

The yeast A kinases differentially regulate iron uptake and respiratory function

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Yeast has three A kinase catalytic subunits, which have greater than 75% identity and are encoded by the *TPK* genes (*TPK1*, *TPK2*, and *TPK3*) [Toda, T., Cameron, S., Sass, P., Zoller, M. & Wigler, M. (1987) *Cell* 50, 277–287]. Although they are redundant for viability, the three A kinases are not redundant for pseudohyphal growth [Robertson, L. S. & Fink, G. R. (1998) *Proc. Natl. Acad. Sci. USA* 95, 13783–13787; Pan, X. & Heitman, J. (1999) *Mol. Cell. Biol.* 19, 4874–4887]; Tpk2, but not Tpk1 or Tpk3, is required for pseudohyphal growth. Genome-wide transcriptional profiling has revealed unique signatures for each of the three A kinases leading to the identification of additional functional diversity among these proteins. Tpk2 negatively regulates genes involved in iron uptake and positively regulates genes involved in trehalose degradation and water homeostasis. Tpk1 is required for the derepression of branched chain amino acid biosynthesis genes that seem to have a second role in the maintenance of iron levels and DNA stability within mitochondria. The fact that *TPK2* mutants grow better than wild types on nonfermentable carbon sources and on media deficient in iron supports the unique role of Tpk2 in respiratory growth and carbon source use.

In yeast, cAMP acting through the A kinases (PKA) provides a key regulatory signal for growth on diverse carbon sources. Growth on fermentable carbon sources (e.g., glucose, fructose, and sucrose) requires a higher basal level of cAMP than does growth on nonfermentable carbon sources (e.g., ethanol, glycerol, and acetate). Therefore, the level of cAMP must decrease in order for cells to switch from growth on fermentable carbon sources to growth on nonfermentable carbon sources (the diauxic shift; ref. 1). Addition of glucose to yeast cells growing on a nonfermentable carbon source or starved for glucose results in a transient peak in intracellular cAMP levels. This transition to fermentation requires both the transient increase of cAMP and the cAMP-dependent PKA (2). Activated PKA shifts the metabolic flux away from gluconeogenesis and toward glycolysis by regulating key enzymes in these processes including fructose-1,6-bisphosphatase and phosphofructokinase-2 (3). Phosphorylation by PKA inactivates the transcription factor Adr1, a positive regulatory factor for the transcription of the respiratory enzyme Adh2 (4). In addition, PKA promotes the breakdown of glycogen and trehalose by inhibiting enzymes involved in synthesis (trehalose synthase and glycogen synthase) and activating enzymes involved in breakdown (trehalase and glycogen phosphorylase) of these storage carbohydrates.

The transcriptional changes occurring in the transition from fermentative growth to respiratory growth have been monitored by genome expression arrays (5). As glucose is depleted, transcription of genes involved in respiration, the tricarboxylic acid cycle, the glyoxylate cycle, gluconeogenesis, and storage carbohydrate synthesis is induced, whereas transcription of genes involved in glycolysis and protein synthesis is repressed (5). Consistent with the shift to respiratory growth, cytoplasmic ribosomal protein genes are repressed, and mitochondrial ribosomal genes are induced. In view of the role of cAMP and the PKAs in the use of carbon sources, these results raise the

question of whether the PKAs function in respiratory growth and whether they are redundant for this function.

To elucidate more broadly the functional differences between the PKA catalytic subunits, we compared the transcriptional profiles of wild-type, *tpk1*, *tpk2*, and *tpk3* strains by using genome-wide scans. We find that PKA transcriptionally regulates processes involved in iron uptake and respiration and that this regulation is specific to individual PKA catalytic subunits. Tpk2, but not Tpk1 or Tpk3, represses transcription of genes involved in high-affinity iron uptake. Tpk1 is required for the derepression of branched chain amino acid biosynthesis genes that seem to have a second role in the maintenance of iron levels and DNA stability within mitochondria. Aside from the previously known role in pseudohyphal development, no genes were identified that would predict functions for Tpk3.

Materials and Methods

Yeast Strains. Genotypes of the strains used are 10560-2B *MATa ura3-52 his3::hisG leu2::hisG*, LRY765 *MATa ura3-52 his3::hisG leu2::hisG tpk1::URA3*, LRY520 *MATa ura3-52 his3::hisG trp1::hisG tpk1::URA3*, LRY590 *MATa ura3-52 his3::hisG leu2::hisG tpk2::HIS3*, and LRY636 *MATa ura3-52 his3::hisG leu2::hisG tpk3::HIS3*. All strains are congenic to the Σ 1278b background.

Growth Media and Plate Phenotypes. Standard yeast genetic techniques and growth media were used (6). For Northern blotting and transcriptional profiling, strains were grown in liquid yeast extract/peptone/dextrose (YPD) at 30°C. For plate phenotypes, 10-fold dilutions of the strains 10560-2B, LRY765, LRY590, and LRY636 were spotted onto an agar plate and grown at 30°C. YPD is a yeast extract, peptone medium supplemented with 2% (wt/vol) glucose. YPEG is a yeast extract, peptone medium supplemented with 2% (wt/vol) ethanol and 2% (wt/vol) glycerol. Low-iron medium is synthetic minimal medium without ferric chloride and buffered to pH 7.0 with Mes-Tris. The iron chelator ferrozine was spread on top of agar medium to a final concentration of 0.5 mM.

Genome-Wide Transcriptional Profiling. The strains 10560-2B, LRY520, LRY765, LRY590, and LRY636 were used in the array experiments. Duplicate cultures for each strain were grown and processed separately. Yeast cultures grown in YPD were harvested during early to mid exponential phase. Total RNA was extracted, and polyadenylated RNA was selected from each sample. Target cRNA was prepared. cRNA was hybridized to high-density oligonucleotide arrays. The arrays were stained,

Abbreviations: PKA, protein kinase A; YPD, yeast extract/peptone/dextrose; YPEG, yeast extract/peptone/2% (vol/vol) ethanol/2% (vol/vol) glycerol.

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washed, and scanned by using the methods of D. J. Lockhart *et al.* (7) and L. Wodicka *et al.* (8). Expression measurements and scaling were done according to the method of Galitski *et al.* (9). The data were analyzed with the web-based tool GEAP developed by Tim Galitski and Alok Saldanha (Whitehead Institute). For the primary data analysis, a quantitative variation filter was applied requiring that the data from at least one strain showed an average difference greater than 100 for a gene to be considered in the data set. This filtered data set was then examined with nearest neighbor analysis with a Euclidean distance metric to find genes whose expression differed in only one mutant strain compared with both wild-type and the other two mutant strains. Random data sets ($n = 10$; within-gene permutations of the experimental data set) were analyzed with the same criteria to determine the distance for each input query that generated on average less than one false positive. This stringent requirement results in high specificity but low sensitivity. Two less stringent approaches were also used for secondary analysis: (i) genes whose expression changed at least 2-fold in a mutant strain compared with wild type and whose expression was consistent in duplicate experiments and (ii) genes from the unfiltered data set that are nearest neighbors as determined by a Euclidean distance metric of genes found in the preliminary analysis.

Northern Analysis. The strains 10560-2B, LRY765, LRY590, and LRY636 were used in the Northern analysis. Strains were grown in liquid YPD at 30°C to an OD₆₀₀ of ≈ 1.0 . Total RNA was harvested, and 10 μg of RNA was loaded per lane (10). Northern blots were probed with *FET3*, *FTR1*, *FRE1*, and *SIT1*. The entire open reading frames of these genes were PCR amplified from genomic DNA by using GENPAIRS obtained from Research Genetics (Huntsville, AL). The exposure times and temperatures were 45 min at -80°C for *FET3*, 15.5 h at -80°C for *FRE1* and *FTR1*, and 12 days at room temperature for *SIT1*.

Results

To identify genes regulated specifically by only one of the PKA catalytic subunits, we used deletion mutants lacking only one of the subunits but expressing the other two. Target cRNA from these strains was hybridized to high-density genomic arrays. Data analysis identified those genes whose expression had changed in one of the mutant strains but whose expression remained constant in the other data sets. This approach eliminated nonspecific PKA effects such as functions required for viability and functions mediated redundantly by the PKAs.

Expression Array Data. For each of the three mutant strains (*tpk1*, *tpk2*, and *tpk3*), the percentage of the genome affected was relatively small; expression increased at least 2-fold compared with that in the wild type for approximately 4% of the genome and decreased at least 2-fold for approximately 4%. This effect is similar to that of some transcription factors such as Gcn5, Swi2, or Srb10 but less than the overall change in transcription associated with the diauxic shift (5, 11). As glucose is depleted, transcription of approximately 12% of the genome is increased and decreased $\approx 18\%$ (5). Deletion of one catalytic subunit does not lead to increased expression of the other two subunits, showing that cells do not compensate for the loss of one catalytic subunit by overexpression of another. For each of the mutants, ≈ 50 different genes were identified that showed a unique *tpk*-specific expression profile.

Our array data confirmed several previously made observations. In particular, the *FLO11* expression pattern obtained from arrays was very similar to that previously uncovered by Northern blot analysis (12): *FLO11* expression was essentially unchanged in a *TPK1* mutant ($0.8\times$ that of wild type), drastically reduced in a *TPK2* mutant ($0.05\times$ that of wild type), and increased in a *TPK3* mutant ($2.6\times$ that of wild type). Another cell-surface

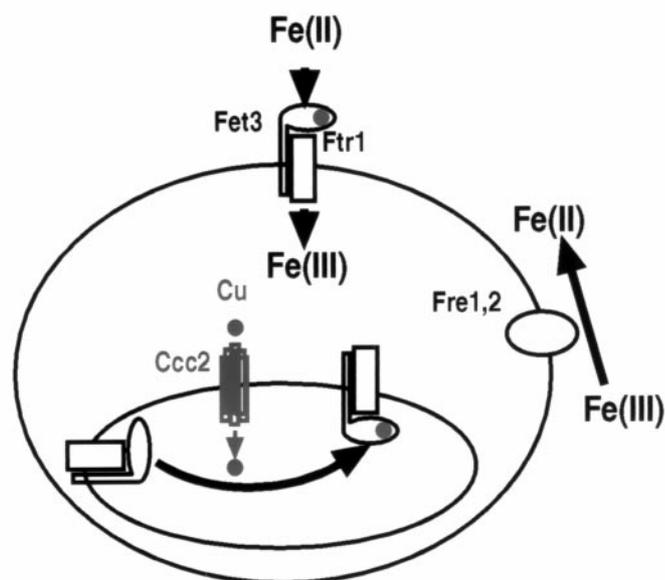


Fig. 1. High-affinity iron uptake in yeast. Insoluble, extracellular Fe(III) is reduced to Fe(II) by the plasma membrane ferric reductases Fre1 and Fre2. Iron is then transported into the cell by a plasma membrane complex consisting of the multicopper oxidase Fet3 and the iron permease Ftr1. Ftr1 transports Fe(III), and Fet3 oxidizes Fe(II) to Fe(III) to allow transport by Ftr1. Fet3 requires copper for activity. The copper transporter Ccc2 is required for the copper-loading of Fet3 in the late Golgi.

flocculin gene, *FLO10*, showed a very similar expression pattern: 1.3-fold increase in *tpk1* strains, an approximately 3-fold decrease in *tpk2* strains ($0.3\times$ that of wild type), and a 12.7-fold increase in *tpk3* strains. The expression of other known flocculin genes, *FLO1*, *FLO5*, *FLO8*, and *FLO9*, was essentially unchanged in the *TPK* mutants.

Tpk2 Regulates Iron Uptake. Tpk2 function is required for transcriptional repression of the entire high-affinity iron-uptake pathway (Fig. 1 and Table 1). The array data show that expression of *FTR1* and *FRE2* is more than 2-fold higher and that expression of *FET3* and *CCC2* was also higher in a *TPK2* mutant. Fre1 and Fre2 are plasma membrane ferric reductases (13, 14) that reduce insoluble, extracellular iron Fe(III) to soluble Fe(II). The soluble iron is transported into the cell via a high-affinity system consisting of the plasma membrane complex of the multicopper oxidase Fet3 (the yeast homolog of ceruloplasmin) and the transporter Ftr1. Ftr1 transports the oxidized form Fe(III); this reoxidation of Fe(II) to Fe(III) is catalyzed by Fet3 (15, 16). The P type ATPase Ccc2 (the yeast homolog of Menkes–Wilson protein) is required for loading copper onto Fet3 (17).

The connection of Tpk2 to iron uptake was strengthened by the finding that expression of *SIT1* (siderophore iron transport), a gene whose function is required for the uptake of the siderophore ferrioxamine B (18), is also increased in *tpk2* strains. *SIT1* is one of four genes in the major facilitator super family/multidrug resistance family that have greater than 2-fold increased expression in *TPK2* mutants. This large family of putative permeases and transporters is predicted to include 186 yeast proteins; however, these four genes (*YOL158c*, *SIT1*, *YHL047c*, and *ARN1*) are closely related and form a distinct subgroup based solely on sequence (19). Because transcription of *YOL158c*, *YHL047c*, and *ARN1* is regulated in a fashion similar to that of the high-affinity iron-uptake pathway and *SIT1*, these three genes may also be involved in iron uptake. The regulation

Table 1. Tpk2 regulates the high-affinity iron-uptake pathway

Gene	Function
High-affinity iron uptake	
Fre1	Ferric reductase, plasma membrane
Fre2	Ferric reductase, plasma membrane
Fet3	Multicopper ferroxidase, plasma membrane
Ftr1	Iron permease, plasma membrane
Ccc2	Copper transporter
Low-affinity iron uptake	
Fet4	Low-affinity Fe(II) transporter
Vacuole iron transport	
Fth1	Vacuolar iron transporter, <i>FTR1</i> homolog
Fet5	Multicopper oxidase, vacuole
Mitochondrial iron homeostasis	
Atm1	ABC transporter, mitochondria
Yfh1	Yeast frataxin homolog
Putative iron transporters	
Sit1	Siderophore iron transport
Arn1	MFS/MDR family
Yol158c	MFS/MDR family
Yhl047c	MFS/MDR family

Proteins involved in iron transport are listed above. Those shown in bold are negatively regulated by Tpk2. ABC, ATP-binding cassette; MFS/MDR, major facilitator super family/multidrug resistance.

of iron-uptake genes by Tpk2 was confirmed by Northern blotting (Fig. 2).

Tpk1 Regulates Genes of the Branched Chain Amino Acid Pathway That Also Function in Mitochondrial Iron Homeostasis, Mitochondrial DNA Stability, and Exit from Stationary Phase. Tpk1 is required for the derepression of both *BAT1* and *ILV5*, because expression of *BAT1* is reduced 2.4-fold and expression of *ILV5* is reduced 1.4-fold in a *tpk1* strain compared with that in wild type. Bat1 is the mitochondrial branched chain amino acid transaminase (20, 21). Ilv5 is a keto acid reductoisomerase that catalyzes an early step in the biosynthesis of valine, isoleucine, and leucine. In addition to their roles in branched chain amino acid biosynthesis, *BAT1* seems to be involved in exit from stationary phase. On exit from stationary phase, there is a transient spike in cAMP, activation of the Tpk, and cellular reprogramming of transcription that mediates the return to growth. Cells that are deficient in one of the two *BAT* genes have no obvious growth reduction during exponential growth but are slow to leave stationary phase in comparison with wild-type cells (21).

Bat1 may also play a role in maintaining mitochondrial iron homeostasis and mitochondrial DNA stability via an interaction with Atm1. Atm1, an ABC transporter required for iron homeostasis, is located in the mitochondrial inner membrane (22).

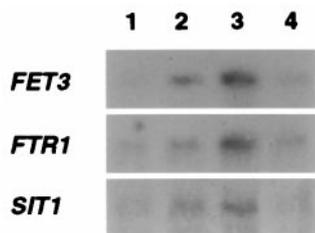


Fig. 2. Tpk2 negatively regulates genes involved in iron uptake. Total RNA was probed with *FET3*, *FTR1*, or *SIT1* by Northern blotting. (Lane 1) 10560-2B (*TPK*). (Lane 2) LRY765 (*tpk1*). (Lane 3) LRY590 (*tpk2*). (Lane 4) LRY636 (*tpk3*).

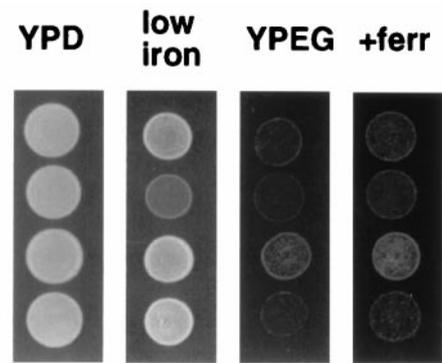


Fig. 3. Growth of *TPK* mutants on different media. Strains were grown on YPD (iron-rich, glucose medium), high-glucose/low-iron media (low iron), YPEG (ethanol glycerol medium), and YPEG containing the iron chelator ferrozine (+ferr). From the top, the strains are 10560-2B (wild type), LRY765 (*tpk1*), LRY590 (*tpk2*), and LRY636 (*tpk3*).

Cells lacking Atm1 accumulate very high levels of iron in their mitochondria and are unable to grow on nonfermentable carbon sources (23). Overexpression of *BAT1* is believed to stabilize the temperature-sensitive Atm1 at the nonpermissive temperature (21). One model that explains these data is that, in the absence of Tpk1, *BAT1* expression is reduced and the level of iron in the mitochondrion rises. High levels of iron result in increased loss of mitochondrial DNA and consequently loss of mitochondrial function.

Null mutations in *ILV5* result in the ρ^- petite phenotype in which large segments of the mitochondrial genome are deleted (24). Overexpression of *ILV5*, but not *ILV2*, another branched chain amino acid pathway gene, suppresses the mutant phenotype of *abf2* strains (24). Abf2 is a DNA-binding protein required for the maintenance of mtDNA on glucose (25, 26). *ABF2* null mutants are deficient in respiration (25). Together, these facts suggest that Ilv5 is required for the stability of the mitochondrial genome and that Tpk1 regulates mitochondrial proteins key to this process.

Respiratory Growth Phenotypes of *TPK* Mutants. Wild-type, *TPK1*, *TPK2*, and *TPK3* mutant strains were grown on ethanol/glycerol medium in the presence and absence of the iron chelator ferrozine to test for phenotypic consequences of the transcriptional effects seen in the array experiments. Because iron is required as a cofactor for several respiratory enzymes, *TPK2* mutants, which overexpress these iron-uptake genes, might show enhanced growth on medium that forces yeast to respire. Reciprocally, *TPK1* mutants may have a growth defect on medium that forces yeast to respire, because Tpk1 is required for derepression of *BAT1* and *ILV5*, genes that may regulate respiratory function through their role in maintenance of mitochondrial iron levels and mitochondrial DNA.

Remarkably, *TPK2* mutants grow better than wild types on ethanol/glycerol medium, and this difference is enhanced in the presence of ferrozine (Fig. 3). In contrast, *TPK1* mutants have a growth defect on ethanol/glycerol, ethanol/glycerol containing ferrozine, and low-iron/glucose media. These phenotypes support opposing functions for Tpk1 and Tpk2 in respiration.

Expression of the Neutral Trehalase *NTH1* Is Decreased in a *TPK2* Mutant. Expression of *NTH1*, whose product breaks down trehalose into its constituent glucose molecules, is reduced ≈ 3 -fold in a *TPK2* mutant. Trehalose is a storage carbohydrate also involved in resistance to stress. Activity of Nth1 decreases 95% at the diauxic shift at the same time that Nth1 is dephosphory-

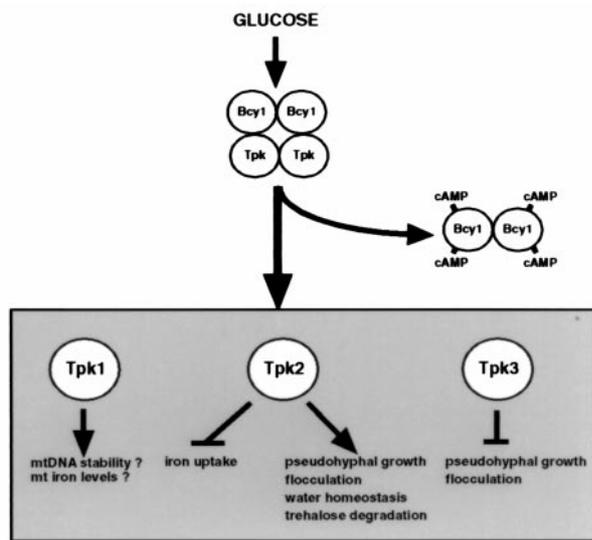


Fig. 4. Model showing functional specificity of the three PKAs. See text for details.

lated (27). Nth1 contains two consensus PKA phosphorylation sites (RRxS), and its activity *in vitro* is stimulated by cAMP (28). We have shown that in addition to this possible posttranslational activation by PKA, transcription of *NTH1* is also positively regulated by Tpk2.

Expression of the Aquaporin *AQY2* Is Decreased in a *TPK2* Mutant.

AQY2 expression is reduced ≈ 3 -fold in a *TPK2* mutant. Aquaporins are involved in the maintenance of water homeostasis in cells. There are four aquaporin family genes in yeast: two aquaglyceroproteins permeable to both water and glycerol, *FPS1* and *YFL054*, and two orthodox aquaporins permeable only to water, *AQY1* and *AQY2*. The two orthodox aquaporins Aqy1 and Aqy2 are both nonfunctional in the laboratory strain S288c but are functional water channels in the strains we profiled ($\Sigma 1278b$; refs. 29 and 30). In mammalian cells, aquaporins have been shown to be regulated both transcriptionally and posttranslationally by PKA (31). We have shown that *AQY2*, but not *AQY1*, is transcriptionally regulated by the Tpk2 kinase. Although the subcellular localization of these aquaporins is not known, it is intriguing to consider that Aqy2 may be responsible for uptake of water into the vacuole. As the vacuole is an important organelle for iron and copper detoxification (32), it is reasonable to posit that the regulation of *AQY2* by Tpk2 is another signature of the iron-uptake pathway.

Discussion

The use of expression arrays coupled with mutants has permitted us to parse the differences between the three PKAs. This analysis emphasizes that Tpk1, Tpk2, and Tpk3 are not functionally redundant, despite a high level of sequence identity at the amino acid level and overlapping roles in viability and many other functions. Use of expression arrays allowed us to identify those genes that are differentially regulated: Tpk2 specifically regulates genes involved in iron uptake, trehalose breakdown, and water homeostasis; Tpk1 specifically regulates a distinct set of genes with a putative role in respiration (Fig. 4). The ensemble of genes affected by mutations in the Tpk3 is consistent with previous studies implicating these proteins in carbon use and respiration.

Expression of the high-affinity iron-uptake pathway (*FRE2*, *FET3*, *FTR1*, and *CCC2*) as well as expression of a family of genes related to the siderophore-uptake gene *SITI* are increased in *TPK2* mutants. This finding is consistent with the growth

phenotypes we uncovered—*TPK2* mutants grow better than wild types on ethanol/glycerol medium, and this differential growth is enhanced further by the addition of ferrozine. Our data also support the finding that strains whose only active PKA is Tpk2 (in *tpk1 TPK2 tpk3 bcy1* strains) are defective for growth on acetate (as compared with wild types or strains whose only active PKA is Tpk1 or Tpk3; ref. 33). Thus, Tpk2 seems to inhibit respiratory growth through the negative regulation of iron uptake.

Data obtained from transcriptional profiling of the mutants are consistent with the following model that connects respiration and iron metabolism. During fermentative growth on glucose, Tpk2, activated by cAMP, represses genes involved in iron metabolism. As glucose is depleted, Tpk2 activity is inhibited, thereby relieving the repression of the iron transport systems. Derepression results in transport of iron into the cell where it is incorporated into respiratory enzymes that permit growth on nonfermentable carbon sources accumulating in the medium as the cells transit into diauxie. Iron transport must be carefully regulated, because excess intracellular iron results in the generation of hydroxyl radicals that are toxic.

Our model supports previous work suggesting that the iron-regulated transcription factor Aft1 regulates high-affinity iron uptake. *FRE2*, *FET3*, *FTR1*, and *CCC2* are transcriptionally activated by Aft1 under conditions of iron deprivation, and *FRE2* mRNA is undetectable in an *aft1* mutant (34). *FRE2*, *FET3*, *FTR1*, and *CCC2*, which are under Tpk2 control, contain Aft1 consensus binding sites in their promoter regions, and Aft1 has been shown to bind these sites (35). *AFT1* null mutants grow on fermentable carbon sources but not on nonfermentable carbon sources (34). This inability to grow on nonfermentable carbon sources is suppressed by the addition of ferrous iron to the growth medium, suggesting that the *aft1* growth defect is due to poor iron uptake (34). The simplest explanation for this circuitry would be that Tpk2 negatively regulates Aft1 activity, which is required for expression of these high-affinity iron-uptake genes after the switch to respiratory growth.

This model is in apparent conflict with the report that transcription of *FRE1*, *FET3*, and *FTR1* decreased at least 2-fold as glucose is depleted (5), which would suggest that iron uptake decreases when cells have exhausted the available fermentable carbon sources. However, our experiments are not strictly comparable with this previous work. Our array experiments were done with mid exponential growth cultures ($OD_{600} = 1.0$) of *TPK* mutants, as opposed to high-density cultures ($OD_{600} = 4.0$ – 8.0) of wild-type strains. Moreover, it is possible that these high-density cells have not yet depleted their internal iron pools. It is known that after a shift to conditions of external iron deprivation, cells do not increase iron uptake until their internal supplies of iron are depleted, up to four doublings after the shift (14). In the time course carried out to study the diauxic shift (5), the cells start to respire at a high density and do not double many times thereafter. Under these conditions, internal pools of iron are probably adequate to support respiration in the absence of increased iron uptake.

Transcriptional profiling of mutants is a way to distinguish among genes with apparently redundant functions. Previously, redundancy was assumed if the members of a gene family had significant amino acid identity, carried out the same biochemical reaction, and could supply the function missing in a strain defective for the function of one of the members. However, the functional universe was limited to a few phenotypes. Transcriptional profiling of each family member mutant provides a unique signature of thousands of phenotypes. Profiles of mutants for each family member can be compared to determine similarities or differences. Thus, transcriptional profiling redefines “redundancy:” two family members are not redundant if they display different transcriptional profiles.

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