**Metabolic cycling without cell division cycling in respiring yeast**

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Metabolic cycling without cell division cycling in respiring yeast

Nikolai Slavov,a,b,1 Joanna Macinskasb, Amy Caudyb, and David Botsteinb,1

*Departments of Biology and Physics, Massachusetts Institute of Technology, Cambridge, MA 02139; bDepartment of Molecular Biology, Princeton University, Princeton, NJ 08540; and 1Donnelly Centre for Cellular and Biomolecular Research, University of Toronto, Toronto, ON, Canada M5S 3E1

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Despite rapid progress in characterizing the yeast metabolic cycle, its connection to the cell division cycle (CDC) has remained unclear. We discovered that a prototrophic batch culture of budding yeast, growing in a phosphate-limited ethanol medium, synchronizes spontaneously and goes through multiple metabolic cycles, whereas the fraction of cells in the G1/G0 phase of the CDC increases monotonically from 90 to 99%. This demonstrates that metabolic cycling does not require cell division cycling and that metabolic synchrony does not require carbon-source limitation. More than 3,000 genes, including most genes annotated to the CDC, were expressed periodically in our batch culture, albeit a mere 10% of the cells divided asynchronously; only a smaller subset of CDC genes correlated with cell division. These results suggest that the yeast metabolic cycle reflects a growth cycle during G1/G0 and explains our previous puzzling observation that genes annotated to the CDC increase in expression at slow growth.

T
wo kinds of periodic behavior have been characterized in slowly growing yeast cultures. The first, the classical cell division cycle (CDC), consists of four phases (G0/G1, S, G2, and M) that are easily distinguished by morphological criteria. When the growth rate of budding yeast is slowed by mutations or chemicals inhibiting growth, the duration of the G1/G0 phase increases relative to the durations of the S, G2, and M phases (1). Recently, we confirmed and quantified this CDC trend (Fig. 1A) in chemostat cultures whose steady-state growth rate was controlled by limiting natural nutrients (2, 3). The second kind of cycle, the yeast metabolic cycle (YMC), was first observed more than four decades ago (4) as periodic oscillations in the oxygen consumption of continuous, glucose-limited cultures growing in a chemostat. Like the CDC, the YMC can be divided phenomenologically into two phases: the low oxygen consumption phase (LOC), when the amount of oxygen in the medium is high because the cells consume little oxygen, and the high oxygen consumption phase (HOC), when the reverse holds (SI Appendix).

These changes in the relative durations of the phases affect the composition of asynchronous cultures, because single cells from asynchronous cultures cycle metabolically (5, 6) and thus the fraction of cells in a particular phase is proportional to the duration of that phase relative to the entire cycle period. The increase in the relative duration of a phase results in the increase in the fraction of cells in that phase, and thus an increase in the population-average expression levels of genes peaking during that phase. Consider, for example, a ribosomal gene that peaks in expression during the HOC phase; at slow growth rate, most cells are in the LOC, expressing the ribosomal gene at low levels (Fig. 1C) and thus resulting in low population-average levels, and vice versa at high growth rate. We will refer to this dependence between the composition of an asynchronous culture and its population-average gene expression as “ensemble average over phases” (EAP). In what follows, distinguishing between the population behavior and the behavior of individual cells is critical, because we present evidence that the population behavior represents at least two physiologically nonidentical subpopulations.

Metabolically synchronized populations, manifested by oscillations in oxygen consumption, have been observed in batch yeast cultures grown on trehalose media (7), as well as in yeast cultures starved for a carbon source (glucose or ethanol) and subsequently fed continuously with either glucose- or ethanol-limited media (8). DNA microarray studies of continuous glucose-limited cultures showed that very many genes are expressed periodically during the YMC (9, 10). Biosynthetic genes peak during the HOC phase, whereas autophagy and vacuolar genes peak during the LOC. In a continuous YMC culture, a fraction of the culture divides synchronously during each YMC period (4, 10, 11), indicating a coupling between the YMC and the CDC. However, the mechanism of this coupling remains unclear. Previous work has shown that during each YMC period, the YMC culture has at least two distinct subpopulations: dividing and nondividing. It had previously been suggested that DNA replication is restricted to what Tu et al. (10) defined as the reductive phase of the YMC (9, 12, 13), but we found that, on a population basis, DNA replication can coincide with the HOC phase (3). These results left open the question of the link between the CDC and the YMC.

In this article, we describe growth conditions in which nondividing cells in the G1 phase of the CDC go through multiple metabolic cycles. We also find that many genes whose expression previously has been associated with the CDC in rich media (14) oscillate with the YMC in the absence of cell division, whereas others do not. These results suggest that the YMC is occurring during the G1 phase of slowly growing cells, and that at least some of it may be relevant to cells growing more rapidly. Furthermore, the oscillation of genes annotated to the CDC even in nondividing cells resolved the puzzling observation that many of these genes increase in expression as the growth rate decreases (2, 3), although the fraction of dividing cells decreases. The discovery of the G1-phase growth cycle and the associated periodic expression of genes previously annotated to the CDC in the absence of cell division can account for the increased expression of such genes at slow growth rates, and enabled us to characterize each of the genes expressed periodically with each cycle.

Results

Experiment and Physiology. We discovered that wild-type diploid prototroph (DBY12007, S288c background) synchronizes spon-

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1To whom correspondence may be addressed. E-mail: nslavov@alum.mit.edu or botstein@princeton.edu.

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Simultaneously when grown in batch in phosphate-limited medium contacting 100 mM ethanol as the only source of carbon and energy. We measured continuously the dissolved oxygen in the medium (Fig. 2A), which tracks oxygen consumption very closely, as can be verified by adding a pulse of a carbon source, which causes the measured level of dissolved oxygen to fall immediately (15, 16). After inoculation, the dissolved oxygen decreased exponentially (Fig. 2A), indicating exponential growth and increase in the oxygen consumption of the culture. Then the dissolved oxygen in the culture media returned to the level before inoculation (100%), indicating sudden and complete cessation of respiration. Subsequently, the oxygen level in the media and thus the consumption of the culture oscillated for many hours, just as seen in continuous cultures (4, 10). We reproduced this synchronization in several independent cultures (SI Appendix, Fig. S1). At the indicated time (red arrow in Fig. 2A), 20% of the culture was diluted with fresh medium, resulting in substantial decrease in cell density and therefore low oxygen consumption. After a growth period, the culture resynchronized spontaneously and was sampled for DNA content, gene expression, cell size, and cell density at 67 time points (marked with red circles in Fig. 2A).

Fig. 1. Growth-rate (GR) changes in the yeast metabolic cycle and the cell division cycle. Changes in the relative duration of the phases of (A) the CDC and (B) the YMC (3). (C) Distributions of the number of mRNAs per cell of a ribosomal gene in an asynchronous culture growing at three growth rates.

Fig. 2. Oxygen, biomass, and DNA content data in a metabolically cycling nondividing culture. (A) Dissolved oxygen in the medium reflecting the oxygen consumption. The culture was sampled at the positions indicated by red circles. (B) Cell density at each of the sampled points. (C) Distribution of DNA content obtained by FACS counting 150,000 cells labeled with SYTOX Green. (D) Fraction of cells in the CDC phases as inferred from the DNA content.
Fig. 2 C and D shows that despite the periodic oxygen consumption, only about 10% of the cells divided initially, after which the DNA content remained constant at the level expected for diploid cells with unreplicated DNA as measured by FACS (Fig. 2C). Thus, as shown in Fig. 2D, most of the culture was in the G0/G1 phase of the CDC for the entire interval that we sampled, during which there were three metabolic cycles and a slight increase in the distribution of cell sizes (SI Appendix, Fig. S4). During the last sampled metabolic cycle, more than 99% of the culture was in G1/G0, indicating that the few cells with replicated DNA (G2/M phase) had divided (Fig. 2D). These results are fully consistent with the small increase in cell density during cycling (Fig. 2B). The dividing subpopulation S/G2/M is unlikely to cause periodic oxygen consumption because its fraction decreases monotonically 10-fold so that by hour 85 it is nearly zero. However, the oxygen consumption continues to oscillate robustly. Indeed, we followed the culture and observed that it continued consuming oxygen periodically for 12 h after the last sample (Fig. 2A), long after the S/G2/M fractions had fallen below 1%, and without a significant increase in cell density.

Global View of Gene Expression. To display global patterns in gene expression, we hierarchically clustered the gene-expression data from the culture characterized in Fig. 2 and from a metabolically synchronized, glucose-limited, continuous culture (10). In our batch culture, genes that peak in the LOC phase generally have higher mean expression levels relative to the reference, a glucose-limited, asynchronous culture growing exponentially at growth rate $\mu = 0.25$ h$^{-1}$. The reverse holds for genes peaking in the HOC phase. A possible reason for this observation is that a fraction of the batch culture is already fully quiescent, that is, arrested in an LOC-like state (resulting in the high levels of LOC genes), and only the remaining fraction of the culture cycled metabolically, resulting in the periodic gene expression and consumption of oxygen. The hierarchically clustered mRNA data show that many periodic genes are in-phase, that is, they peak in the same phase both in the batch and continuous cultures (Fig. 3A). To characterize such genes, we ordered them by phase using correlation analysis (Materials and Methods) (Fig. 3B). These 3,000+ genes include the genes with carbon source- and limitation-independent growth-rate response that can be explained by the EAP mechanism introduced above applied to the growth-rate (GR) changes in the YMC (Fig. 1B) (3), and have similar amplitudes of oscillation in the two cultures (SI Appendix, Fig. S5).

Cell Division Cycle. To define better the relationship of the YMC and the CDC, we explored the expression of genes annotated to the CDC with two questions in mind: (i) Which genes are expressed with the division cycle, and thus decrease monotonically in our batch culture, and which genes are expressed with the growth cycle, and thus oscillate in our batch culture? (ii) Can we connect the periodic expression of genes with either the division or the growth cycle to the growth-rate changes in the expression of these genes in asynchronous cultures?
answer these questions, all 468 genes annotated to the CDC in yeast (biological process “cell cycle”; Gene Ontology accession no. GO:0007049; for brevity, we will refer to these genes as “CDC genes” throughout the article) were plotted, portioned into three clusters based on their expression profiles in our batch culture (Fig. 4).

The first cluster (Fig. 4, CDC genes, Top) is composed of genes whose expression steadily decreases, with minimal evidence of cycling. The cluster includes genes essential for CDC progression, such as cyclins (CLN1, CLB1, CLB2), CDC20, which is a component of the anaphase-promoting complex/cyclosome, CDC12, which is required for cytokinesis, and other essential classical CDC genes such as MCD1 and CDC5. The genes in this cluster tend to have positive growth-rate slopes [as defined previously by Brauer et al. (2)], with a mean of +0.81. This is entirely consistent with the idea that, at higher growth rates, the increase in the fraction of cells that are dividing and thus expressing those genes results in higher population-average expression levels of these genes, that is, the EAP mechanism introduced above applied to the growth-rate (GR) changes in the CDC (Fig. 1A).

The second and largest cluster of genes annotated to the CDC (Fig. 4, CDC genes, Middle) is expressed periodically with the metabolic cycle. Because there are so few dividing cells (less than 1% after hour 85) in this culture, we are forced to conclude that the genes in this second cluster were expressed in the subpopulation of cells that cycled metabolically (SI Appendix). This conclusion is fortified by the observation that the GR response of these genes is predicted by the EAP mechanism applied to the GR changes in the YMC, not the CDC (Fig. 1B). The genes that peak during the HOC tend to have positive GR slopes (mean of +0.79), whereas the genes peaking during the LOC tend to have negative GR slopes (mean of −0.76); the difference between the distributions of GR slopes is very significant (P < 10−21, rank-sum test).

The third and smallest cluster (Fig. 4, CDC genes, Bottom) consists of genes that increase in expression during the course of the oscillations. Consistent with the results from Fig. 2, this cluster includes genes repressing (negatively regulating) the CDC and resulting in CDC arrest. These genes have mostly negative GR slopes that can be explained by the EAP mechanism applied to the GR changes in the CDC, not the YMC (Fig. 1A). Thus, we conclude that the genes from the first and third clusters are expressed by the cells that divided, whereas the genes from the second cluster are expressed by the cells that cycled metabolically.

Discussion

Coupling of the Metabolic and Cell Division Cycles. Our goal was to understand better the relationship between the CDC and the YMC. Our gene-expression data from a metabolically cycling, largely nondividing culture of budding yeast indicate that a very large fraction of CDC genes cycles even when the majority of the culture is not dividing, whereas a smaller subset of CDC genes is expressed only in dividing cells. These periodic genes likely comprise what has been described as a free-running oscillator driving G1 events (17) and are consistent with previous reports of periodic expression of CDC genes in the absence of mitotic cyclins (18). Furthermore, the phase of the metabolic cycle in which the cycling CDC genes are expressed correlates strongly with the GR slope (change in gene expression with growth rate) in asynchronous cultures (2, 3), fortifying the relevance of the autonomous YMC to single cells from asynchronous cultures.

As we stressed before, the GR response of CDC genes in asynchronous cultures could not be accounted for simply by the change in the CDC with growth rate. Taking into account the

![Fig. 4. Genes annotated to the CDC are periodically expressed in a nondividing culture in correlation with the metabolic cycle. (Left) Expression levels relative to the reference (glucose limitation at μ = 0.25 h−1). (Center) The same data centered to mean zero. (Right) The growth-rate (GR) slopes of the corresponding genes in asynchronous cultures (2). We previously computed GR slopes by regressing transcript levels of asynchronous cultures against the growth rate (2, 3); positive GR slopes indicate increasing expression with growth rate, and negative GR slopes indicate decreasing expression.](image-url)
metabolic cycling of CDC genes, however, we can largely explain their GR responses by extending our model of changes in the relative durations of the HOC and LOC phases of the YMC with growth rate (3). A possible explanation for the periodic expression of CDC genes in nondividing cells is that genes thought to be involved in the CDC are tied to metabolism and growth. Importantly, about half of the periodically expressed CDC genes, such as histone genes, are expressed at very low levels relative to the reference (even during their peak expression), indicating that nondividing cells may not be affected strongly by the periodic expression, especially if the mRNAs are not translated. The other half of the CDC genes expressed periodically in our culture, however, are expressed at high levels.

The smaller subset of CDC genes whose expression decreases during the course of the oscillations is likely to be the genes ultimately regulating CDC progression. These genes are likely expressed only in dividing cells (S/G2/M phases), and have positive GR responses (increasing in expression with growth rate in asynchronous cultures) that can be explained only by the growth rate changes in the CDC without considering the YMC. As growth rate increases, the fraction of budded cells also increases, and thus genes expressed only in budding cells increase in population-average expression. This mechanism can account for both the expression levels in our batch culture and for the GR response in asynchronous cultures (2,3).

The link between the YMC and CDC can be conceptualized by thinking of two related but distinct cycles: the cycle of cell growth (i.e., biomass increase) and the cycle of cell division. The link, then, is an expression of the requirement to couple cell growth and cell division so that the cell size can be maintained in a physiologically functional range. At fast growth in rich media, growth and cell division are always coupled in the same way and have been characterized as the classical CDC. In contrast, our studies (2,3) in a variety of growth conditions and growth rates have enabled us to observe both the cycle of growth and the cycle of division and to begin characterizing the link between the two cycles across growth rates.

One attractive way to think about the relationship of the CDC and the YMC is to recognize that at slow growth rates, when exponentially growing cells spend much of their time in G0/G1 (2,3), the barrier of entry to the CDC likely involves some measures of cell mass or size and nutritional status in addition to DNA integrity. Cells that are too small or unable to pass the G1/S checkpoint for other reasons remain in G0/G1, where they continue to cycle metabolically and grow. What our work emphasizes is that this metabolic dependence of the CDC appears to require the oscillatory behavior of the YMC. Once one accepts this, then much of the literature on growth rate and metabolic oscillation is reconcilable quantitatively. Previous work has shown that the YMC functions in individual cells from unsynchronized (5) as well as synchronized populations. Thus, we emerge with a picture of the YMC and CDC in continuous cultures as cartooned in Fig. 5.

By finding conditions in which the YMC oscillates robustly without cell division, we have been able to parse out two kinds of oscillating genes: those that oscillate with the YMC and those that oscillate only with the CDC. Every slowly growing culture of yeast should then be seen as a mixed population with respect to the phases of these cycles.

**Why Cycle?** The suggestion that the biological role of the YMC is to restrict DNA replication to the reductive phase of the YMC (9,12,13) is not supported by our previous observation that DNA replication can coincide with the peak of oxygen consumption in a wild-type strain (3). Here we report again a behavior that cannot be accounted for by the suggestion that the function of the YMC is to protect DNA replication; we found robust periodic oscillations in oxygen consumption and gene expression, although less than 1% of cells replicated DNA without measurable periodicity. Both our data from continuous YMC cultures (3) and from this paper suggest that the change in oxygen consumption seen at the level of the culture does not directly reflect the change in oxygen consumption of the subpopulation of cells committed to division (S/M/G2 phases). Our data do not indicate directly the rate of respiration in the dividing subpopulation in continuous cultures; rather, the data show that the measured changes in oxygen consumption are due to the G1 subpopulation (Fig. 5). If a requirement for a reductive environment for DNA replication is not the primary driver of the YMC, what is it? A possible explanation could be that at slow growth, when most genes are expressed at very low average levels (SI Appendix, Fig. S6), on the order of one mRNA per cell (5), concentrating the expression of biosynthetic genes and active metabolism into short pulses provides a more reliable mode of regulation compared with constant low expression levels throughout the CDC period. In fact, it is rather unlikely for a cell to make all subunits of a macromolecular complex simultaneously if the probability of making each subunit is low and uniformly distributed throughout the CDC period. Considering that the biosynthesis of the subunits also needs energy (ATP) and precursors (such as amino acids), one can easily see why the coordination of many biosynthetic reactions can be beneficial in ensuring timely synthesis and assembly of macromolecular complexes. Another possible and not mutually exclusive role for the YMC could be the periodic degradation of mistranslated and damaged proteins, because autophagy and vacuolar genes peak during the LOC, right after the biosynthesis phase (HOC). The accumulation of reserved carbohydrates during the LOC, which was reported with the discovery of the YMC (4) and confirmed recently (19), likely imposes a fundamental limit on the duration of the LOC required for accumulating enough trehalose in glucose-limited conditions, and on the duration of the HOC given the pool size of reserved carbohydrates. The metabolic synchrony at the level of the culture likely requires cell–cell communication, which we do not yet understand.

**Regulation and Predictions.** The majority of the genes cycling in phase both in our batch culture and in the continuous glucose culture have universal growth-rate response (3) and are regulated by the target of rapamycin (TOR) and protein kinase A (20). Therefore, we expect periodic changes in the activity of at least one of these master regulators. This expectation is bolstered by the report that TOR regulates the acetylation of histones of ribosomal proteins (21) and by the recent finding that for many HOC genes (including the ribosomal proteins), the time of peak expression coincides with the acetylation of their
Material and Methods

Culture Conditions and Gene Expression. The culture was grown in 500-mL fermenter vessels (Sixfors; Infors) containing 500 mL of culture volume, stirred at 400 rpm, and aerated with humidified and filtered air. We used the phosphate-limited medium that we described previously (3). Gene expression was measured with 8 x 15k Agilent microarrays as described previously (3).

Ordering Genes by Phase of Peak Expression. Among the many possible approaches for identifying the phase of peak expression of each gene, we chose correlation analysis with the following algorithmic steps:

- For each experiment (three cycles), the data for oxygen consumption and for the ith gene were interpolated at 150 equally spaced time points resulting in two vectors, o and g, for oxygen and gene expression, respectively: o ∈ R150 and g ∈ R150.
- A vector of correlations (r ∈ Rm) was computed by sliding the two interpolated vectors (o and g) relative to each other, one element at a time for the first 40 elements.
- The index (position) of the largest element of r was selected to represent the phase (φ) of peak expression of the ith gene: φ = argmaxi r(ψ), j = 1…40.
- The genes were ordered by sorting the phases φ of all genes for which the maximum correlation between o and g exceeded a threshold.

See SI Appendix for more details.

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