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Characterization of Differentiated Quiescent and Nonquiescent Cells in Yeast Stationary-Phase Cultures

Anthony D. Aragon,* Angelina L. Rodriguez,* Osorio Meirelles,† Sushmita Roy,‡ George S. Davidson,§ Phillip H. Tapia,* Chris Allen,‖ Ray Joe,* Don Benn,* and Margaret Werner-Washburne*

Departments of *Biology, †Math and Statistics, ‡Computer Science, University of New Mexico, Albuquerque, NM 87131; §Sandia National Laboratories, Albuquerque NM 87185; and ‖Department of Cytometry, University of New Mexico Health Sciences Center, Albuquerque, NM 87131

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Cells in glucose-limited *Saccharomyces cerevisiae* cultures differentiate into quiescent (Q) and nonquiescent (NQ) fractions before entering stationary phase. To understand this differentiation, Q and NQ cells from 101 deletion-mutant strains were tested for viability and reproductive capacity. Eleven mutants that affected one or both phenotypes in Q or NQ fractions were identified. NQ fractions exhibit a high level of petite colonies, and nine mutants affecting this phenotype were identified. Microarray analysis revealed >1300 mRNAs distinguished Q from NQ fractions. Q cell-specific mRNAs encode proteins involved in membrane maintenance, oxidative stress response, and signal transduction. NQ-cell mRNAs, consistent with apoptosis in these cells, encode proteins involved in Ty-element transposition and DNA recombination. More than 2000 protease-released mRNAs were identified only in Q cells, consistent with these cells being physiologically poised to respond to environmental changes. Our results indicate that Q and NQ cells differentiate significantly, with Q cells providing genomic stability and NQ cells providing nutrients to Q cells and a regular source of genetic diversity through mutation and transposition. These studies are relevant to chronological aging, cell cycle, and genome evolution, and they provide insight into complex responses that even simple organisms have to starvation.

INTRODUCTION

Most cells on earth exist in a nondividing, quiescent state, often typified by low metabolic activity and arrest in an unbudded, relatively unstudied state (Gray *et al*., 2004). In eukaryotes, this state is also referred to as G0, and it is exemplified by stem cells, neurons, eggs, and spores. We know less about the quiescent state than about dividing cells, because both this state and the biogenesis of cells in this state have been difficult to study. However, understanding this state is important, because it plays a critical role in normal metazoan development and disease, including the development of cancer stem cells in solid tissues (Kim *et al*., 2005; Suda *et al*., 2005; Abbott, 2006) and the persistence of long-lived spores in infections such as tuberculosis, cryptosporesis, and anthracis (Murray, 1999; Gray *et al*., 2004). Finally, the quiescent state is important in the environment, including the 99.9% of all microbes that exist in an unculturable, quiescent state (Kaeberlein *et al*., 2002). Fortunately, several recent developments, including the ability to use genome-scale tools to understand this state, have facilitated studies into the quiescent state, especially in yeast (Allen *et al*., 2006; Yang *et al*., 2006).

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Address correspondence to: Dr. Margaret Werner-Washburne (maggieww@unm.edu).

Abbreviations used: NQ, nonquiescent; Q, quiescent; SP, stationary phase.

We recently reported the isolation of quiescent (Q) and nonquiescent (NQ) cells from stationary phase (SP) yeast cultures (Allen *et al*., 2006). These cells were also observed in long-term, unfractionated, SP cultures by electron microscopy (Yang *et al*., 2006). In cultures grown in rich, glucose-based medium, Q cells are dense, more thermotolerant, and can be separated from NQ cells 1–2 d after glucose exhaustion. Q cells are also predominantly daughter cells that are synchronous upon reentry into the cell cycle. NQ cells, in contrast, retain viability but rapidly lose the ability to reproduce, with ~50% of the viable cells unable to produce daughters at 7 d. By days 14 and 21, almost 90% of the viable NQ cells have lost their ability to reproduce, and ~50% are apoptotic or necrotic.

To increase our understanding of this differentiation, we carried out comprehensive analyses Q and NQ cells from SP cultures, by using wild type, parental, and 101 mutants from the yeast deletion set (Winzeler *et al*., 1999). We report the identification of 11 mutants that affect viability and/or reproductive capacity of NQ cells and/or Q cells. Microarray analysis led to the identification of >1300 mRNAs that differentiate these two cell types, ~1100 more than in our previous study (Allen *et al*., 2006). Finally, we determined that most mRNAs released by protease treatment, identified previously in unfractionated SP cultures (Aragon *et al*., 2006) were present in Q cells. The largest single group of mRNAs released by proteinase K treatment in Q cells encodes proteins required for Ty-element transposition and DNA recombination. This and previous data (Aragon *et al*., 2006) suggest that Q cells are poised for a variety of physiological responses to environmental conditions, including apoptosis, which is likely to be a long-term response of Q cells to

ongoing starvation. These results provide a critical foundation for beginning to understand the differentiation of Q and NQ cells in yeast and the role of each of these cell types in species survival.

MATERIALS AND METHODS

See http://biology.unm.edu/biology/maggieww/Public_Html/aragon/Compendium.htm for all Supplemental Materials.

Strains

Yeast strains used in this study include S288c (*MAT α gal2mal2*) and the parental strain for the deletion set, BY4742 (*MAT α his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*). In this study *MATα* yeast deletion strains (Winzeler *et al*., 1999) were used for 101 mutants whose mRNAs were previously identified as being abundant in SP cultures (Martinez *et al*., 2004). See the Supplemental Material for list of mutants.

Growth Conditions

Wild-type and parental strains were grown in YPD + A (1% yeast extract, 2% peptone, 2% D-glucose, and 0.04 mg/ml adenine) with aeration at 30°C to SP (7 d OD_{600} = 20–25). Yeast deletion strains (Winzeler *et al*., 1999) were grown in the same media with the addition of 200 μg/ml G-418 (Geneticin; Invitrogen, Carlsbad, CA). Cells in the exponential phase of growth were collected at OD_{600} = 1–2, after growth overnight.

Cell Separation and Harvest

Q and NQ cells were separated from SP cultures by using Percoll (GE Healthcare, Little Chalfont, Buckinghamshire, United Kingdom) density gradients as described previously (Allen *et al*., 2006). Briefly, 9 parts of Percoll was added to 1 part of 1.5 M NaCl and centrifuged at 19,240 × *g* for 15 min at 20°C to form a density gradient. For RNA isolations, yeast cells (200 OD_{600}) were pelleted and resuspended in 1 ml 50 mM Tris buffer, pH 7.5. Cells were overlaid on the 25-ml Percoll gradient and centrifuged at 400 × *g* for 1 h at room temperature. Q and NQ fractions were removed with a pipette, and Percoll was removed by washing with 45 ml of 50 mM Tris buffer, pH 7.5. Cell pellets were frozen at −70°C. To obtain cells (OD_{600} = 10) for flow cytometry assays, gradients were formed as described above using 2-ml tubes. Cells were pelleted, resuspended in 100 μl of 50 mM Tris buffer, pH 7.5, overlaid on a 1.75-ml gradient, and centrifuged at 400 × *g* for 1 h. Percoll was removed by washing with 1.5 ml 50 mM Tris buffer, pH 7.5, and cells were then divided for flow assays.

Assays for Viability (FungaLight staining) and Reproductive Capacity (Colony-forming Units [CFUs])

For these assays, parental and mutant strains were grown to SP and separated into Q and NQ fractions as described above. To determine viability, cells were stained using the LIVE/DEAD FungaLight yeast viability kit (Invitrogen) according to the manufacturer's protocol. Cells from an exponential culture were used as a "live" control. A "killed" control was prepared by incubating cells from an SP culture in 70% isopropanol for 1 h at room temperature before staining. Unstained cells were used as an autofluorescence control, and these emissions were confined to the region between the upper and lower gated regions. All cell samples were diluted to 1 × 10^6 cells/ml in 500 μl of 50 mM Tris buffer, pH 7.5, stained, and incubated at 30°C for 30 min. For flow cytometry, 30,000 cells per sample were analyzed with a flow cytometer (FACScan; BD Biosciences, San Jose, CA) by using 488-nm excitation and collecting fluorescent emission with filters at 530/30 nm for FL-1 parameter and 585/42 nm for FL-2 parameter. CellQuest software (BD Biosciences) was used for data collection and analysis. Gates were established such that 99.9% of the emissions from the "live" control cells were contained in the lower gated area and 99.9% of the emissions from the "killed" control were contained in the upper gated area (see Supplemental Material). To determine reproductive capacity via CFUs, automated plating was performed using the MoFlo cell sorter (Dako Denmark, Glostrup, Denmark) as described previously (Allen *et al*., 2006).

RNA Isolation and Microarray Procedures

For microarray analysis, all strains were grown to SP, and Q and NQ fractions were separated as described above. Total RNA was isolated using a modified Gentra protocol, labeled, and hybridized as described previously (Aragon *et al*., 2006). To avoid confounding factors, all RNA preps, labeling, and hybridizations were carried out in random order and rerandomized for each of the three steps. Analysis of hybridized arrays was carried out using GenePix 6.0 and Acuity 4.0., as described previously (Aragon *et al*., 2006). VxOrd 1.58 was used to cluster by both gene expression and arrays, and VxInsight 2.165 was used for visualization (Davidson *et al*., 2001). Clustering was done using force-directed placement of 20 and 15 nearest neighbors, for cluster by gene and array, respectively, by using the *t*-statistic of Pearson's R (Werner-Washburne *et al*., 2002). Gene lists were then queried using Gene Ontology (GO) Term Finder. All microarrays analyzed in this study are available at Gene Expression Omnibus by using accession number GSE8624.

Reproducibility

Experimental reproducibility was determined using in-house generated MATLAB (Mathworks, Natick, MA) scripts (Slide Compare and Set Compare), as described previously (Martinez *et al*., 2004), and they are available by request. To determine biological reproducibility, five biological replicates were hybridized in duplicate for a total of 10 microarrays for both Q and NQ fractions from wild-type and parental strains. Correlation plots and histograms of the deviation were examined using Slide Compare, with the R^2 value and proportional error estimate determined for each replicate. The R^2 values were also visualized using Set Compare, by using an all-against-all plot (Martinez *et al*., 2004). Technical and biological samples were highly correlated for both the Q and NQ fractions (see Supplemental Material). Within Q and NQ fractions, Pearson's correlation coefficients were 0.97 for both Q and NQ cell fractions from the wild-type strain (see Supplemental Material) and 0.94 and 0.95 for Q and NQ fractions, respectively, from the parental strain (see Supplemental Material). In contrast, the correlation between Q and NQ fractions was 0.90 and 0.86 for the wild-type and parental strains, respectively (see Supplemental Material). Although still reasonably correlated, this difference was significant, with $p < 1 \times 10^{-30}$ for both wild-type and parental strains and allowed identification of hundreds of genes whose mRNA abundance is significantly different between Q and NQ cell fractions.

Statistical Ranking

A previously described statistical ranking method was used to determine the mRNAs whose abundance was significantly different in the Q versus NQ fractions (Allen *et al*., 2006). Briefly, the mean difference in mRNA abundance for each gene between the Q and NQ fraction in all replicate microarrays was determined. If the difference was positive, the mRNA was highly abundant in the Q fraction; if it was negative, the mRNA was more highly abundant in the NQ fraction. A *t*-distribution was calculated and only mRNAs with p value cutoffs of $\leq 1 \times 10^{-5}$ were considered significantly different. Gene lists were queried using GO Term Finder and by manual curation.

Protease Treatment

Protease digests during RNA isolations were carried out as described previously (Aragon *et al*., 2006). Briefly, after bead beating samples were centrifuged at 13,000 × *g* for 3 min at 4°C to remove cell debris. Cell-free lysates were divided into two tubes and incubated on ice for 1 h with 14 mg/ml proteinase K (QIAGEN, Valencia, CA) in 10 mM Tris base, pH 7.5, or 10 mM Tris, pH 7.5, alone. To ensure mixing, samples were inverted every 10 min during the incubation. After incubation, the RNA isolation for both samples was completed as described previously (Aragon *et al*., 2006).

RESULTS

Identification of Genes Required for Viability and Reproduction of Q and NQ Cells

Q and NQ fractions from 101 mutants in genes whose mRNA was identified as induced in SP (Martinez *et al*., 2004) were tested for viability, by using FungaLight staining (Invitrogen), and reproductive capacity, by using CFUs. We had previously demonstrated that at 7 d postinoculation, ~50% of the viable NQ cells were unable to reproduce (Allen *et al*., 2006). We identified five categories of mutants that exhibited defects in viability and/or reproductive capacity the cell fractions (Table 1). Parental (BY4742) strains, on which percentage increase or decrease were based, showed 92 ± 2.2 and 87 ± 3.4% viability of Q and NQ cells, respectively, and 87 ± 3.4 and 38 ± 3.1% reproductive capacity (CFUs) for Q and NQ cells, respectively. The ability of less than half (44%) of the viable parental NQ cells to reproduce was consistent with our previous findings in wild-type cells (S288c) (Allen *et al*., 2006).

One mutant, *etr1*, exhibited a significant loss in Q cell reproductive capacity (Table 1, group 1). *etr1* mutants lack a mitochondrial thioester reductase that likely functions in fatty acid synthesis (Torkko *et al*., 2003). In addition to reduced Q cell reproductive capacity, *etr1* strains also showed a marked decrease in the typical 1:1 Q:NQ cell abundance (see Supplemental Material). The role of Etr1p in Q cells is consistent with our finding that mRNAs involved

Table 1. Change in viability and reproductive capacity of mutants from the parental strain

Group	Strain	Q viability, %	Q CFU, %	NQ viability	NQ CFU, %	Function[a]
1	*ETR1*[b]	NC[c]	↓ 33 ± 1.4	NC	NC	Thioester reductase[d]
2	*ATP2*[b]	NC	↓ 87 ± 4.5	NC	↓ 92 ± 7.8	ATP synthesis[d]
	FMP45[b]		↓ 34 ± 20.2		↓ 42 ± 21.5	Membrane protein[d]
	QCR7[b]		↓ 71 ± 21.1		↓ 59 ± 24.7	Cytochrome-c reductase[d]
	ARD1[b]		↓ 29 ± 6.6		↓ 46 ± 23.3	Acetyltransferase
	NAT1[b]		↓ 24 ± 5.3		↓ 29 ± 2.0	Acetyltransferase
3	*POR1*[b]	NC	NC	NC	↓ 93 ± 5.5	Mitochondrial porin[d]
	DDR2				↓ 28 ± 6.3	Stress response
4	*ICL1*[b]	NC	NC	NC	↑ 80 ± 45.5	Glyoxylate cycle
	SPG3				↑ 116 ± 57.3	Unknown
5	*DOA4p*[b]	↓ 48 ± 36.2	↓ 95 ± 4.4	↓ 32% ± 2.2	↓ 91 ± 2.4	Ubiquitination

[a] *Saccharomyces* Genome Database.

[b] Reduced Q cell production.

[c] NC, no change.

[d] Mitochondrial.

in fatty acid and membrane metabolism are abundant in Q cells (Allen *et al*., 2006).

Five mutants exhibited loss of reproductive capacity in both Q and NQ cells (Table 1, group 2). These included strains carrying deletions in the mitochondrial gene *QCR7* encoding subunit 7 of the ubiquinol cytochrome-c reductase complex (De Haan M. *et al*., 1984); *NAT1* and *ARD1*, encoding a subunits of N-terminal acetyltransferase, previously reported to be required for "viability" in SP (Park and Szostak, 1992); and *FMP45*, which encodes a highly conserved, cell cortex protein (MIPS) that has been shown to affect sphingolipid biosynthesis and sporulation (Young *et al*., 2002).

Mutants lacking *ATP2*, encoding the β-subunit in the F1 portion of mitochondrial F1F0 ATP synthase (Devenish *et al*., 2000), also showed reproductive defects. To determine whether other F0F1-ATPase components exhibit this phenotype, we also examined *atp1*, *atp17*, and *atp18 mutants*. Although *atp1* and *atp2* mutants frequently exhibited a similar effect on both Q and NQ cells, all of the *atp* mutants exhibited a high degree of variability in terms of their effects on reproductive capacity and the changes over time (data not shown). This suggests to us that these strains are capable of accumulating suppressors, possibly in an epigenetic manner. For example, *atp17* strains from the deletion set typically produced both small and large colonies. When restreaked, cells from small colonies produced both large and small colonies. We conclude from this result that, although ATPase mutants have an effect on the reproductive capacity of Q and NQ cells, there are additional, possibly epigenetic mechanisms that promote a high degree of variability in these cells after glucose starvation.

All of the strains identified in group 2 affected the production Q cells, i.e., whether a band was visible, although for some, the effect is variable. We previously reported that *atp1* and *qcr7* mutants did not form a lower band (Allen *et al*., 2006); however, these and *atp2* strains usually form a smear of cells that extends from the upper, NQ band to where Q cells typically band. These intermediate cells were evaluated. *atp 17* and *atp18* strains, which did not reduce reproductive capacity of either cell type also showed a variable reduction in the Q cell fraction (see Supplemental Material).

Two groups of mutants affected the reproductive capacity of NQ cells only (Table 1, groups 3 and 4). One group was composed of two mutants that exhibited reduced NQ cell replicative capacity, and the second group of two mutants exhibited increased reproductive capacity. In the first group were strains carrying deletions in *POR1*, encoding mitochondrial porin (Lee *et al*., 1998); and *DDR2*, encoding a *Saccharomyces*-specific stress-response protein of unknown function (Kobayashi *et al*., 1996). Mutants that resulted in an increase in NQ reproductive capacity carried deletions in *ICL1*, encoding isocitrate lyase, a key enzyme in the glyoxylate cycle and required for growth on ethanol (Fernandez *et al*., 1992), and *SPG3*, encoding a protein of unknown function. There were several other mutants that were not included in this table because they were highly variable in their ability to reduce or increase NQ cell reproductive capacity (see Supplemental Material). We hypothesize that induction of proteins involved in DNA transposition and recombination in NQ cells may make this cell fraction phenotypically more variable (Allen *et al*., 2006). We conclude from the identification of mutants that had an *increase* in the reproductive capacity of NQ cells (Table 1, group 4) that the loss of reproductive capacity in NQ cells at 7 d is genetically regulated (Allen *et al*., 2006).

Viability and reproductive capacity of both Q and NQ cells was reduced in *doa4p* strains, lacking ubiquitin isopeptidase, required for ubiquitin recycling (Amerik *et al*., 2000). *DOA4* was previously shown to be required for maintenance of reproductive capacity in SP cultures (Swaminathan *et al*., 1999). To determine whether this was due to a decrease in proteasome function or the concentration of free ubiquitin, we examined mutants lacking *UBI4*, encoding polyubiquitin and known to be required for survival in SP (Finley *et al*., 1987), and *UMP1* and *RPN10*, encoding proteasome proteins. *ubi4* mutants exhibited a reduction in reproductive capacity of NQ cells, similar to the observation with *doa4* mutants, whereas *rpn10* and *ump1* mutants showed a slight increase in reproductive capacity of NQ cells and no effect on Q cell reproduction (see Supplemental Material). Examination of these mutants grown on solid medium revealed that *ubi4* and *doa4* mutants produce a relatively high number of elongated and, sometimes, branched cells, whereas *ump1* and *rpn10* mutants do not (see Supplemental Material). We conclude from these results that *ubi4* and *doa4* deletions have similar effects on NQ and Q cells; thus, free ubiquitin, not proteasome function, is important for survival and reproduction of Q and NQ cells.

Table 2. Change in petite colonies of mutants from the parental strain

Group	Strain	Q petite, %	NQ petite, %	Function[a]
1	*ATP1*[b]	NC	↓ 99 ± 3.3	ATP synthesis[c]
	DOA4[b]		↓ 98 ± 8.9	Ubiquitination
	POR1		↓ 97 ± 1.9	Mitochondrial porin[c]
	XBP1		↓ 95 ± 6.9	Transcriptional repressor
	JEN1		↓ 95 ± 2.2	Lactate transporter
	OM14		↓ 92 ± 9.9	Mitochondrial outer membrane protein[c]
	GPD1		↓ 92 ± 4.1	Dehydrogenase
	MDH1		↓ 89 ± 3.5	Dehydrogenase[c]
2	*ARD1*	↑ 1074 ± 303.0	NC[d]	Acetyltransferase
	GPG1	↑ 157 ± 32.0		Signal transduction
	GTT1	↑ 442 ± 202.0		Glutathione transferase[e]
	YLR312C	↑ 294 ± 9.6		Unknown
3	*SPG3*	NC	↑ 110 ± 9.9	Unknown

[a] *Saccharomyces* Genome Database.

[b] *atp2* and *ubi4* mutants, analyzed separately, exhibit the same phenotype.

[c] Mitochondrial.

[d] NC, no change.

[e] Endoplasmic reticulum.

During this analysis, we noticed that, starting with nonpetite, single colonies from both wild type and mutants, NQ but not Q cell fractions harvested at 7 d postinoculation were more likely to contain cells that produced petite colonies. For parental, BY4742, 6 ± 2.7% of cells in Q cell fractions produced petite colonies, whereas 38 ± 15.9% of the cells in NQ fractions produced petite colonies (n = 10; 432 cells each). For wild type, S288c, the numbers were similar: Q and NQ fractions produced 8 ± 4.1 and 45 ± 18.7% petite colonies, respectively (n = 4; 432 cells each).

During our screen, we identified 13 mutants that showed increased or decreased petite formation (Table 2). Petite formation was selective, in that the production of petites in either Q or NQ cell fractions but not both (Table 2), and most mutants affected petite formation in the NQ fraction. Interestingly, some mutants that affected petite formation, such as *gpg1*, *jen1*, *om14*, *gpd1*, and *mdh1*, had no effect on viability or reproductive capacity, whereas the increase in NQ reproductive capacity shown by the *spg3* mutant was due almost completely to the increased reproductive capacity of petite colonies. We conclude from these results that viability, reproductive capacity, and production of petites are independent phenotypes. However, these phenotypes can interact, for example, when NQ cell reproliferation increases as a result of increases in the ability of petite cells to reproduce or a decrease in apoptosis. Finally, the pleiotropic effects of different mitochondrial mutants again underscore the complexity of mitochondrial function during this phase of the yeast life cycle. We conclude from these results that Q and NQ cells have differentiated significantly but that, for both cell types, reproductive capacity is much more sensitive to perturbation than viability.

Compendium Microarray Analysis

We were interested in identifying a large number of genes whose mRNA abundance distinguishes Q from NQ cells. Previous analysis of mRNA abundance, by using statistical ranking of data from 12 microarrays, revealed slightly >300 transcripts that distinguished Q from NQ cells (Allen *et al*., 2006). To increase our resolution of the differences between Q and NQ cells, we analyzed a total 398 microarrays of isolated cell fractions from wild type (S288c), auxotrophic parental (BY4742), and 101 deletion mutants (Winzeler *et al*., 1999) (358 arrays, including a replicate set of 98 deletion mutants).

Comparison of Q and NQ Cell Fractions

Using the yeast deletion strain data set (358 arrays), 1301 mRNAs were identified ($p < 10^{-5}$) that distinguished Q from NQ cells (683/618 genes for Q/NQ fractions, respectively) (see Supplemental Material). Cluster analysis using VxInsight confirmed that the mRNAs from each group formed distinct domains in the topography (Figure 1A). As observed in previous clustering analysis (Werner-Washburne *et al*., 2002), genes encoding ribosomal proteins formed a tight cluster (Figure 1D, green dots). To determine whether Q cells exhibited a G1-like expression pattern, i.e., that the observed clustering in the Q/NQ topography was similar to that from published cell cycle data (Spellman *et al*., 1998; Werner-Washburne *et al*., 2002), we determined the cluster location of Q- and NQ-specific genes in the cell cycle topography (Figure 1B). It is clear that both Q- and NQ-specific genes are nonrandomly distributed over the entire topography. To determine whether G1-regulated genes were clustered in the Q/NQ VxInsight topography, we selected G1-regulated genes (Figure 1B, yellow dots) and we evaluated their distribution as a function of Q/NQ clusters (Figure 1C). Because G1-regulated genes do not cluster in the Q/NQ topography, we concluded that Q cells are not in a G1 arrest. This is consistent with our previous hypothesis that Q cells are in a G0 state (Allen *et al*., 2006). The clustering of G1-regulated genes with both Q and NQ genes (Figure 1D) further suggests that these genes may affect Q and NQ cells differently; thus, reevaluation of these mutants could provide important insight into the differentiation of Q and NQ cells.

We examined the gene lists from NQ and Q cells by using GO, and we found significant differences between the two cell fractions. For NQ cells, categories of Ty element transposition and DNA recombination were significantly increased ($p \leq 10^{-5}$) (Table 3). The identification of mRNAs encoding proteins involved in DNA recombination is consistent with our hypothesis that futile or nonproductive

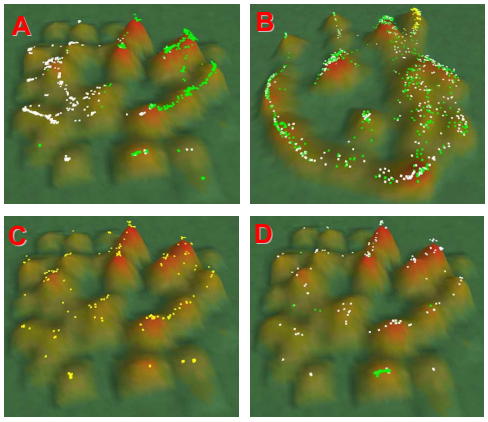

Figure 1. Distribution of gene sets in VxInsight Q/NQ gene-expression and cell cycle topographies (Spellman *et al.*, 1998). Clustering is described in *Materials and Methods*. Hill height is a function of the number of genes in that cluster. (A) Clustering of gene expression for 178 arrays from Q and NQ fractions. Green dots, genes whose mRNAs are significantly increased in Q fractions; white dots, genes that are significantly increased in NQ fractions. (B) Localization of Q (green dots) and NQ (white dots) from A on the gene expression topography from Spellman's cell cycle data set. Yellow spots in upper right are G1-regulated genes. (C) Distribution of G1-regulated genes from B as a function of Q/NQ gene expression topography. (D) Green dots, ribosomal hill; white dots, distribution of the top 200 aging genes (Powers *et al.*, 2006) as a function of the Q/NQ gene-expression topography.

recombination events in these cells eventually results in apoptosis (Allen *et al.*, 2006). In contrast, Q cell-abundant mRNAs were remarkable for the large number of significant GO process categories (Table 3). The most significant GO categories included exocytosis, membrane organization and biogenesis, and vesicle-mediated transport. The latter category was paradoxical, because one of the characteristics of these cells is the apparent absence of endoplasmic reticulum and Golgi (Allen *et al.*, 2006). mRNAs encoding proteins involved in fatty acid oxidation and response to oxidative stress were consistent with the enhanced stress resistance of these cells and the likelihood that these cells survive long periods via lipid catabolism. Finally, signal transduction was a significant GO category in Q but not NQ cells in this analysis.

Table 3. GO processes[a] terms strongly associated with Q or NQ cells ($p \leq 10^{-5}$)

	NQ cells	
Go Term	Gene no.	p value
Ty element transposition	56	4.19^{-29}
DNA transposition	57	3.01^{-27}
DNA recombination	63	1.94^{-19}
DNA metabolism	81	2.48^{-06}
	Q cells	
Vesicle-mediated transport	63	1.29^{-09}
Vesicle docking during exocytosis	9	2.77^{-06}
Oxygen and reactive oxygen species metabolism	18	9.11^{-06}
Vesicle docking	9	9.41^{-06}
Fatty acid oxidation	8	1.22^{-05}
Exocytosis	14	1.55^{-05}
Membrane organization and biogenesis	24	2.05^{-05}
Response to oxidative stress	17	2.07^{-05}
Membrane fusion	17	7.46^{-05}
Lipid metabolism	41	9.20^{-05}
Cellular lipid metabolism	39	9.55^{-05}
Signal transduction	33	9.67^{-05}

[a] Gene Ontology *Saccharomyces* Genome Database.

Analysis of mRNAs Encoding Signal Transduction Proteins in Q and NQ Cells

Conserved signal transduction pathways, including protein kinase (PK) A, target-of-rapamycin (TOR), PKC, SNF1, and PHO85 are known to be required for the transition from fermentative to respiratory growth at the diauxic shift and entry into SP (Herman, 2002; Gray *et al.*, 2004). The SNF1 pathway must be activated and PKC transiently activated during the diauxic shift, whereas inhibition of PKA and TOR is required for this process (Gray *et al.*, 2004).

Of the seven signaling-related genes whose mRNAs are abundant in NQ cells ($p < 10^{-5}$), at least four are involved in the cell cycle or growth (Table 4). These include Sit4p, which up-regulates *CLN1* and *CLN2* expression (Fernandez-Sarabia *et al.*, 1992); Ste20p, involved in pseudohyphal growth under conditions of nitrogen deficiency (Hohmann, 2002); *HSL1*, a kinase required for the degradation of Swe1p, an inhibitor of Cdc28p (McMillan *et al.*, 1999); and Kic1p, a kinase required for cell wall morphogenesis and integrity (Sullivan *et al.*, 1998). Hog1p, required for osmotolerance during exponential growth (Hohmann, 2002), is the only primary signaling kinase whose mRNA is abundant in NQ cells. These data are consistent with the hypothesis that NQ cells are unable to arrest growth and, as a result, they may attempt to maintain a physiological state closer to growing cells in nonstarvation medium.

In contrast, 31 mRNAs abundant in Q cells encode proteins involved in every major signaling pathway ($p < 10^{-5}$) (Table 5). Strikingly, these included the central protein kinases for TOR, PKA, and SNF1 pathways, including *TPK1* and *TPK2*, encoding two of three catalytic subunits of PKA (Toda *et al.*, 1987b), and *TOR1* and *TOR2*, encoding the Tor kinases, positive regulators of ribosome biogenesis, cell cycle, and other growth-related processes (Raught *et al.*, 2001). mRNA encoding Pho85p, a cyclin-dependent kinase required for proper gene regulation at the diauxic shift (Nishizawa *et al.*, 2004) accumulated in Q cells, but it was present in <80% of our arrays (see *Materials and Methods*). These results suggest that Q cells have the capacity to respond rapidly to environmental stimuli.

Regulation of these signal transduction pathways is critical for survival. To evaluate the potential for Q cells to activate or repress signaling, we examined mRNAs for other proteins in these pathways. Snf1p needs to be activated for cells to transit the diauxic shift, and, consistent with Snf1p activation, mRNAs encoding Snf1p activators Gal83p and Sak1p (Vincent and Carlson, 1998) were abundant in Q cells. In contrast, for the progrowth PKA pathway, mRNAs were abundant that encode negative regulators or antagonists, including Rim15p kinase, required for survival after glucose exhaustion and requiring down-regulation of PKA, TOR, and Pho85p for activation (Cameroni *et al.*, 2004; Gray *et al.*, 2004; Wanke *et al.*, 2005); Yak1p, a negative regulator of PKA required for the transient growth arrest after glucose exhaustion (Garrett *et al.*, 1991; Moriya *et al.*, 2001); and Bcy1p, the evolutionarily conserved regulatory subunit of PKA (Toda *et al.*, 1987a). Similarly, for the TOR pathway, *PPH22* mRNA was abundant, encoding protein phosphatase 2A, an antagonist of

Table 4. Nonquiescent fraction signaling pathway genes[a]

Gene	Pathway	Conservation[b]	Functional annotation[b]
HOG1	HOG	HC[c]	Regulates osmoresponsive genes
HSL1	HOG	HC	Relays nutrient conditions to Cdc28p, negative regulator of *SWE1*
STE20	HOG	HC	Involved in invasive and pseudohyphal growth pathways
KIC1	PKA	HC	Serine/threonine protein kinase involved in cell separation
YGK3	PKA	HC	Phosphorylates Bcy1p
SKM1	PKA	HC	Acts on Cdc42p in the pseudohyphal/invasive growth pathways
SIT4	TOR	HC	Involved in mitotic cell cycle regulation

[a] Statistical ranking analysis.

[b] Proteome database.

[c] Highly conserved.

TOR activity. These results suggest that signal-transduction pathways in Q cells are highly regulated; thus, Q cells are poised for long-term survival of starvation and rapid response when conditions become favorable for growth.

mRNAs Released after Protease Treatment Are Found Predominately in the Q Fraction

We previously demonstrated the presence of thousands of mRNAs released after protease treatment during RNA isolation in wild-type cells in unfractionated SP cultures and that these mRNAs can be released in a stress-specific manner (Aragon *et al*., 2006). To determine whether Q or NQ cell fractions preferentially contain protease-released mRNAs, lysates from Q and NQ cell fractions and unfractionated cells from parental SP cultures were incubated with or without proteinase K (Figure 2). Cells from SP culture contained 1570 protease-released transcripts that increased 1.5- to 128-fold. Lysates from Q and NQ cell fractions exhibited similar increases in 1957 and 223 transcripts, respectively. Thus, there were almost 9 times more protease-released mRNAs in

Table 5. Signal transduction genes associated with the quiescent fraction[a]

Gene	Pathway	Conservation[b]	Annotation[b]
PTP3	HOG	HC[c]	Negative regulator of HOG pathway
RCK2	HOG	HC	Activated by Hog1p and activates elongation factor EF-2
SSK1	HOG	Yeast only	Involved with osmosensing
STE11	HOG	HC	Pheromone response, pseudohyphal and invasive growth pathways
STE50	HOG	Yeast only	Mating response, invasive/filamentous growth, and osmotolerance
VPS36	HOG	Yeast only	Part of the ESCRT-II complex
FMP48	HOG	HC	Serine/threonine protein kinase of unknown function interacts with *HOG1*
PRR2	HOG	HC	Mitogen-activated protein (MAPK) kinase signaling in the pheromone response pathway
CDC55	HOG	HC	Required for DNA replication and spore production
MSG5	HOG	HC	Inactivates Fus3p
CKA1	PHO	HC	PHO pathway transporting inorganic phosphate
TPK1	PKA	HC	Catalytic subunit of cAMP-dependent protein kinase *BCY1*
TPK2	PKA	HC	Activates filamentous growth, regulates unipolar budding and invasive growth
BCY1	PKA	HC	PKA regulatory subunit
BMH2	PKA	HC	RAS/MAPK cascade signaling during pseudohyphal development
CYR1	PKA	HC	Adenylate cyclase
IRA1	PKA	HC	Negatively regulates GTP-Ras2p basal level with Ira2p
PKH3	PKA	HC	Member of the PKA-related protein kinases
RIM15	PKA	HC	Positive regulator of entry into SP
YAK1	PKA	HC	Negative regulator of cell growth in opposition to PKA
LSP1	PKC	Yeast only	Down regulates PKC pathway
MKK2	PKC	HC	Mitogen-activated kinase kinase involved in PKC signaling
RHO1	PKC	HC	Required to activate PKC pathway
TUS1	PKC	HC	Regulates Rhop1 activity
GAL83	SNF	HC	Activates Snf1p
SNF1	SNF	HC	Response to glucose starvation and derepression of glucose-repressed genes
SAK1	SNF	HC	Activates Snf1p
SIP1	SNF	Yeast only	Proposed regulator of Snf1p
TOR1	TOR	HC	Reg of replicative life span, translation initiation, starvation response, etc.
TOR2	TOR	HC	Actin cytoskeleton organization
PPH21	TOR	HC	Catalytic subunit of PP2A-1, interacts with Tap42p

[a] Statistical ranking analysis.

[b] Proteome database.

[c] Highly conserved.

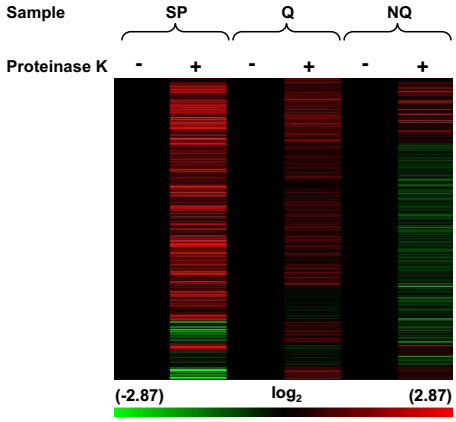

Figure 2. mRNA abundance in Q and NQ samples treated with or without proteinase K. Unsupervised hierarchical clustering (Pearson's centered, average-linkage) was used to cluster ~2400 transcripts. Samples include, unseparated SP culture (SP) and Q and NQ cell fractions. Cell lysates from samples were incubated with buffer alone (−) or proteinase K (+). Before clustering, treated samples were normalized to untreated samples (black lanes). The color scale at the bottom indicates the $\log_2$ values for changes in mRNA abundance.

Q as in NQ cells. The overlap between protease-released mRNAs present in both SP cells and Q cells was 85% ($p < 1 \times 10^{-50}$) and between SP and NQ cells was 70% ($p < 1 \times 10^{-40}$) (Figure 3). We hypothesize that the protease-released mRNAs found in Q or NQ cells but not in cells from SP cultures are likely to be mRNAs that are relatively abundant in the other cell fraction so they do not increase significantly in protease-treated lysates from SP cultures.

Protease-released mRNAs in Q cells included almost half of the mRNAs identified as abundant in NQ cells (299/618), whereas only 26 of 683 Q mRNAs were found among protease-released mRNAs from NQ cells. We conclude from these results that during differentiation of Q and NQ cells, Q cells selectively sequester the mRNAs that can be released in a stress-specific manner (Aragon *et al*., 2006). This makes Q cells uniquely capable of responding to environmental changes through the rapid release of groups of mRNAs.

We identified significant GO process groups for protease-released mRNAs from SP cultures and Q and NQ cells. Protease-released mRNAs in cells in SP culture encoded proteins involved in Ty element transposition and carbohydrate metabolism (Table 6). There were no significant GO categories in the protease-released NQ gene list ($p < 10^{-5}$). The largest group was annotated as hypothetical open reading frames, again, consistent with the lack of information about these cells, and this stage of the yeast life cycle. In Q cells, the most significant groups of protease-released mRNAs ($p < 10^{-5}$) encode proteins involved in Ty-element transposition and DNA recombination and a large number of *HSP70*-family genes (Table 5). Strikingly, these are the same mRNAs that are constitutively abundant in NQ cells (Table 3). This result suggests that Q cells are able not only to respond to progrowth signals but, should starvation continue for very long periods, they are also poised to enter apoptosis.

Table 6. GO processes[a] associated with protease-treated cell lysates ($p < 10^{-5}$)

	Cells in stationary phase culture	
Go Term	Gene no.	p value
Generation of precursor metabolites and energy	92	9.64^{-13}
Main pathways of carbohydrate metabolism	44	3.94^{-12}
Ty element transposition	47	4.78^{-10}
DNA-mediated transposition	49	7.30^{-10}
Energy derivation by oxidation of organic compounds	74	9.35^{-10}
Cofactor metabolism	62	2.04^{-09}
Coenzyme metabolism	53	2.44^{-09}
Organic acid metabolism	97	1.77^{-08}
Carboxylic acid metabolism	97	1.77^{-08}
Carbohydrate metabolism	76	1.10^{-07}
Acetyl-CoA metabolism	17	1.59^{-07}
Tricarboxylic acid (TCA) cycle intermediate metabolism	16	4.12^{-07}
Cellular carbohydrate metabolism	69	5.03^{-07}
TCA cycle	14	6.56^{-07}
Acetyl-CoA catabolism	14	6.56^{-07}
Coenzyme catabolism	14	2.75^{-06}
Cofactor catabolism	14	5.23^{-06}
Pyruvate metabolism	20	8.83^{-06}
	Q cells	
Ty element transposition	74	2.21^{-28}
transposition	75	5.54^{-27}
transposition, DNA-mediated	75	5.54^{-27}
DNA recombination	82	3.15^{-15}
DNA metabolism	132	4.82^{-06}

[a] Gene Ontology *Saccharomyces* Genome Database.

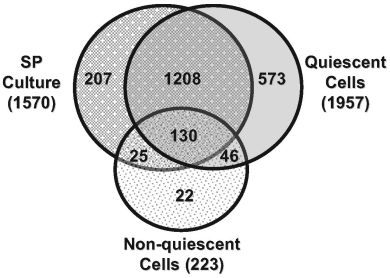

Figure 3. Venn diagram of transcripts that increased after proteinase K treatment of cell lysates. Transcripts were evaluated that had a ≥1.5-fold increase in abundance after proteinase K treatment of SP culture and Q and NQ cell fraction lysates. The transcripts that were evaluated were required to be present in 80% of the data points.

DISCUSSION

This study expands on our previous characterization of the distinctions between Q and NQ cells in yeast SP cultures (Allen *et al*., 2006) and reinforces the importance of studying specific cell types in a heterogeneous mix of cells and distinguishing viability/metabolic activity from reproductive capacity. The mutants identified here are the first known to selectively affect viability or reproductive capacity in Q and/or NQ cells and to demonstrate the significant physiological differentiation of these cell types. Mitochondrial function is important for both Q and NQ reproduction and petite formation and plays a complex role in the differentiation of these cells. The ability of deletions in mitochondrial genes, especially those in the oxidative phosphorylation or

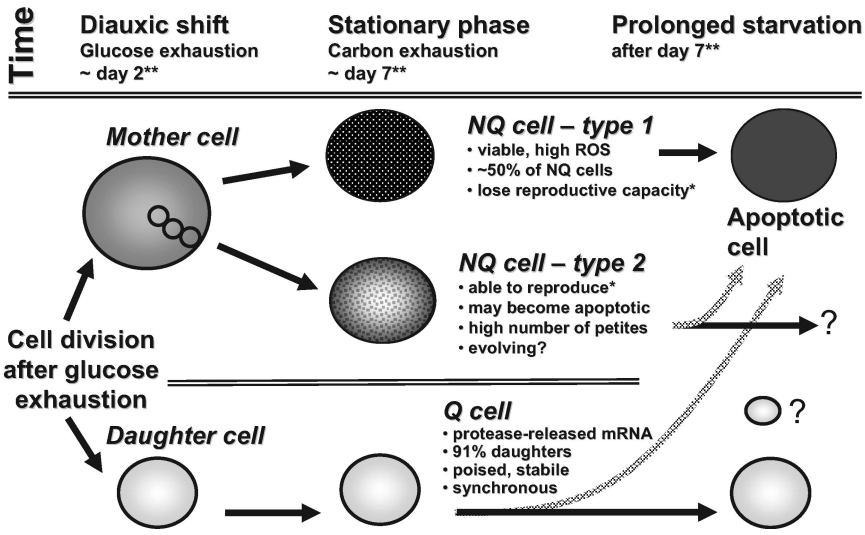

Figure 4. Model for cell differentiation in yeast grown in rich, glucose-based medium (YPD) after glucose and carbon starvation. Glucose exhaustion leads to the formation of daughter cells, which will become quiescent, and mother cells, 50% of which will become apoptotic by day 14 after inoculation. The NQ cells rapidly lose the ability to reproduce, and many of the NQ cells that can reproduce lose mitochondrial function, i.e., are petite. We hypothesize that this is a result of genomic instability in these cells. Q cells contain large P-bodies (Ray, unpublished) and the majority of protease-released mRNAs. In addition, based on abundant mRNAs, these cells are also poised to respond to a variety of environmental changes. Because a major group of protease-released mRNAs includes those encoding proteins involved in recombination and transposition, it seems likely that these cells are also poised to become apoptotic. We hypothesize that the differentiation of these cells, including the group of NQ cells that undergoes genomic rearrangements, is an evolutionarily conserved process that serves to provide both stability in the quiescent cells and innovation in the nonquiescent cells during times of nutrient limitation.

ATP synthesis pathways to cause different phenotypes, including inhibiting the formation of Q cells (Allen *et al*., 2006), reveals the importance and complexity of mitochondrial function during differentiation, consistent with the pleiotropic role of mitochondria in human health (Steinmetz *et al*., 2002; Barrientos, 2003). The finding that free ubiquitin and not proteasome function is important for survival and reproduction of both cell types may be relevant to other types of cells, including stem cells, where ubiquitin has also been shown to be important for regulation of differentiation (Naujokat and Saric, 2007).

Although nuclear genes have been identified that cause differences in the production of petite or respiratory incompetent yeast cells (Contamine and Picard, 2000), the cell type that produces petite cells has not previously been known. It has been suggested that petite formation (reduction of mitochondrial function) protects cells experiencing high levels of reactive oxygen species (ROS) (Davermann *et al*., 2002). Our data showing that NQ cells exhibit much higher levels of ROS within days after glucose exhaustion (Allen *et al*., 2006) is consistent with this hypothesis. However, our results also show that many mitochondrial mutants do not form petites, consistent with other studies showing mutants, including ATPase mutants, that cannot tolerate loss of mitochondrial DNA (Dunn *et al*., 2006).

The results presented in this study are relevant to chronological aging studies using SP yeast cultures (Longo *et al*., 1996; Ashrafi *et al*., 1999; Gershon and Gershon, 2000; Jakubowski *et al*., 2000; Chen *et al*., 2005). The identification of the NQ cell (mother cell) population as producing petite colonies is especially relevant to the question of genomic instability during aging (Laun *et al*., 2006). Because clustering analysis revealed that the top 200 aging genes were randomly distributed (Figure 1D), we hypothesize that genes differentially affecting either Q or NQ cells could contribute to an aging phenotype in cultures, either by extending the life of Q cells or allowing NQ cells to better protect Q cells or extend the life of a subpopulation of NQ cells. Our results suggest that more precise evaluation of phenotypes based on fractionated cells could provide important insights into chronological aging.

The identification of mRNAs encoding proteins in "progrowth" signaling pathways in Q cells is consistent with previous work showing an eightfold increase in Bcy1p, the regulatory subunit of PKA, and Tpk1p, one of three PKA catalytic subunits, as cultures entered SP (Werner-Washburne *et al*., 1991). We hypothesized that regulation of these pathways was dynamic and that they were poised for rapid activation under favorable growth conditions (Peck *et al*., 1997). To our knowledge, similar experiments have not been carried out with TOR pathway components, but the role of TOR in mRNA turnover (P-bodies) and translation (De Virgilio and Loewith, 2006) suggest that this pathway is also dynamic at this time.

We summarize these results in our model (Figure 4). Quiescent daughter and nonquiescent cells differentiate after glucose exhaustion in cultures grown in rich, glucose-based medium (YPD). Mother cells continue to divide during the postdiauxic phase, producing unbudded, NQ daughter cells, and both budded and unbudded NQ cells rapidly lose their ability to divide (Allen *et al*., 2006). NQ cells are heterogeneous, with at least two types of NQ cells—those that enter apoptosis (type 1) and those that retain the capacity to reproduce and produce a high proportion of petite colonies (type 2). Q cells contain protease-released mRNA, in P-bodies or other RNA granules (Wickens and Goldstrohm, 2003; Parker and Sheth, 2007). The very tiny Q cell in Figure 4 represents cells that we see in some very old yeast cultures that are extremely small but seem to be yeast (Werner-Washburne, unpublished data). They are included to indicate that the complete story of the diversification of cell types in stationary-phase cultures is not yet completely understood.

The differentiation of Q and NQ cell types in yeast begs the question of the relationship of this process to the production of quiescent cells in other eukaryotes. Localized or "protease-released" mRNAs are found in quiescent cells from higher eukaryotes, including neurons and stem cells (Kedersha and Anderson, 2002; Anderson and Kedersha, 2006). mRNAs encoding a variety of highly conserved and regulated signaling pathways are also highly abundant during stem-cell renewal and differentiation (Ramalho-Santos *et*

al., 2002; Arbouzova and Zeidler, 2006) and stem cells, like Q cells, are poised to activate any number of signaling pathways (Molofsky *et al*., 2004; Moore and Lemischka, 2006). Regulation of the process of Q cell and NQ cell differentiation involves the same signaling pathways, including Pho85p and TOR, that regulate nutrient-regulated stem cell proliferation in *C. elegans* and *Drosophila* (Narbonne and Roy, 2006). Apoptosis observed in NQ cells is commonly observed during the asymmetrical cell divisions leading to neuronal and stem cell biogenesis (Yoshikawa, 2000; Cai *et al*., 2004; Lynch, 2004). Finally, the potential for Q cells to become apoptotic, based on the presence of protease-released mRNAs in Q cells, is thought to be the mechanism by which the body removes most stem cells with oncogenic mutations (Lynch, 2004). We hypothesize, therefore, that Q and NQ cells are analogous to undifferentiated stem cells and differentiated niche cells, respectively, and that the process of formation of these two cell types is related to biogenesis of stem cells in higher eukaryotes.

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