

# Influence of genotype and nutrition on survival and metabolism of starving yeast

Viktor M. Boer, Sasan Amini, and David Botstein\*

Lewis-Sigler Institute for Integrative Genomics and Department of Molecular Biology, Princeton University, Princeton, NJ 08544

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**Starvation of yeast cultures limited by auxotrophic requirements results in glucose wasting and failure to achieve prompt cell-cycle arrest when compared with starvation for basic natural nutrients like phosphate or sulfate. We measured the survival of yeast auxotrophs upon starvation for different nutrients and found substantial differences: When deprived of leucine or uracil, viability declined exponentially with a half-life of <2 days, whereas when the same strains were deprived of phosphate or sulfate, the half-life was  $\approx 10$  days. The survival rates of nongrowing auxotrophs deprived of uracil or leucine depended on the carbon source available during starvation, but were independent of the carbon source during prior growth. We performed an enrichment procedure for mutants that suppress lethality during auxotrophy starvation. We repeatedly found loss-of-function mutations in a number of functionally related genes. Mutations in *PPM1*, which methylates protein phosphatase 2A, and target of rapamycin (*TOR1*) were characterized further. Deletion of *PPM1* almost completely suppressed the rapid lethality and substantially suppressed glucose wasting during starvation for leucine or uracil. Suppression by a deletion of *TOR1* was less complete. We suggest that, similar to the Warburg effect observed in tumor cells, starving yeast auxotrophs wastes glucose as a consequence of the failure of conserved growth control pathways. Furthermore, we suggest that our results on condition-dependent chronological lifespan have important implications for the interpretation and design of studies on chronological aging.**

auxotrophy starvation | chronological aging | Warburg effect

To grow and survive, all cells must sense and respond to the availability of nutrients in their environment. In budding yeast (*Saccharomyces cerevisiae*), the means by which cells sense and respond to the availability of sources of nitrogen, phosphate, sulfate, and fermentable sugars are well studied. The nutrient sensor and assimilation pathways are known to dynamically modify activities of diverse cellular functions, including nutrient utilization, ribosome biosynthesis, and stress responses (1–4). The onset of cell division is directly dependent on the output of these pathways: When inadequate carbon, nitrogen, sulfur, or phosphorus is detected in its environment, it arrests at the start ( $G_0/G_1$ ) of the cell cycle (5–7). Only under favorable conditions will progression through the cell division cycle proceed (8).

The failure to commit to the cell division cycle, and entering instead a resting or quiescent state, has always been assumed to be a survival strategy in the face of inadequate nutrition (6). This assumption is entirely consistent with findings that pathways that stimulate growth and division in nutrient-rich conditions, like target of rapamycin (TOR) and protein kinase A (PKA), repress entry into a resting state. Moreover, pathways that activate entry into a resting state, such as protein kinase C (PKC) and Snf1, are repressed by glucose, arguably the strongest activator of growth (4, 9). Thus, it came as no surprise that mutations in genes involved in these signaling pathways and their downstream targets have been found to affect long-term viability (9–12).

In a comparison with several nutrients, Saldanha *et al.* (13) observed that cells respond differently to starvation for a nutrient made necessary by mutation (leucine in a leucine

auxotroph or uracil in a uracil auxotroph), compared with a “natural” starvation for phosphate or sulfate. Notably, in the latter cases, nearly all of the cells in the culture appeared to have arrested in  $G_0/G_1$  (unbudded), whereas there was no similarly rapid, coordinated arrest upon starvation for leucine and uracil in the respective auxotrophs. In contrast, when cultures of exponentially growing cells in nutrient-limited chemostats were compared with the same limiting nutrients (2), no difference in the budding index between the natural and auxotrophic limitations was observed. Brauer *et al.* (2), however, observed that chemostat cultures limited for uracil or leucine differed from those limited by phosphate, sulfate, or ammonium in another way: The latter conserved much of the excess glucose available to them, whereas the former completely fermented the excess glucose to ethanol.

Both the lack of a uniform arrest during starvation of auxotrophs and the wasting of excess glucose suggest a failure of regulation. Specifically, they raise the possibility that one or more signals given during starvation for the natural nutrients, leading to cell-cycle arrest and cessation of glucose fermentation, are lacking when a nutrient made necessary only by a mutation runs out. It seemed likely that this failure of regulation might have consequences for the viability of cells that were starved of auxotrophic nutrients.

Here we show that starvation does indeed have differential effects on viability depending on the nature of the missing nutrient. Several auxotrophic strains we tested die exponentially when deprived of their cognate nutrients, whereas the same strains survive when deprived of phosphate or sulfate; the degree of the difference depends on the carbon source. We selected mutants that survive auxotrophic starvation for leucine and repeatedly found null mutations in *PPM1* and *TOR1*. Our data further point to a major involvement of protein phosphatase 2A (PPA2) as a central component in the response upon auxotrophy starvation.

## Results

We determined the viability of yeast cells in dilute, nonsaturating media, where only a single nutrient limits growth. The survival of cells starved for phosphate, sulfate, uracil, or leucine was tested in triplicate in a single strain (DBY8863) doubly auxotrophic for leucine and uracil. In each case, this strain was growing in the appropriate nutrient-limited medium before being completely deprived of the nutrient by dilution, as described in *Materials and Methods*. Fig. 1 shows that the cells

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\*To whom correspondence should be addressed at: Carl Icahn Laboratory, Lewis-Sigler Institute, Princeton University, Washington Road, Princeton, NJ 08544. E-mail: botstein@genomics.princeton.edu.

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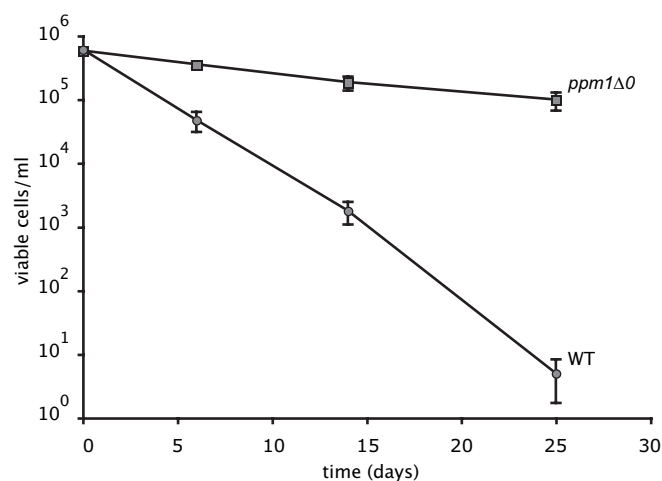


**Table 1. Viability during nutrient starvation of wild-type and mutant strains**

Strain	Limitation	Carbon source	Half-life, days
DBY8863	Phosphate	Glucose	11
	Sulfate	Glucose	9.4
	Leucine	Glucose	1.9
	Uracil	Glucose	0.8
	Leucine	Galactose	2.8
BY4742	Leucine	Ethanol plus glycerol	11
	Leucine	Glucose	2.0
<i>ppm1</i> Δ0	Uracil	Glucose	0.7
	Leucine	Glucose	8.6
<i>tor1</i> Δ0	Uracil	Glucose	2.6
	Leucine	Glucose	2.4
	Uracil	Glucose	1.0

strains, we found frameshift mutations introducing premature STOP codons (a single-bp deletion at bp 1,333,843 introducing a STOP codon at L49 in DBY11202 and a 71-bp deletion starting at bp 1,333,461 introducing a STOP codon at L146 in DBY11203).

Another 12 independent mutants were isolated, and all were resequenced by using tiling arrays and Sanger sequencing (15). The mutations that were found in annotated genes are listed in Table 2. Mutations in some genes were found more than once and thus are likely to be causative. In addition to the two truncating *ppm1* mutations, we found three independent truncating mutations in *TOR1*, one truncating and one missense mutation in *SCH9*, one nonsense and one missense mutation in *HRD1*, and one nonsense and one missense mutation in *HAP1*. Initial tests of the ability of these spontaneous mutant strains to survive leucine starvation suggested that the *ppm1* mutations had the strongest effect of restoring survival to levels found with sulfate or phosphate starvation, followed by *sch9* and *tor1* mutations; the *hap1* and *hrd1* mutations had very minor effects. Below, we further characterize the effect of loss of function of *ppm1* and *tor1*, recognizing that it is likely that many of the other genes in which we recovered mutations also may be involved in starvation survival. Specifically, the *sch9* mutants seem to confer significant resistance (half-life on leucine-limited glucose me-



**Fig. 4.** Survival of strain DBY8863 (circles) and isogenic *ppm1*Δ0 derivative (squares) during leucine starvation, with mean and SD of six independent replicates.

dium is >5 days); however, the severe growth defect of the *sch9* deletion strain (16) compromises a straightforward comparison.

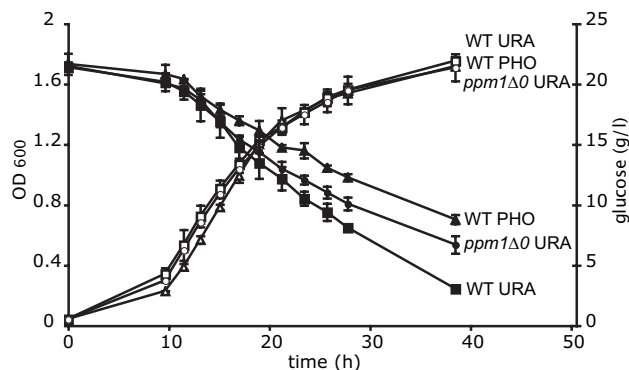
To confirm more rigorously that the *ppm1* and *tor1* mutations and not other bp differences (whether detected or not) in the strains we recovered are responsible for the enhanced survival, we tested the survival of constructed *ppm1*- and *tor1*-deletion mutants. Fig. 4 shows survival during leucine starvation in a *ppm1*-deletion mutant and the isogenic parent DBY8863. Fig. S2 shows results for a *ppm1*- and *tor1*-deletion strain from the systematic yeast-deletion project (17), tested for survival upon leucine and uracil starvation; the half-lives also are given in Table 1. The isogenic parent strain (BY4742) dies at about the same rate as the closely related strain DBY8663, which was the parental strain in our selection (2.0 days for leucine and 0.7 day for uracil). Survival of the *ppm1*Δ0 mutant is considerably higher than wild type (half-life of 8.6 days for leucine and 2.6 days for uracil). Survival of the *tor1*Δ0 mutant is only slightly higher than wild type (half-life of 2.4 days for leucine and 1 day for uracil). These results strongly support a causal role for *ppm1*-null mutations in survival upon auxotrophy starvation, which is

**Table 2. Mutations found in isolated strains with enhanced survival upon leucine starvation**

Gene	Mutation (chr:bp)	Protein	Parent	Strain	Other mutations in
<i>PPM1</i>	4:1333843 A to Δ	L49X	1	DBY11202	CPR3, GCD1, ACF4, KEX1
<i>PPM1</i>	4:1333461 71bpΔ	L146X	1	DBY11203	
<i>TOR1</i>	10:559755 C to Δ	S116X	1	DBY11204	
<i>TOR1</i>	10:563920*	S1509X	1	DBY11205	
<i>TOR1</i>	10:565483 C to G	Y2025X	1	DBY11206	ADP1
<i>SCH9</i>	8:511331 G to A	G657S	2	DBY11211	SSK22
<i>SCH9</i>	8:509689 C to AA	N125X	2	DBY11214	
<i>HRD1</i>	15:302637 C to T	Q134X	1	DBY11207	
<i>HRD1</i>	15:301850 G to T	C396F	1	DBY11208	CDC60
<i>HAP1</i>	12:646559 C to G	S48X	2	DBY11213	AUS1
<i>HAP1</i>	12:647879 T to G	F488C	2	DBY11215	
<i>KOG1</i>	8:480255 T to C	S140 P	2	DBY11212	
<i>TPD3</i>	1:126722 G to C	D615H	1	DBY11210	FLO1, FLO5
<i>HRD3</i>	12:557609 G to T	G274C	1	DBY11210	FLO1, FLO5
			1	DBY11209	UBR2, 15:1042k to end

Genes contributing to the gene neighborhood (Fig. 6) are given in the first column. Parent 1, FY strain DBY8863, parent 2, SK1 strain NKY1060. These 14 strains were randomly chosen from 27 independent selections.

\*TTACGA to CTTCTTA



**Fig. 5.** Growth and glucose consumption during phosphate and uracil starvation. Triangles, strain DBY8863 during phosphate starvation; squares, strain DBY8863 during uracil starvation; circles, isogenic *ppm1Δ0* deletion strain during uracil starvation; open symbols, OD<sub>600</sub>; closed symbols, glucose concentration. Values represent averages and SD of two (DBY8863) or four (*ppm1Δ0* deletion strain) independent experiments.

consistent with the repeated recovery of null mutations in the selection. The situation for *tor1* mutants is less clear. The three independent spontaneous *tor1* mutants we recovered had half-lives of 3.9 ( $\pm 0.3$ ) days on leucine-limited media. The modest suppression of the *tor1* deletion in the BY background could be due to strain differences (e.g., because of the relative contribution of Tor2p in TOR complex 1) or, alternatively, mutations that were not detected in our screen.

Finally, we tested whether the *ppm1* mutations had any effect on the glucose-wasting phenomenon previously associated with starvation for leucine and uracil, compared with starvation for phosphate or sulfate. Fig. 5 shows that, during uracil starvation, our *ppm1*-deletion strain consumes significantly less excess glucose than its isogenic parent, but more compared with exponential growth of the parent strain during phosphate starvation. Fig. S3 shows the same for leucine starvation.

## Discussion

**Survival of Nongrowing Cells Depends on the Nutritional Limitation and the Carbon Source.** Our results show that starvation for auxotrophic requirements is substantially more lethal than starvation for phosphate or sulfate. Just by changing the limiting nutrient, we found  $\geq 10$ -fold differences in exponential survival rate (e.g., between phosphate and uracil starvation in Fig. 1). Hence, an important aspect of chronological aging has gone largely unnoticed so far: Yeast viability depends on which nutrient is limited first. Therefore, it seems clear that rigorous control of conditions is required while studying chronological aging specifically because gene effects previously found to affect chronological aging (10, 12) are often smaller than the conditional effects we have found here. Moreover, all studies on chronological aging in *S. cerevisiae* published so far have made use of auxotrophic strains (usually including leucine, histidine, and uracil as auxotrophic requirements). Assays that have been used in determining chronological lifespan almost uniformly involved growing auxotrophic strains to high density in YPD or synthetic complete medium and subsequent starvation in the same medium. Alternatively, cells were transferred to water after growth to saturation (reviewed in refs. 18 and 19). In both experimental designs, one cannot tell which nutrient runs out first, and it is unclear to the absence of what nutrient(s) the cells responds. As we have shown, the nature of the limiting nutrient determines lifespan. Thus, the techniques of measurement of chronological aging require a rigorous reassessment.

The rapid loss of viability during starvation for leucine is, in part, dependent on the carbon source(s) available during star-

vation. Changing the carbon source from glucose to galactose or ethanol/glycerol, which are less preferred sources of carbon, drastically increased the lifespan during starvation. These results strengthen the notion that growth-stimulating pathways that are activated by glucose (TOR and PKA) have a negative effect on lifespan (9, 12, 20), whereas glucose represses pathways that activate entry into a resting state (4, 9). It is significant that the carbon source matters only during the starvation: The carbon source available before starvation makes no detectable difference, ruling out prior metabolic activity or growth rate as a factor influencing viability.

**Amino Acid Starvation and the Cell Division Cycle.** Survival during starvation of auxotrophs has been studied previously in a number of organisms, including *Neurospora* (21), *Escherichia coli* (22), and *S. cerevisiae* (23, 24), with varying survival rates. In *S. cerevisiae*, Henry (24) reported very rapid loss of viability of *S. cerevisiae* inositol auxotrophs (hundreds of fold per day) and only a modest loss of viability upon lysine, adenine and tryptophan starvation. The rates of loss of viability found by Henry (24) for amino acid starvation appear to be comparable to our observation for leucine and uracil; for inositol starvation, the rate is markedly higher. We also tested histidine auxotrophs with very similar results (data not shown). An exception to the pattern that yeast auxotrophs die rapidly upon starvation seems to be methionine. When Unger and Hartwell (5) observed that methionine starvation of a methionine auxotroph results in G<sub>0</sub>/G<sub>1</sub> arrest, they reported no loss of viability. They proposed that methionine or a product of methionine (e.g., methionyl-tRNA) acts as a more general signal for starvation and also is involved in signaling sulfate starvation (5).

Following the reasoning of Unger and Hartwell (5), the lethality observed with auxotrophic starvations results, in part, from a failure to execute a cell-cycle arrest; the prompt arrest of the cell cycle during starvation for methionine or the natural nutrients phosphate or sulfate serves to put cells into a "safe" quiescent state. This idea is in accord with our observation that auxotrophs starved for phosphate or sulfate results in low lethality. We suppose further that the lethality depends on metabolic activities (e.g., initiation of DNA replication) inappropriate to the circumstances that happen to a greater degree when good carbon sources are available.

**Loss-of-Function Mutations in a Number of Genes Can Suppress Lethality During Auxotrophy Starvation.** Identifying the genetic basis of new phenotypes is a serious challenge in biological studies. With the use of recently developed microarray technology, we were able to identify causal mutations in strains with acquired longer lifespans. The mutations that we recovered that suppress the lethality of auxotrophic starvation provide clues to the identity of regulatory pathways involved. We repeatedly isolated mutations in three genes (*PPM1*, *TOR1*, and *SCH9*) that significantly suppress the lethality phenotype. Two independently derived *ppm1* deletions suppress lethality, resulting in survival over time close to that observed for phosphate starvation. The *ppm1* deletion also suppresses the elevated glucose consumption during auxotrophy-limited growth, although not completely. In the case of *TOR1*, a deletion suppresses the lethality phenotype, but significantly less well.

In addition to the use of new microarray technology, we also made use of recently developed bioinformatic tools. The bioPIXIE program (25) summarizes evidence for gene interactions by using Bayesian analysis of diverse kinds of experimental data, including coexpression, synthetic lethality, and more direct biochemical protein interaction data. Fig. 6 summarizes the genes in which we found mutations and the closest associated genes, as inferred by using the bioPIXIE program. We found that, in many mutants, the suppression of lethality is the result



manganese, and zinc sulfate, and, hence, time 0 was set 2 days after the last transfer.

**Enrichment for Mutants with Enhanced Survival upon Leucine Starvation.** Strains DBY8863 and NKY1060 were grown in leucine-limited medium, and viability was assayed over time. When the total fraction of viable cells was  $<5 \times 10^{-4}$ ,  $\approx 100$ –1,000 viable cells were transferred to phosphate-limited medium to limit selection for strains that simply grow faster or recover from arrest more quickly in the selection media. After several days, this culture was used as an inoculum for a new round of leucine starvation. Between five and seven rounds of enrichment were performed, after which single colonies were isolated and assayed for viability.

**Mutation Detection.** DNA of one clone of each enrichment was hybridized to Affymetrix yeast tiling arrays and analyzed for mutations as described previously (15). SNP predictions in ORFs were confirmed by PCR and Sanger sequencing (in duplicate).

**Linkage Analysis.** Linkage analysis was performed by using a strategy described previously (14). In short, strain DBY11202 was backcrossed twice to get a 2:2 segregation (resistant; high viability upon leucine starvation, sensitive; low viability). One of the resistant segregants was crossed to the sensitive, divergent SK1 derivative strain NKY1060. After sporulation, segregants were scored for viability upon leucine starvation. DNA was isolated from the

resistant and sensitive pools (segregants with intermediate resistance were omitted) and hybridized to Affymetrix tiling arrays (15). For estimation of the linkage peak, only the probes were used that have known differences between FY and SK1 strains (38).

**Assembly of the Gene Neighborhood (Fig. 6).** To place the mutations found in the genetic screen into a biological context, we used a program called bioPIXIE (25). All genes affected by mutations (Table 2, excluding *CPR3*, *GCD1*, *ACF4*, and *KEX1* because in this strain it was established that *PPM1* is causal) were used as input in bioPIXIE to assemble the gene neighborhood and to generate the accompanying gene rank list (Table S1). The top nine ranking genes of the gene neighborhood remained identical when only using a subset of all genes (Table 2, column 1) as input. Hence, this subset contributes most strongly to the gene neighborhood. The graph obtained from bioPIXIE was redrawn retaining the gene interaction information between the top-ranking genes in the neighborhood and the genes in Table 2. The connection between TOR1 and SCH9 was added based on evidence published previously (39).

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