SYNTHETIC LETHAL AND BIOCHEMICAL ANALYSES OF NAD AND NADH KINASES IN SACCHAROMYCES CEREBVIAE ESTABLISH SEPARATION OF CELLULAR FUNCTIONS*

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Production of NADP and NADPH depends on activity of NAD and NADH kinases. Here we characterized all combinations of mutants in yeast NAD and NADH kinases to determine their physiological roles. We constructed a diploid strain heterozygous for disruption of POS5, encoding mitochondrial NADH kinase, UTR1, cytosolic NAD kinase, and YEF1, a UTR1-homologous gene we characterized as encoding a low-specific activity cytosolic NAD kinase. pos5 utr1 is a synthetical lethal combination rescued by plasmid-borne copies of the POS5 or UTR1 genes or by YEF1 driven by the ADH1 promoter. Respiratory-deficient and oxidative damage-sensitive defects in pos5 mutants were not made more deleterious by yef1 deletion and a quantitative growth phenotype of pos5 and its arginine auxotrophy were repaired by plasmid-borne POS5 but not UTR1 or ADH1-driven YEF1. utr1 haploids have a slow growth phenotype on glucose not exacerbated by yef1 deletion but reversed by either plasmid-borne UTR1 or ADH1-driven YEF1. The defect in fermentative growth of utr1 mutants renders POS5 but not POS5-dependent mitochondrial genome maintenance essential because rho- utr1 derivatives are viable. Purified Yef1 has similar nucleoside triphosphate specificity but substantially lower specific activity and less discrimination in favor of NAD versus NADH phosphorylation than Utr1. Low expression and low intrinsic NAD kinase activity of Yef1 and the lack of phenotype associated with yef1 suggest that Utr1 and Pos5 are responsible for essentially all NAD/NADH kinase activity in vivo. The data are compatible with a model in which there is no exchange of NADP, NADPH or cytoplasmic NAD/NADH kinase between nucleocytoplasmic and mitochondrial compartments but the cytoplasm is exposed to mitochondrial NAD/NADH kinase during the molecule’s transit.

NADP is required by the pentose phosphate pathway and NADPH is required for resistance to oxidative stresses. Deletion mutants in the gene encoding mitochondrial matrix NADH kinase, POS5, are sensitive to growth in hyperoxia, H2O2, and methyl viologen and are also respiratory-deficient (1,2). Interestingly, transforming freshly prepared pos5 mutants with a POS5 plasmid restores resistance to reactive oxygen species and growth on nonfermentable carbon sources but older pos5 mutants lose the ability to have their respiratory defect restored by Pos5 expression. These data suggest that mitochondrial NADP-dependent anti-oxidant reductases are required to protect mitochondria from reactive oxygen species damage and that this damage can lead to irreparable lesions to the mitochondrial genome (1,2). In the cytosol, NADP is used by pentose phosphate pathway enzymes glucose-6-phosphate dehydrogenase Zw1 and 6-phosphogluconate dehydrogenase Gnd1,2 to produce ribulose-5-phosphate and NADPH (3). The yeast glucose-6-phosphate dehydrogenase mutant zwf1 is consequently sensitive to oxidizing agents that deplete cytosolic pools of reduced glutathione and thioredoxin, which depend on cytosolic NADPH (4,5). In
addition, the zwf1 mutant requires sulfur to be supplied in organic form (6).

Beyond the requirement of Zwf1 and Gnd1,2 for NADP, cytosolic NADP is required by NADP-specific isocitrate dehydrogenase Lpd2, glutamate dehydrogenase Gdh1, and aldehyde dehydrogenase Ald6 (7). Thus, a cytosolic NAD kinase mutant would be expected to produce pleiotropic phenotypes consistent with deficiencies in multiple enzymes. In yeast, Utrl was originally reported as a transcript of unknown function (8), and then shown to encode a component of ferrireductase (9) with NAD kinase activity (10). Though the utr1 deletion mutant was recently shown to exhibit poor growth in low iron media (11) consistent with function of Utrl within ferrireductase (9), a more general phenotype has not been reported.

In many eukaryotes, NAD is synthesized from a de novo pathway from tryptophan (12,13), an import pathway from nicotinic acid (14,15), and a salvage pathway from nicotinamide riboside (16). NAD is not only a co-enzyme for enzymes such as inosine monophosphate dehydrogenase, which use NAD as a hydride acceptor, producing NADH (17), but also a substrate of NAD glycohydrolases. While NAD glycohydrolases were once considered mysterious enzymes that cleave NAD to a nicotinamide and an ADPribosyl product for no obvious reason (18), we now understand that a class of protein lysine deacetylases related to yeast Sir2 (Sirtuins) (19-21) as well as cADPribose synthetase and poly(ADPribose) polymerases (22) are NAD-dependent enzymes. Because these enzymes convert copious amounts of NAD to nicotinamide, organisms benefit from enzymes that salvage nicotinamide to either nicotinic acid or nicotinamide mononucleotide for reentry into NAD biosynthetic pathways. Phylogenetic analysis suggests that the former enzyme, nicotinamidase, found in fungi (23), and the latter enzyme, nicotinamide phosphoribosyltransferase, found in vertebrates (24), entered eukaryotic lineages via horizontal gene transfer (25). The final steps in eukaryotic NAD synthesis are catalyzed by either glutamine-dependent NAD synthetase (26) for the de novo, nicotinic acid import and nicotinic acid salvage pathways that go through NaMN, or by NaMN/NMN adenyllyltransferase (27) for the nicotinamide riboside kinase and nicotinamide phosphoribosyltransferase pathways that go through an NMN intermediate. In the nucleus and cytosol, NAD-dependent hydride transfer enzymes such as inosine monophosphate dehydrogenase, alcohol dehydrogenase and glyceraldehyde-3-phosphate dehydrogenase reduce NAD to NADH.

NADH is imported into mitochondria by the voltage-dependent anion channel (porin isoform 1) (28,29) and possibly by the translocase of the outer membrane complex (30). NAD is imported into mitochondria by the products of the YIA6 (NDT1) and YEAE6 (NDT2) genes, which have essentially no transport activity on NADP or NADPH (31). Thus, unless NADP or NADPH is imported into mitochondria by a process that has not been described, mitochondrial NADP and NADPH should depend entirely on a mitochondrial NAD/NADH kinase activity. Similarly, unless NADP or NADPH can flow back from mitochondria to the cytosol, cytosolic NADP and NADPH are expected to depend on a cytosolic NAD/NADH kinase or mislocalization of the corresponding mitochondrial enzyme. Because two of three NAD/NADH kinase genes had been analyzed with respect to phenotypes and biochemical characteristics, we wished to perform a genetic and biochemical analysis of the remaining NAD/NADH kinase gene in yeast and to analyze phenotypes of double and triple mutants. During preparation of this manuscript, Shi and coworkers purified the third yeast NAD/NADH kinase, YeI, and concluded, as we do, that YeI is a cytosolic NAD kinase (11). However, they claim to produce viable cells deleted for pos5, utr1 and yeI and suggested existence of an additional NAD kinase unrelated to the sequence of NAD/NADH kinases (11). In constrast, we report that pos5 utr1 is a synthetic lethal combination, with or without deletion of yeI.

Our analysis confirms that pos5 mutants have mitochondrial (i.e., respiratory) defects (1,2) and establishes that Utrl has cytoplasmic (i.e., fermentative) defects, indicating that neither enzyme is significantly mislocalized and that triphosphopyridine nucleotides do not exchange between nucleocytoplasmic and mitochondrial compartments. We also demonstrate that pos5 and utr1 produce a synthetic lethal combination. Moreover, our analysis shows that YeI, encoded
by the YEL041W gene, is a low-specific activity NAD kinase. While deletion of the yef1 gene did not exacerbate either the pos5 or the utr1 phenotypes, the pos5 utr1 double mutant was synthetically lethal, indicating that there is not enough NADP produced by Yef1 to support the fermentation that must occur in pos5 mutants. Finally, though the pos5 utr1 mutant is inviable, we find that rho- utr1 mutants are viable, suggesting that the viability of utr1 mutants depends on cytosolic Pos5-dependent NAD/NADH kinase activity prior to or during this molecule’s transport to the mitochondrion.

**EXPERIMENTAL PROCEDURES**

**Enzyme expression and purification**—Expression plasmids were designed for purification of His-tagged versions of Utr1 and Yef1 from *E. coli*. Primer sequences are provided in Supplementary Table 1. The UTR1 open reading frame was amplified from *S. cerevisiae* genomic DNA with primers 7125 and 7126 and cloned into vector pMR103 (32) using *Neo I* and *Bam HI* restriction sites to create plasmid pB414. An N-terminal His-tag was added by annealing oligonucleotides 7300 and 7301 and ligating to *Neo I*-digested plasmid pB414 to produce plasmid pB421. The YEF1 coding sequence was amplified with primers 7318 and 7319, digested with *Nde I* and *Bgl II*, and cloned into vector pSGA04 (33) cleaved with *Nde I* and *Bam HI* to create plasmid pB430. *E. coli* BL21 cells transformed with either plasmid pB421 or pB430 were used to express the His-tagged NAD kinases. Bacterial cells were lysed by sonication in Buffer A (20 mM Na phosphate pH 8, 100 mM NaCl, 10% glycerol, 1 mM β-mercaptoethanol, 10 mM imidazole and protease inhibitor cocktail (Roche)). Clarified lysates were loaded on cobalt chelate affinity columns. Columns were washed with 15 volumes of Buffer A, and enzymes were eluted with Buffer A supplemented with 100 mM imidazole. Purified enzymes were concentrated and dialyzed against Buffer A without imidazole.

**Enzymatic assays**—To determine the specific activities of the Utr1 and Yef1 kinases, 100 μl pre-mixes containing 100 mM Tris-HCl pH 7.1, 5 mM MgCl2, 1 mM NTP or dNTP, and 1 mM NAD, NADH or NaAD were incubated with either Utr1 or Yef1 enzyme (0.5 μg) for 20 min at 37 °C. EDTA (10 μl of 0.5 M) was added to stop the reactions and NADP, NADPH or NaADP products were separated and quantified by HPLC using a strong anion exchange column in a 10 to 750 mM gradient of Na phosphate, pH 2.6. Mean specific activities of three separate experiments, expressed as μmol of NADP, NADPH or NaADP min⁻¹ mg⁻¹, were calculated from reactions in which no more than 10% of substrates were converted into products.

**Yeast strains**—DNA fragments for disruption of UTR1, POS5 and YEF1 genes were constructed as described (34). For UTR1, primers 7127 and 7128 were used to amplify the HIS3 marker of plasmid pRS413 (35). Correct integration of HIS3 at the UTR1 locus was verified by PCR using primers 7125 and 7126. For POS5, primers 7343 and 7345 were used to amplify the geneticin-resistance marker of plasmid pRS401 (35). Correct integration was verified with primers 7342 and 7344. For YEF1, primers 7312 and 7313 were used to amplify the TRP1 marker of plasmid pRS414 (35). Correct integration was verified with primers 7314 and 7315. Diploid yeast strain SEY6210.5 was subjected to three consecutive steps of gene replacement and verification using these disruption cassettes. The resulting strain, BY274, triply heterozygous for deletion of pos5, utr1 and yef1, was incubated on sporulation media. Following tetrad formation, spores were separated by micromanipulation and the genotypes of the resulting haploid isolates were tested by growth on appropriate selective media (Table 1). UTR1, YEF1 and POS5 were amplified from the *S. cerevisiae* genome using primers 776 and 777, 778 and 779, and 780 and 781, respectively. The UTR1 gene PCR product and plasmid pRS416 (35) were digested with *Eco RI* and *Spe I* and ligated to produce pB534. The YEF1 open reading frame PCR product was similarly digested and ligated into p416ADH1 (36) to produce pB535. The POS5 gene PCR product and pRS416 were digested with *Bam HI* and *Xho I* and ligated to produce pB532. Diploid strain BY274 heterozygous for pos5, utr1 and yef1 disruption was transformed with pB532, pB534 and pB535 and, after sporulation and tetrad dissection, haploids were isolated with each plasmid present in each combination of NAD/NADH kinase gene disruption. Strains were then plated on 5-fluororotic acid (5FOA) media in order to recover
and characterize viable haploid genotypes. Media used to grow yeast cultures and protocols for transformations and tetrad dissection have been described (37).

**Yeast growth assays**—Triplicate growth curves of all viable haploid strains were obtained at 28°C in YPD in 10 time points over 50 hours. Triplicate growth curves of utr1 and pos5 strains transformed with pRS416 (empty vector), pB532, pB534 or pB535 were obtained at 28°C in YPD in seven time points over 13 hours. Plate assays of the pos5 strain transformed with pRS416, pB532, pB534 or pB535 were completed on YPD, YPglycerol and synthetic dextrose media without arginine. Respiratory deficient (rho-) yeast strains were obtained by passage on ethidium bromide media (38).

**RESULTS AND DISCUSSION**

The NAD/NADH kinase reaction with ATP as phosphoryl donor is schematized in Figure 1. Sequence analysis of **YEF1** (YEL041W) indicates the presence of an NAD kinase signature (39) motif (residues 311 to 333) and a conserved GGDG motif (residues 190 to 193), which has been shown to be part of the ATP-binding site in highly divergent metabolite kinases (39-43). As shown in Figure 2, at the level of primary sequence, Yef1 is more similar to the NAD kinase Utr1 than to NADH kinase Pos5. To compare their biochemical activities, we purified Utr1 and Yef1 proteins expressed in *E.coli* as N-terminally His-tagged fusions. Because yeast NAD kinase is a relatively unstable enzyme (44), our rapid, one-step purification produced higher specific activities than those previously reported (10,45).

As shown in Table 1, Utr1 is an effective NAD kinase with ATP, dATP and dCTP as phosphoryl donors and lesser activity with CTP, GTP and dGTP as phosphoryl donors. Utr1 phosphorylates NADH with ~2% of the specific activity of its NAD kinase activity, preferring ATP as the phosphoryl donor for formation of NADPH. The maximal activity for Yef1 is with NAD and ATP, though it possesses ~33% of this activity with NADH and ATP. In terms of NAD-ATP kinase activity, Yef1 exhibits only about 6% of the specific activity of Utr1. However, with the low activity of Utr1 on NADH and the relative nonspecificity of Yef1 for phosphoacceptors, Yef1 has about as much NADH kinase specific activity as does Utr1.

To test whether either NAD kinase might have a role in formation of the calcium-mobilizing second messenger, NaADP (46), we examined Utr1 and Yef1 as ATP-dependent NaAD kinases (Table 1). The specific activity of Utr1 was 0.4 ± 0.01 µmol NaADP min⁻¹ mg⁻¹ and that of Yef1 was 0.5 ± 0.02 µmol NaADP min⁻¹ mg⁻¹. These values represent less than 1% of the specific activity of Utr1 as an ATP-dependent NAD kinase.

Enzyme activity in vivo is a function of enzyme localization, enzyme concentration and intrinsic activity. Global analysis of protein expression indicates that Yef1 is expressed at a level of only about 300 molecules per cell, compared to approximately 5000 molecules of Utr1 and Pos5 (47). Thus, with the important caveat that our recombinant preparations might have missed a factor that contributes to the activity of Utr1 or Yef1 in vivo, our data suggest that Yef1 contributes very slightly to total NAD and NADH kinase activities in vivo.

To determine the roles of NAD and NADH kinase genes in living cells, we created diploid yeast strain BY274 with confirmed heterozygous deletions of the **POS5**, **UTR1** and **YEF1** genes. Following sporulation and dissection, the resulting haploids were analyzed. As shown in Table 2, of the eight possible genotypes that could potentially be recovered from the triheterozygous diploid, we recovered six genotypes ten to nineteen times. No strain was recovered with utr1 and pos5 deleted or with all three genes deleted. Synthetic lethality of utr1 and pos5 has not been previously reported. Because **UTR1** and **POS5** are not genetically linked, the nonrecovery of the double mutant is the first indication that either genetic loss renders the second gene to be essential.

To further characterize the viability and growth characteristics of NAD/NADH kinase deletion mutants, strain BY274 was transformed with pB532 (**POS5**), pB534 (**UTR1**) or pB535 (**ADH1** promoter driving **YEF1**) and haploids containing each of these plasmids in the triple delete background were recovered. Though the pos5 utr1 double mutant and the pos5 utr1 yef1 triple mutant could not be recovered by simple dissection of strain BY274 or BY274 carrying the empty vector pRS416, we recovered both of these
genotypes in the presence of each of the NAD/NADH kinase plasmids, pB532, pB534 and pB535. Whereas pB532 and pB534 transformants would be expected to confer a viable utr1 yepl phenotype and a pos5 yepl phenotype to the pos5 utr1 yepl chromosomal genotype, the viability of pB535 transformants indicates that overexpression of YEF1 suppresses the lethal phenotype of pos5 utr1. As shown in Figure 3, upon plating each of the transformants on 5FOA media, none of the strains could be recovered as plasmid-free pos5 utr1 yepl triple mutant strains. Taken together, Table 2 and Figure 3 indicate that pos5 utr1 and pos5 utr1 yepl are synthetically lethal mutant combinations that can be suppressed by overexpression of Yef1.

Pos5 has been characterized as a mitochondrial NADH kinase that phosphorylates NAD to a lesser extent (1,2) whereas Utr1 has been characterized as a cytoplasmic NAD kinase that phosphorylates NADH to a lesser extent (10). Mutants within these genes display distinct phenotypes. Mutants lacking Pos5 exhibit a near normal rate of growth on glucose (Figures 4A and 4C) but are unable to grow on non-fermentable carbon sources, such as glycerol (Figure 4B). Since NADPH is used as a cofactor by enzymes involved in the detoxification of peroxide, superoxide and hydroxyl radicals that are generated in mitochondria (48-50), depletion of the mitochondrial NADPH pool would be expected to lead to an increased level of oxidative damage to mitochondrial proteins and DNA, consistent with the mitochondrial genome instability that results from pos5 mutation (2). In contrast to the respiratory incompetence of pos5 mutants, utr1 mutants displayed wild-type growth on glycerol (Figure 4B) and slow growth on glucose (Figures 4A and 4C).

As shown in Figure 4, growth of yepl mutant strains on glucose and glycerol was indistinguishable from that of wild type strains. Unlike mutants in glucose-6-phosphate dehydrogenase that are deficient in NADP, yepl mutants exhibited no increased sensitivity to methyl viologen and H2O2 (4,5), and did not exhibit methionine and/or cysteine auxotrophy (6). The double mutants, pos5 yepl and utr1 yepl, did not demonstrate any additional growth defects on glucose or glycerol, relative to single pos5 and utr1 mutants. Similarly, yepl deletion did not enhance reactive oxygen sensitivity or auxotrophic requirements of pos5 or utr1 (data not shown). These results indicate that the Yef1 protein does not have a major role in production of triphosphopyridine nucleotides in vivo. Based on the synthetic lethality of utr1 and pos5, it is apparent that Yef1 activity under its own promoter is insufficient to support the UTR1-requirement of pos5 mutants or the POS5-requirement of utr1 mutants.

These experiments established three facts that could be interpreted through at least two mutually exclusive mechanisms. First, a cell cannot live if it is devoid of the mitochondrial NAD/NADH kinase Pos5 and the cytosolic NAD/NADH kinase Utr1. Second, ADH1-driven Yef1 suppresses the Pos5-dependence of utr1 mutants. Third, ADH1-driven Yef1 suppresses the Utr1-dependence of pos5 mutants. The most distinctive explanations for these facts are that 1) the poorly fermenting utr1 mutant is totally dependent on Pos5-dependent respiration or some specific mitochondrial function of Pos5; or 2) the poorly fermenting utr1 mutant is dependent on at least transient cytosolic NADP/NADPH production by Pos5. Based on experiments with plasmid-borne copies of POS5, UTR1 and YEFL in pos5 and utr1 backgrounds, and experiments in which rho- derivatives of utr1 were prepared and characterized, we were able to eliminate the first mechanism and gather evidence for the second.

Plasmid pB535, encoding YEFL driven by the ADH1 promoter, has the property of an overexpression-based suppressor of one phenotype of pos5, namely synthetic lethality with utr1. To test whether plasmid-borne copies of either POS5, UTR1 or YEFL would suppress specific mitochondrial phenotypes of pos5, we tested BY274-derived haploids of genotype pos5 UTR1 YEFL carrying pB532, pB534 and pB535 for growth on a nonfermentable carbon source and arginine prototrophy. As reported earlier (1), the pos5 mutant is arginine auxotrophic, due to lack of NADPH needed for the NADPH-dependent step of arginine biosynthesis catalyzed by mitochondrial N-acetyl-γ-glutamyl-phosphate reductase (51). As shown in Figure 5, the glycerol- and arg- phenotypes of pos5 were only rescued by POS5 expression and not by plasmid-borne copies of UTR1 or YEFL. Because the ADH1-driven YEFL construct allows a pos5
deletion strain to survive 

utr1 disruption but does not improve its respiratory or mitochondrial function, the mechanism of ADH1-driven YEF1 as a suppressor of synthetic lethality with 

utr1 appears to be production of cytoplasmic rather than mitochondrial NADP/NADPH.

As shown in Figure 4, pos5 mutants are incapable of growth on glycerol while 

utr1 mutants have a slow growth phenotype on glucose and 

pos5 mutants have a modest slow growth phenotype. To test whether the slow growth phenotypes of 

pos5 or 

utr1 might be suppressed by any of the NAD/NADH kinase genes, we compared the growth of 

pos5 and 

utr1 haploid isolates carrying empty vector pRS416 with those carrying plasmids pB532, pB534 and pB535. As shown in Figure 6A, normal growth of a 

pos5 strain can be restored by a plasmid-borne copy of 

POS5 but not by either of the genes encoding cytosolic NAD/NADH kinases. In contrast, as shown in Figure 6B, the slow growth phenotype of 

utr1 can be fully reversed by plasmid-borne 

UTR1 and by ADH1-driven YEF1 and can be improved by plasmid expression of 

POS5. Thus, while expression of cytosolic NAD/NADH kinases have no effect on the phenotype of the mitochondrial NAD/NADH kinase mutant, expression of mitochondrial NAD/NADH kinase appears to reduce the phenotype of a deficiency in cytosolic NADP/NADPH.

Because ADH1-driven YEF1 suppresses the 

pos5 dependence on 

UTR1 without restoring the mitochondrial function to 

pos5, we considered the possibility that 

utr1 is not synthetically lethal with the respiratory defect of 

pos5 but rather with the lack of at least a transient exposure of the Pos5 active site to the cytosol. As a test of this hypothesis, we set out to obtain rho- derivatives of 

utr1 by growing cells overnight in YPD medium containing 10 µg/ml ethidium bromide and streaking resulting cells on glucose medium (38). As shown in Figure 7, such mutants were easily obtained and proved to be respiration-incompetent by failure to grow on glycerol medium. However, as judged by plate assays, the rho- 

utr1 mutants grown on glucose possessed the same growth rate as 

rho+ 

utr1 mutants. Because there is not a respiratory component to the growth of 

utr1 mutants but 

utr1 mutants depend on 

POS5, we conclude that the viability of 

utr1 mutants depends on cytosolic Pos5 enzymatic activity expressed during Pos5 transit to the mitochondria.

In conclusion, Utr1 is responsible for essentially all of the NAD/NADH kinase activity resident in the cytoplasm while Pos5 is responsible for all mitochondrial NAD/NADH kinase activity and consequent mitochondrial genome maintenance. Yef1 can substitute for Utr1 when overexpressed. Because 

utr1 is synthetically lethal with 

pos5 but not with loss of respiration, the data indicate that transitory exposure of Pos5 to the cytoplasm is required for the viability of 

utr1 mutants.

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FIGURE LEGENDS

Figure 1. **Reaction scheme of NAD kinase.** NAD and NADH kinases catalyze phosphoryl transfer to the adenosine 2’ position of NAD and NADH, forming NADP and NADPH. Depicted is ATP as the phosphodonor and NAD as the phosphoacceptor.

Figure 2. **Sequence Alignment of Yef1, Utr1 and Pos5.** Black letters on a grey background denote residues that are similar. White letters on a grey background denote positions at which two of three sequences are identical. White letters on a black background denote residues that are identical. Signature sequences for NAD/NADH kinases are boxed in red and blue (39).

Figure 3. **Inviability of pos5 utr1 yef1.** Triheterozygous diploid strain BY274 was transformed with plasmids pB532, pB534 or pB535 containing POS5, UTR1 or YEF1 (under the control of the ADH1 promoter), respectively, and the URA3 gene. Upon sporulation and dissection of the diploid transformants, pos5 mutants (left) carrying each plasmid and pos5 utr1 yef1 mutants (right) carrying each plasmid were streaked on 5FOA media to select for loss of the URA3 plasmids. Whereas each plasmid carries a dispensable gene in the pos5 strain, each plasmid carries an indispensable gene in the triple mutant.

Figure 4. **Phenotypic analysis of the six viable NAD/NADH kinase genotypes.** Yeast strains with the indicated genotypes were grown on complete media containing glucose (A) or glycerol (B) as the sole carbon source. pos5 mutants are respiratory-incompetent while utr1 mutants have a slow-growth phenotype on fermentable carbon sources. yef1 mutants are aphenotypic and not additive with either pos5 or utr1. In YPD liquid cultures (C), the growth rate of an isogenic yef1 mutant strain was indistinguishable from wild-type, while a modest slow growth phenotype for pos5, not made more deleterious by yef1, and the slow growth phenotype of utr1, not made more deleterious by yef1, were apparent. Average optical densities were plotted ± standard deviations, which in many cases were smaller than the plotting symbols.

Figure 5. **Phenotypic analysis of pos5.** pos5 yeast strains recovered by tetrad dissection of strain BY274, carrying plasmids pRS416, pB532, pB534 or pB535, were serially diluted on complete media containing glucose or glycerol as the carbon source, and on glucose-containing -arg media. Though UTR1 and ADH1-driven YEF1 plasmids suppress the lethality of pos5 utr1, only POS5 expression repairs the mitochondrial phenotypes of pos5.

Figure 6. **Quantitative growth characteristics of utr1 and pos5 strains in glucose and analysis of plasmid-based complementation and suppression.** Growth curves of a pos5 haploid strain (A) and a utr1 haploid strain (B) transformed with pRS416, pB532, pB534 or pB535. Whereas the quantitative growth phenotype of pos5 is only complemented by plasmid-based expression of POS5, the slow growth phenotype of utr1 is complemented by UTR1, fully suppressed by ADH1-driven YEF1, and partially suppressed by POS5. Average optical densities were plotted ± standard deviation, which in many cases were smaller than the plotting symbols.

Figure 7. **utr1 is not synthetically lethal with lack of mitochondrial genome but with pos5 mutation.** A rho- derivative of a utr1 haploid strain, obtained by passage on ethidium bromide media, is respiratory incompetent yet viable. Because utr1 is synthetically lethal with pos5 but not with mitochondrial dysfunction, the utr1 mutant must depend on at least transient exposure of Pos5 to the cytoplasm for viability.
Table 1. Specific activities of Utr1 and Yef1 (µmol of NADP, NADPH or NaADP min⁻¹ mg⁻¹)

| phosphodonor |    |    |    | phosphoacceptor |    |    |    |
|--------------|----|----|----|-----------------|----|----|----|
|              | NAD | NADH | NaAD | Utr1            | NAD | NADH | NaAD |
| ATP          | 51.5 ± 2.7 | 1.2 ± 0.05 | 0.4 ± 0.01 | 3.3 ± 0.12 | 1.2 ± 0.05 | 0.5 ± 0.02 |
| CTP          | 7.3 ± 0.34 | 0.5 ± 0.04 | ND | 0.2 ± 0.04 | 0.5 ± 0.04 | ND |
| GTP          | 5.1 ± 0.41 | 0.1 ± 0.01 | ND | 0.3 ± 0.02 | 0.1 ± 0.01 | ND |
| dATP         | 20.0 ± 1.2 | ND | ND | 3.2 ± 0.24 | ND | ND |
| dCTP         | 15.6 ± 0.78 | ND | ND | 0.3 ± 0.02 | ND | ND |
| dGTP         | 3.9 ± 0.53 | ND | ND | 0.3 ± 0.04 | ND | ND |

ND, not determined.
Table 2. Synthetic lethality of *utr1* and *pos5*

| Genotype                  | Number of independent isolates |
|---------------------------|---------------------------------|
| POS5 UTR1 YEF1            | 14                              |
| pos5 UTR1 YEF1            | 16                              |
| POS5 utr1 YEF1            | 12                              |
| POS5 UTR1 yef1            | 15                              |
| pos5 UTR1 yef1            | 10                              |
| POS5 utr1 yef1            | 19                              |
| pos5 utr1 YEF1            | 0                               |
| pos5 utr1 yef1            | 0                               |
Figure 3

|         | pos5Δ | utr1Δ | pos5Δ | yef1Δ |
|---------|-------|-------|-------|-------|
| pBS32   |       |       |       |       |
| pBS34   |       |       |       |       |
| pBS35   |       |       |       |       |
Figure 4

A

pos5 yef1
utr1 yef1
WT
yef1
pos5

B

pos5 yef1
utr1 yef1
WT
yef1
pos5

C

\[ \text{OD}_{600} \]

\[ \text{time [h]} \]

- pos5
- pos5 yef1
- utr1
- utr1 yef1
- yef1
- wild type

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Figure 5

| POS5 | UTR1 | YEF1 | Vector |
|------|------|------|--------|
| Glucose | Glycerol | - Arginine |
Figure 6

A

OD$_{600}$ vs. time [h]

- Vector
- POS5
- UTR1
- ADH1-YEF1

B

OD$_{600}$ vs. time [h]

- Vector
- POS5
- UTR1
- ADH1-YEF1
Figure 7

glycerol

utr1 rho-

utr1

utr1 rho-

glucose

utr1
Synthetic lethal and biochemical analyses of NAD and NADH kinases in Saccharomyces cerevisiae establish separation of cellular functions
Pawel Bieganowski, Heather F. Seidle, Marzena Wojcik and Charles Brenner

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