm on chromosome 9 (orange bar), centromere (circle), *Bam*HI cut sites (black triangles), *Bgl*II sites (tall red triangles), fragment sizes detected by probes (brackets), Ty LTRs flanking 50 kb region encoding *FIS1* (pink triangles). (b) Representative Southern blots of genomic DNA hybridized with the indicated probes. (c) Southern blot analyses for colonies of each of the indicated strains tested in independent experiments. A colour version of this figure is available online

putative knockouts analyzed from 3 independent experiments had normal tubular mitochondria, rather than the netted morphology characteristic of *fis1Δ* strains, indicating retention of the *FIS1* gene despite the presence of the selection marker used to replace *FIS1* (Figure 6c, data not shown). The presence of *FIS1* was confirmed by CGH analysis, and by three different PCR primer pairs covering ~200 to 500 bp flanking sequences (data not shown). These results raise the possibility that attempted deletion of *FIS1* may have selected for duplication of the *FIS1* locus or entire chromosome, and that only one copy of duplicated *FIS1* was replaced by the selection marker (Figure 6c). Similar to our observation, aneuploidy has been observed in the YKO haploid collection during initial construction.^{32,33}

Given these difficulties, we considered the possibility that haploid *fis1Δ* strains in the YKO collection could also have undergone gene/chromosome duplications. Results from our marker-swap (MS) experiment are consistent with this possibility. In this experiment, we replaced the *KanMX4* cassette at the *FIS1* locus in the *fis1Δd₁* strain from the YKO collection with a *URA3* cassette by homologous recombination as described³⁴ (Figure 6d and e). Only two clones were obtained from several independent experiments and both retained their ability to grow on uracil-minus medium when retested (Figure 6f, right). Strikingly, one of them retained resistance to G418, confirming the presence of *KanMX4* in addition to the *URA3* cassette (Figure 6e and f left). This finding suggests a prior duplication of *FIS1*, one of which is now occupied by the *URA3* cassette. PCR analysis confirmed that both the *KanMX4* and *URA3* cassettes are associated with at least ~500 bp of *FIS1* flanking sequences (not shown).

Duplication of *FIS1* can be mediated by segmental or chromosomal duplication. The evidence presented above suggests that gene or chromosome duplication may occur with relative frequency when attempting to delete the *FIS1* gene. Hughes *et al.*³³ examined gene duplication using whole-genome DNA microarray, and found that 8% of ~300 homozygous and haploid YKO strains have either chromosomal or segmental duplication, and suggested that duplication was selected for by dosage/functional compensation of the deleted gene.

To determine if *FIS1* duplication is mediated by chromosomal or segmental duplication, the genome copy number was analyzed by Southern blot analysis using intergenic probes both proximal and distal to the *FIS1* locus on chromosome 9 (Figure 7a). All results from three different colonies each for *fis1Δd₁* and *fis1Δd₂* strains confirmed the absence of a *FIS1* gene as expected based on fragment sizes detected with the proximal probe (Figure 7b and c). However, a comparison of signal intensities for proximal and distal probes relative to the actin probe suggests one of three events, no detectable duplication (*fis1Δd₂* colonies 1 and 3), segmental duplication (*fis1Δd₁* colonies 1 and 3, *fis1Δd₂* colony 2) or chromosome duplication (*fis1Δd₂* colony 2). These variants are presumably a reflection of an unstable rare preexisting event (before *FIS1* deletion) that was selected as a consequence of *Fis1* deficiency and then subsequently evolved, perhaps more as a reflection of instability, but potentially also a consequence of *FIS1* deletion. Taken together, the evidence suggests negative selection for *de novo* deletions of *FIS1* and indicates that *WHI2* mutations are not preexisting, but arise by selection when *FIS1* is deleted.

Discussion

Loss of *FIS1* imposes a selective pressure for genetic alterations in yeast. We report here that three independently constructed *FIS1*-knockout strains of *S. cerevisiae* acquired a loss-of-function mutation in the same gene, *WHI2*. Although *FIS1* is not classified as an essential gene, our findings suggest that depletion of Fis1 is detrimental. However, the long-term survival function of Fis1 may be independent of a fission function mediated through Dnm1 and Mdv1, as deletion of *FIS1*-interacting genes *DNM1* or *MDV1* does not select for cell death sensitivity, overgrowth on low amino acids or for secondary mutations that alter these functions. Thus, Fis1 may have additional functions not shared with Dnm1 or Mdv1. Consistent with this possibility, Fis1 appears to be required for maintenance of mitochondrial DNA-encoded function, as *FIS1* knockouts (with wild-type *WHI2*) exhibit increased petite frequency (loss of mitochondrial respiratory function). Although mitochondrial fusion factors are known to be required for maintenance of mitochondrial DNA in yeast and mammals,² this is unexpected upon loss of a fission factor. Importantly, loss-of-function mutations in *WHI2* specifically suppress increased petite frequency caused by *FIS1* deletion, although the mechanism is not known.

WHI2 is a fungi-specific gene that was first identified for its involvement in cell size and cell cycle, but was not known to have an involvement in programmed cell death. Others found that deletion of *WHI2* causes a failure to undergo cell-cycle arrest in G₁ when nutrients become depleted, resulting in continuous division/growth, reduced cell size and eventual arrest at random stages of the cell cycle upon exhaustion of nutrients.^{35,36} In addition, *WHI2* is important for mounting stress responses against H₂O₂, heat shock and high salt,²³ consistent with our observation that loss of *WHI2* provides a growth advantage during amino-acid deprivation and confers sensitivity to cell death when stressed. By binding to the protein phosphatase Psr1, Whi2 was suggested to stimulate expression of stress-response genes through transcription factors Msn2/Msn4.²³ Thus, although *WHI2* mutants can outgrow wild-type cells during nutrient limitation, any growth advantage could potentially be offset by the propensity of *FIS1*-knockout strains to undergo cell death.

A model of tumorigenesis? Attempts to generate new *FIS1* knockouts can also select for *FIS1* gene or chromosome duplication, presumably because the cells that contain these preexisting events have an advantage when *FIS1* is deleted, and because these events are more common than acquisition of inactivating point mutations in *WHI2*. Because *WHI2* mutations did not arise immediately after losing *FIS1*, it is also possible that a step-wise progression of mutations eventually allows fixation of *WHI2* loss-of-function. This situation may be analogous to the process of tumorigenesis in mammals, where the selection for cell growth and survival functions may be governed by some of the same evolutionarily conserved mechanisms operating in yeast. Although secondary mutations are confounding for genetic studies in both yeast and mammals, these events are likely to be far more common

than currently appreciated, but will be much easier to dissect in yeast than in mammals. We anticipate that identification of such mutations in the YKO collection will reveal new genetic interactions relevant to cell death and survival pathways.

Cryptic secondary mutations. Several additional studies have identified a genetic linkage between *FIS1* and *WHI2*. Two independent genome-wide screens for mutants (homozygous diploids) with altered sporulation efficiencies identified both *FIS1* and *WHI2* knockouts.^{38,39} A third genome-wide screen for synthetic lethal interactions with the Hsp82 chaperone identified both *FIS1* and *WHI2*.⁴⁰ These screens may reflect *FIS1*-*WHI2* genetic interactions, but a cryptic *WHI2* mutation in the *FIS1* knockout could also explain these results. However, the mere presence of a cryptic *WHI2* mutation in *FIS1* knockouts implies a genetic interaction, even though the phenotypes being scored in these screens may directly reflect only the function of *WHI2*, rather than *FIS1*. The presence of a *WHI2* mutation in the *CAF4* knockout, but not in other knockout strains, including *DNM1* and *MDV1*, further fuels the hypothesis that the secondary mutations are specifically selected to compensate for an undetermined function of Fis1. Fis1 can bind Caf4 to promote mitochondrial fission in the absence of Mdv1,³¹ but it is not evident how this would occur independently of Dnm1/Drp1, suggesting additional functions for Fis1.

The cryptic secondary mutation conundrum is a major inconvenience to yeast geneticists, as it compromises applications of the YKO collection to epistasis analyses and other strategies, though case reports are generally lacking. The prevalence of cryptic mutations is also underappreciated because these mutations lack phenotypes detected by the assays being applied. Secondary mutations may account for many examples of inconsistent phenotypes, but the underlying mutation is rarely pursued, in part because these mutations are generally thought to be more artifact than of biological significance. To the contrary, these secondary mutations will likely yield insightful new genetic interactions. Application of tiling microarray techniques developed by Gresham *et al.*²⁸ makes it feasible to rapidly identify these cryptic mutations.

Materials and Methods

Yeast strains, plasmids and media. Genotypes of all yeast strains used in this study are listed in Supplemental Table S1. The 3 kb *SalI/KasI* fragment containing the *WHI2* gene and regulatory sequences was subcloned from plasmid pWhim3, provided by PE Sudbery, into vector YCplac33, a CEN-URA3 plasmid (accession number X75456) provided by J Heitman. Synthetic media SCD_{CSH} (or SCD_{CSH}-URA, minus uracil)²⁶ and SCD_{ME} (or SCD_{ME}-URA),²⁷ and rich media (2% peptone, 1% yeast extract)²⁷ with 2% dextrose (YPD) or 3% glycerol (YPG), and 2% agar for solid media, were prepared as described.

Cell death assay. Yeast strains were seeded at low density into 5 ml liquid YPD or uracil dropout media (SCD_{CSH}-URA), incubated overnight at 30°C with rotation until reaching 0.5 OD₆₀₀, and 2 ml of culture were treated 4 h with the indicated concentrations of acetic acid. Treated and untreated cultures were serially diluted fivefold in media and 5 μl of each dilution were plated on solid media starting with undiluted sample. Plates were incubated at 30°C in a humidified chamber for 2–3 days.

Quantification of growth on SCD_{CSH} and SCD_{ME} media. Quantification of yeast growth in Figure 1 was performed using ImageJ (Rasband, W.S., ImageJ, US National Institutes of Health, Bethesda, Maryland,

USA, <http://rsb.info.nih.gov/ij/>, 1997–2007). TIFF images of plates were imported into ImageJ and converted from RGB color to 8-bit. Using the tool functions 'subtract background', 'selection' and 'measure', the ratios of measured area intensities were calculated for each dilution spot. Dilutions with the most similar intensities between strains/treatments were used to calculate a ratio, and subsequently multiplied by the dilution factor to represent fold differences relative to WT (*y* axis).

CGH and yeast tiling microarray. Yeast CGH and tiling microarrays were processed and analyzed as described²⁹ to identify single nucleotide differences between WT and *fis1Δ*-derived spores, including spore 2a, derived from sporulation of a heterozygous *FIS1*-knockout strain that was created by mating WT₂ and *fis1Δ*Δ₁, and spore 3b, derived by sporulation of a heterozygous *FIS1*-knockout strain created by mating WT₁ and *fis1Δ*Δ₂.

Mitochondrial function (petite frequency). Overnight cultures (YPD) started from single colonies were diluted to 0.2 OD₆₀₀, incubated (~16 h) at 30°C until mid-log phase (~0.5 OD₆₀₀), diluted 8000-fold, and 200 μl was spread onto duplicate/triplicate YPD plates. Colonies were counted after 3 days of incubation, replica plated onto YPG plates and incubated for 3 days at 30°C. The percent of respiration-incompetent colonies, defined as petites, were calculated as the percent of total colonies (YPD) that failed to grow on YPG.²⁴

Southern blotting. Genomic DNA from each strain was purified as described,²⁶ and 10 μg was digested with *Bam*HI and *Bgl*II, separated on a 0.7% agarose gel and hybridized with [³²P]radiolabeled probes using the High Prime DNA Labeling Kit (Roche). Signal intensities on autoradiographs were quantified from multiple exposures using the ImageGauge Version 4.22 software (Fuji Photo Film). Probes correspond to chromosome 9 nucleotide numbering for proximal probe (243 013–242 416) and distal probe (424 383–424 999), and chromosome 6 for the act1 probe (52 937–53 520).

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