

Regulation of yeast oscillatory dynamics

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When yeast cells are grown continuously at high cell density, a respiratory oscillation percolates throughout the population. Many essential cellular functions have been shown to be separated temporally during each cycle; however, the regulatory mechanisms involved in oscillatory dynamics remain to be elucidated. Through GC-MS analysis we found that the majority of metabolites show oscillatory dynamics, with 70% of the identified metabolite concentrations peaking in conjunction with NAD(P)H. Through statistical analyses of microarray data, we identified that biosynthetic events have a defined order, and this program is initiated when respiration rates are increasing. We then combined metabolic, transcriptional data and statistical analyses of transcription factor activity, identified the top oscillatory parameters, and filtered a large-scale yeast interaction network according to these parameters. The analyses and controlled experimental perturbation provided evidence that a transcriptional complex formed part of the timing circuit for biosynthetic, reductive, and cell cycle programs in the cell. This circuitry does not act in isolation because both have strong translational, proteomic, and metabolic regulatory mechanisms. Our data lead us to conclude that the regulation of the respiratory oscillation revolves around coupled subgraphs containing large numbers of proteins and metabolites, with a potential to oscillate, and no definable hierarchy, i.e., heterarchical control.

metabolic regulation | respiratory oscillation | temporal structure | transcriptional regulation | self-organization

As we obtain a greater understanding of systems dynamics, it is becoming apparent that biological oscillators play critical roles in the organization of physiology in all time scales, ranging from milliseconds to years (1, 2). At the end of yeast growth in batch culture, there is series of cycles in respiratory activity that occur before the culture entering stationary phase (3). When continuous culture is initiated, the culture can be maintained in this oscillatory state [40 min to 5 h; see [supporting information \(SI\) Fig. 5](#)] for months (4–6). These dynamics result in the temporal separation of many essential cellular functions, including redox biochemistry (7, 8), transcription (9, 10), energetics (11), chromosome cycle (1), and mitochondrial function (5). Although respiration is always active, the cellular redox state cycles between an oxidative phase and reductive phase. It is known that at least two redox active compounds, acetaldehyde and H₂S, mediate cell–cell communication (12), but little is known regarding how synchrony is generated within the cell and how the network is regulated.

A major problem in elucidating the oscillatory mechanism is the extent to which the cellular network is entrained, i.e., in a system where the majority of parameters oscillate how does one pull out core mechanisms. Fortunately, *Saccharomyces cerevisiae* has been used extensively as a proving ground for many of the new low- and high-throughput tools in modern molecular biology, leading to yeast having the best characterized cellular network among all eukaryotes. We combined this information with our data to address the regulation of oscillatory dynamics. Initially we used GC-MS data and statistical analyses of transcriptional data to identify the operation of a defined biosynthetic program during the oscillation. We then developed a

simple fast Fourier transform-based algorithm to calculate a parameters correlation to a sine wave and phase of peak concentration for data from a number of experimental sources and the statistical analyses of data (13). The top oscillating components were visualized by using a reconstructed large-scale network that encompassed much of the current understanding of yeast physiology. We then tested one of the most significant network features that coordinates amino acid biosynthesis, cellular reduction, cell division and respiration, by perturbation.

Results

Previously we have shown that many metabolites oscillate with a phase relationship to respiration (5, 7, 12). We expanded these data by using GC-MS to show that this oscillation is metabolome-wide (Fig. 1*a*). Furthermore, most metabolite concentrations (70%) peak during the transition from the oxidative to reductive phase (Fig. 1*a*), in coincidence with the maximum in NAD(P)H concentration and the maximum DNA synthesis rate (14). The transcripts whose abundance peaked during the oxidative phase were involved in biosynthesis. Statistically grouping these transcripts according to ontology (Fig. 1*b*) revealed that there was a defined sequence of events or biosynthetic program between 35° and 120° (Fig. 1*b*). During the reductive phase of the cycle transcript, abundance was enriched with transcripts responsible for respiration, mitochondrial biogenesis, and actin arrangement. This production was compatible with our previous observation that mitochondrial cristae oscillate between orthodox (oxidative phase) and a condensed (reductive phase) state during a respiratory cycle (11). From these data we estimate that the metabolite concentrations peak 100–150° (12–18 min) after the peak in transcriptional activation of the pathway.

Using fold changes to gauge how an individual parameter influences a system underestimates the importance of certain key molecules, such as transcription factors on cell physiology. Furthermore, it is difficult to integrate data from numerous experiments to produce a coherent view of complex phenomena. However, when one has densely populated time series data, one can use signal-processing techniques to derive dynamics. Here, we use a simple Fourier technique to derive two measures from a variety of raw data sets (SI Fig. 6). The peak production time of the waveform is represented by fast Fourier transform-derived phase angles (φ), where 0° represents the minimum first derivative of the dissolved oxygen measurements, where one cycle

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Abbreviations: CMtr complex, Cbf1–Met⁴–Met²⁸–Met³¹–Met³² transcription regulatory complex; PKA, protein kinase A; TOR, target of rapamycin.

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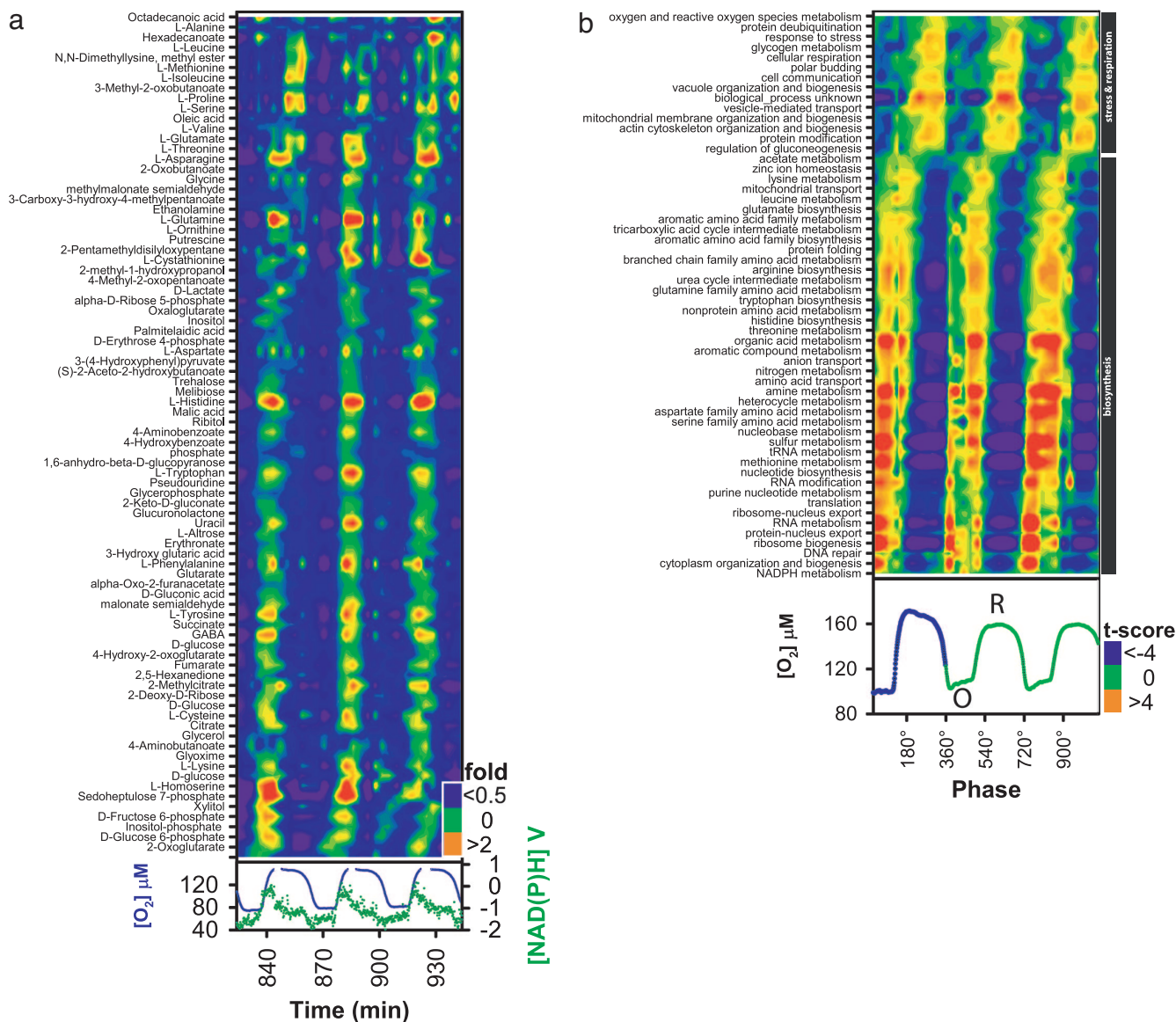


Fig. 1. Metabolic orchestration in oscillatory yeast continuous cultures. (a) The GC-MS analysis of the respiratory oscillation observed during continuous culture indicates the entire metabolome oscillates. For visualization, the data have been scaled to show the fold change. (Lower) Dissolved oxygen trace (blue) and the [NAD(P)H] trace (green) for the sample period. (b) (Lower) The respiratory oscillation consists of the cyclic switching between phases of low [oxidative (O), high oxygen uptake rate] and high [reductive (R), low oxygen uptake rate] oxygen concentrations. (Upper) The transcriptional program was visualized as a heat map constructed from the statistical analyses of gene functional ontology (13). The values were then plotted against oscillation phase. Biosynthetic processes and respiratory/stress events were clearly separated during the oscillation (black boxes), occurring (120–180°) out of phase with their respective phenotype of biosynthetic (a) and respiratory events (b Lower). Both heat maps were ordered according to the phase angle (φ) of the measurements' peak production.

typically lasted ≈ 40 min. The correlation to sine wave or oscillation strength (O) for each measured parameter was also calculated by dividing the amplitude derived by fast Fourier transformation by the integrated raw data (SI Fig. 7). We reasoned that if the parameter had a high O , then it was likely to be coupled more closely to the oscillation regulatory loop, i.e., the further the target is away from the oscillating loop, the more the transcript would deviate from a sinusoidal waveform.

Transcriptional Core Involved in Respiratory Dynamics. Next we asked what factors regulate the operation of the biosynthetic program. To approach this question, we reconstructed a network consisting of transcriptional regulatory and protein interactions (for details on the construction of this network, see SI Methods) that was filtered according to the oscillation strength of the

transcript ($O \geq 0.75$; 861 transcripts; 33 transcriptional regulators; Fig. 2a and SI Table 1) and the oscillation strength of the transcription factor activities ($O \geq 0.75$; 21 transcriptional regulators; Fig. 2b) derived from statistical analyses of their targets. The filtered connected network of 312 nodes and 519 edges showed strongest oscillatory behavior. The network was further filtered to focus on the core connected transcriptional regulatory nodes and complexes (33 nodes, 74 edges; Fig. 2c). This regulatory network consisted of two out-of-phase subgraphs whose transcripts were produced in tight temporal windows.

The gene network fanning out from the transcriptional regulatory network was then analyzed statistically for 2- and 3-node gene network motifs (15) (see SI Methods and SI Table 1). These small gene networks, in combination, are thought to regulate

Although we have focused on the regulation of the biosynthetic program, the majority ($\approx 90\%$) of the mRNA species concentrations peaked during the reductive phase (9). The major oscillating cohort in this phase ($\approx 1,500$ genes) had no defined function (Fig. 1*b*). This poor functional classification may be partly explained by the utilization of glucose-repressed growth conditions where mitochondrial activity was greatly reduced for the majority of yeast studies (3). The core regulatory network coexpressed with this event includes an array of transcriptional regulatory hubs that were highly interconnected (Fig. 2*c*, gray circle). At its core are four transcription regulators (Fig. 2*c*) implicated in the regulation of complex phenotypic responses. The Phd1p is implicated in pseudohyphal regulation (32). Sok2p is implicated in sporulation and long-term yeast adaptation (33). Cin5p and Msn4p have a more general stress-sensing role (34). Msn4p activity by localization in the nucleus is influenced by protein kinase A (PKA) (35), and it has been suggested that Sok2p and Phd1p are involved in the regulation of cAMP metabolism (32, 36). In addition the cyclic mobilization of glycogen and oscillation in cAMP concentrations observed during oscillatory dynamics during growth on glucose media, supplies evidence that the PKA pathway is involved in the regulation of the oscillation (6). It is an attractive theory that cyclic mobilization of the reserve carbohydrate glycogen regulated by PKA in a cAMP-dependent manner, thus controlling energy metabolism, is the central oscillatory loop. However, the oscillation persists during growth on respiratory carbon sources, such as ethanol and acetaldehyde (37), whereas glycolytic activity was abolished. This persistence provides strong evidence that respiratory dynamics, not fermentative dynamics, are critical for oscillation. Also, although cAMP and glycogen oscillated when the cells were grown on glucose, no oscillation was observed in these metabolites when the cells were grown on ethanol media (38 and D.B.M., unpublished data). This finding does not rule out a role for the PKA pathway in controlling the respiratory oscillation because the TOR complex 1 and PKA signaling pathways cross-talk (39), and the PKA pathway can be phosphorylated independently of cAMP (40). The mechanistic elucidation of these transcriptional regulators is needed to elucidate their role during oscillatory dynamics.

Biological networks have been layered into genome, transcriptome, proteome, and metabolome, and these networks are then modularized. Within the cellular context, this hierarchy is at best misleading because intrinsic network fluctuations are quickly communicated throughout the highly interconnected network by molecular interactions, thus the network forms a complex heterarchy (41). The regulatory loops described here have the potential to oscillate, but in isolation they may never show stable oscillation. The regulation of the oscillatory dynamics by metabolic control of enzymes and the modification of proteins were not examined in detail in this work, but this omission does not preclude their contribution. Indeed, a purely metabolic computational model has been shown to reproduce limited aspects of the respiratory oscillation (42). Rather, it is only when metabolic, translational, transcriptional, and protein control are placed within a coupled network context that the depth of the cellular dynamics we observe can be explored. From this work, we conclude that regulation of the oscillator is not the result of a central oscillator, but rather it emerges from numerous subgraphs with the potential to oscillate, i.e., stable periodicity arises from arrays of small genetic feedforward loops, coupled together by metabolic and protein feedback loops to provide a temporal program (such as the events we described in biosynthesis). It has been postulated that this type of control is involved in circadian regulation (43), and it may be general strategy for the robust maintenance of cellular processes in eukaryotes against environmental perturbation. Indeed, there is already evidence from

other eukaryotic systems such as the zebrafish development clock (44) and Protista (14) that this is the case.

Materials and Methods

Culture Techniques and Online Measurements. *S. cerevisiae* strain IFO 0233 was maintained, precultured, and grown continuously on semidefined glucose medium as described previously (5) (for details, see *SI Methods* and *SI Fig. 5*). Continuous partial pressure of oxygen (P_{O_2}) and partial pressure of carbon dioxide (P_{CO_2}) off-gas measurements were carried out by using an Enoki-III analyzer (Figaro Engineering, Osaka, Japan). The partial pressure of hydrogen sulfide (P_{H_2S}) in the off-gas was measured continuously by using an electrode-based gas monitor (HSC-1050HL; Gastec, Ayase-City, Kanagawa, Japan). O_2 uptake rates (q_{O_2}), CO_2 production rates (q_{CO_2}), and H_2S production rates (q_{H_2S}) were derived from these measurements (*SI Methods*). Instruments were calibrated according to the manufacturer's instructions. NAD(P)H was measured as described previously (7), except data were acquired by using a fast-acquisition USB-pod (PMD-1608; Measurement Computing, Norton, MA). Pulse additions (1 ml) of rapamycin (Wako Chemicals, Osaka, Japan) were carried out by syringe/sterile filtering (Teflon 0.2- μm pore; Millipore, Tokyo, Japan) a diluted stock solution (1 mg/ml ethanolic solution). Injections were carried out three times, and the data used show a representative perturbation. Respiratory quotient (q_R) was defined as q_{CO_2}/q_{O_2} . Measurements of CO_2 , O_2 , and H_2S were phase-delayed by 10.2 min to account for the measurement lag caused by the fermentor headspace (≈ 2 liters) and were accomplished by pulse-injecting a known concentration of the gas in question into the air supply line and subtracting the peak maxima from the time of addition.

Metabolite and Protein Measurements. The metabolite data set used previous work (5, 7, 12, 21, 22, 25, 27, 28, 37, 38), and GC-MS analyses (*SI Methods*) of intracellular metabolites were used to construct the metabolite data set. All Western blots were carried out according to the manufacturer's instructions (Atto, Bunkyo-ku, Tokyo, Japan). Antibodies for Gcn4p were supplied by Santa Cruz Biotechnology (Santa Cruz, CA), and the reactions for antibody detection of proteins were carried out according to the manufacturer's instructions (Atto).

Statistical Analyses. The analyses were carried out on each microarray time point (13). Generation of T scores used t tests to analyze for differences between groups of genes and global expression on the YS98 chip (Affymetrix, Santa Clara, CA). Before analysis, the data were scaled by dividing the transcript abundance values by the mean abundance for each experiment. All of the data were imported into MS Excel worksheets (*SI Table 1*). Small gene networks containing 3- and 4-node motifs were analyzed for significance and quality (15). The P value and the Z score were calculated by comparing the frequency of all occurrences of the target motif in a target network with the frequency values of the motif 1,000 random permutations of the target network. The resulting network motifs were imported into MS Excel worksheets (*SI Table 1*).

Signal Processing. All calculations were carried out in MATLAB (version 6.5.1). The Affymetrix data set used was described previously by Klevecz *et al.* (ref. 8; Gene expression Omnibus, GSE2583). The use of the discrete Fourier transform to calculate oscillation strength from raw unscaled periodic data captured the correlation to a sine wave, therefore avoiding skewing data patterns inherent in traditional of normalization and preserving intensity information lost by scaling of high-throughput data and allowing the integration of data sets. These data included measurements of metabolites, low noise Affymetrix transcript profiles (9), and derived statistical anal-

ysis of transcription factor activity (13), online measurements, and in this study GC-MS. Therefore, we summarized waveforms by two measures: the time of maximum production (φ) and its correlation to a sine wave (O). The detailed methods used are shown in the *SI Methods* and *SI Fig. 6*. On the network graphs (Figs. 2 and 3), φ is represented by colored fills where light magenta represents 0° and light green indicates 180° (≈ 20 min after the minimum first derivative).

Visualization. All graphs were constructed by using Sigmaplot 8.0 (Hulinks, Tokyo, Japan). To visualize the transcript and metabolite data, the data were first scaled to each experiment. Two

independent experiments were used for the transcription data, one containing 10 microarrays and one containing 22 microarrays (the blue and green scatter plot in Fig. 1*b Lower*, respectively). Publicly available databases were downloaded and converted to Cytoscape (45) format before visualization (*SI Methods*).

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