

A conserved cell growth cycle can account for the environmental stress responses of divergent eukaryotes

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ABSTRACT The respiratory metabolic cycle in budding yeast (*Saccharomyces cerevisiae*) consists of two phases that are most simply defined phenomenologically: low oxygen consumption (LOC) and high oxygen consumption (HOC). Each phase is associated with the periodic expression of thousands of genes, producing oscillating patterns of gene expression found in synchronized cultures and in single cells of slowly growing unsynchronized cultures. Systematic variation in the durations of the HOC and LOC phases can account quantitatively for well-studied transcriptional responses to growth rate differences. Here we show that a similar mechanism—transitions from the HOC phase to the LOC phase—can account for much of the common environmental stress response (ESR) and for the cross-protection by a preliminary heat stress (or slow growth rate) to subsequent lethal heat stress. Similar to the budding yeast metabolic cycle, we suggest that a metabolic cycle, coupled in a similar way to the ESR, in the distantly related fission yeast, *Schizosaccharomyces pombe*, and in humans can explain gene expression and respiratory patterns observed in these eukaryotes. Although metabolic cycling is associated with the G0/G1 phase of the cell division cycle of slowly growing budding yeast, transcriptional cycling was detected in the G2 phase of the division cycle in fission yeast, consistent with the idea that respiratory metabolic cycling occurs during the phases of the cell division cycle associated with mass accumulation in these divergent eukaryotes.

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INTRODUCTION

Periodic oscillations in the oxygen consumption of metabolically synchronized populations of budding yeast (*Saccharomyces cerevisiae*), termed the yeast metabolic cycle (YMC), were observed decades ago (Finn and Wilson, 1954; Küenzi and Fiechter, 1969). DNA microarray studies of metabolically synchronized cultures showed that most genes in the genome are expressed periodically during the YMC (Klavec et al., 2004; Tu et al., 2005; reviewed by Murray and

Lloyd, 2007; Murray et al., 2011; Laxman and Tu, 2010). The various phases of the YMC can be referred to in many ways based on the identities of the genes that are expressed in waves during the cycle (Tu et al., 2005, introduced such a system). However, variation in the patterns of gene expression in cycling cultures can be introduced by differences in nutrition or growth rate (Slavov and Botstein, 2011). To simplify discussion, we divide the YMC into just two phenomenologically defined phases, based only on oxygen consumption: the low-oxygen consumption phase (LOC), when the amount of oxygen in the medium of a synchronized culture is high because the cells consume little oxygen, and the high-oxygen consumption phase (HOC), when the reverse holds (Slavov and Botstein, 2011; Slavov et al., 2011). Biosynthetic genes peak during HOC, whereas autophagy and stress response genes peak during LOC (Tu et al., 2005; Slavov and Botstein, 2011; Slavov et al., 2011). Comparison of mRNA correlations in single cells and in synchronized populations suggested that the YMC observed in synchronized cultures reflects a cycle also present in single cells from slowly growing asynchronous cultures (Silverman et al., 2010; Wyart et al., 2010). We suggested previously

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Abbreviations used: CDC, cell division cycle; ESR, environmental stress response; HOC, high oxygen consumption; LOC, low oxygen consumption; TOR, target of rapamycin; YMC, yeast metabolic cycle.

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(Slavov *et al.*, 2011) that this single-cell autonomous cycle normally occurs during the G0/G1 phase of the cell division cycle (CDC) of slowly growing budding yeast, leaving open the question of metabolic cycling during other phases of the CDC.

Depending on the conditions of metabolic synchrony, metabolic cycles can vary substantially in length and may be either accompanied by synchronous cell division or not (Klevecz *et al.*, 2004; Tu *et al.*, 2005; Slavov *et al.*, 2011). Yet there are three main factors suggesting the common origin of metabolic cycles observed in different conditions: 1) The expression levels of thousands of genes are correlated with each other and with the oxygen consumption in the same way across different conditions (Slavov *et al.*, 2011). 2) By gradually changing growth parameters, such as growth rate and biomass density, we can gradually change the frequency (period) of metabolic cycling without any abrupt transitions (Slavov and Botstein, 2011). 3) Even when nutrients are so depleted that cells cannot pass through START (enter S phase), we observe that the cyclins driving this transition increase in expression (Slavov *et al.*, 2011) during the HOC phase when the cell division begins in the continuous YMC cultures, albeit nutrient shortage limits the range of cyclin expression and prevents entering the S phase.

As the growth rate of an asynchronous culture increases, so does the duration of the HOC phase relative to the LOC phase (Slavov and Botstein, 2011), resulting in an increase in the fraction of cells in HOC and thus the population-average expression levels of genes whose expression peaks in HOC (Brauer *et al.*, 2008; Slavov and Botstein, 2011). Conversely, the fraction of cells in LOC decreases with increasing growth rate, and so do the population-average levels of genes peaking in the LOC phase (Brauer *et al.*, 2008; Slavov and Botstein, 2011). Given the reported connection between these growth rate changes in gene expression and the transcriptional response to environmental stress (Gasch *et al.*, 2000; Regenbergh *et al.*, 2006; Brauer *et al.*, 2008), we sought to explore whether a similar mechanism, based on changes in the LOC/HOC composition of asynchronous cultures, can account for the environmental stress response (ESR) at the single-cell level. The connection between the ESR and the growth rate is fortified by the recent observation that slowly growing cultures are more resistant to lethal heat shock (Lu *et al.*, 2009), indicating that slow growth protects against heat stress. Such "cross-protection" has been observed previously between different types of stresses: Exposure to a nonlethal stress of one kind increases resistance to the same and to other stress factors (Mitchel and Morrison, 1982, 1983; Berry and Gasch, 2008).

An attractive possibility emerges from all of these observations (Gasch *et al.*, 2000; Brauer *et al.*, 2008; Lu *et al.*, 2009; Slavov and Botstein, 2011; Slavov *et al.*, 2011): changes in the fraction of cells in the HOC and in the LOC phases may underlie the growth rate response, the environmental stress response, and the cross-protection among different stress factors. In this view, when an asynchronous population of budding yeast is stressed, cells in the HOC phase that survive either slow or stop their growth and transition into the LOC phase until growth can resume. On a population level, the result will be that a much larger proportion of the cells will be in the LOC phase, during which we hypothesize that cells have higher stress resistance (Wang *et al.*, 2000), and the observed expression level of genes normally expressed in the LOC phase will increase, whereas the expression level of genes normally expressed in the HOC phase will fall. Such transitions can explain both the stress cross-protection and the growth rate dependence of the heat shock sensitivity. Here we tested this hypothesis based on new and published data. We found that much of the transcriptional response to stress and its change with growth rate can be

explained by our hypothesis; in metabolically synchronized cultures, we observed directly higher resistance to lethal heat shock for cells assayed during the LOC phase compared with cells in the HOC phase.

When growing slowly, *S. cerevisiae* cells accumulate mass mainly during the G0/G1 phase of the CDC; this is the CDC phase in which the metabolic cycle is prominent. The assessment of whether or not to proceed in the CDC is carried out by the system of cyclin-dependent kinases at the G1/S boundary (Hartwell, 1973; Hartwell *et al.*, 1973; reviewed by Reed, 1992). In contrast, fission yeast (*Schizosaccharomyces pombe*) accumulates mass mainly during the G2 phase, and the assessment of whether or not to proceed is carried out at the G2/M boundary by a largely orthologous system of cyclin-dependent kinases (Nurse and Bissett, 1981; Beach *et al.*, 1982; Shiozaki, 2009). If fission yeast has a metabolic cycle, the cycle should be directly evident in the gene expression data of CDC-synchronized cultures (Peng *et al.*, 2004; Rustici *et al.*, 2004; Oliva *et al.*, 2005), since such cells grow mainly in the G2 phase before they divide (Shiozaki, 2009).

Using gene expression data from fast-growing, respiring, CDC-synchronized cultures of fission yeast, we found that oxygen consumption and biosynthetic genes that function during HOC peak in expression in the early G2 phase, followed by genes that function during LOC. This result suggests that fission yeast has a metabolic cycle similar to that of the budding yeast YMC, but in the G2 phase of its CDC. Furthermore, we discovered that this metabolic growth cycle in fission yeast correlates to the ESR in fission yeast (Chen *et al.*, 2003) in the same way as the YMC correlates to the ESR in budding yeast. Thus the same principles and mechanism can explain the growth rate response and the environmental stress response in those divergent genera of fungi.

Finally, we also obtained suggestive evidence of a metabolic cycle in human cells. This result is based on the expectation that if human cells have a metabolic cycle, each cell should express at high levels either genes functioning in HOC or genes functioning in LOC but not both sets simultaneously. Consistent with this expectation, expression of genes serving functions in the HOC and LOC phases of the YMC are negatively correlated, and thus reciprocally regulated, across small subsets of human epithelial cells in a three-dimensional culture model of mammary acinar morphogenesis. Reciprocal regulation of these genes, associated with metabolic cycling in yeasts, suggests the existence of a metabolic cycle that might be shared by all eukaryotes, including humans. This conclusion is bolstered by our observation that various stress factors induce a common transcriptional stress response, involving thousands of genes, in primary human fibroblasts that both resembles the yeast ESR and can be explained in terms of the HOC-to-LOC transition.

RESULTS

The environmental stress response is coupled to the budding yeast metabolic cycle

The transcriptional response of budding yeast to changes in growth rate is strongly correlated with the transcriptional response to environmental stress and with the periodic gene expression during the YMC (Brauer *et al.*, 2008; Slavov and Botstein, 2011; Slavov *et al.*, 2011). The relationship between the ESR and the YMC, however, has not been characterized apart from the observation that some genes peaking in LOC are annotated to the stress response (Tu *et al.*, 2005; Slavov and Botstein, 2011). To explore this relationship for all genes expressed periodically in the YMC and regulated by stress, we plotted the phase-ordered expression profiles of YMC periodic genes during the budding yeast metabolic cycle and the

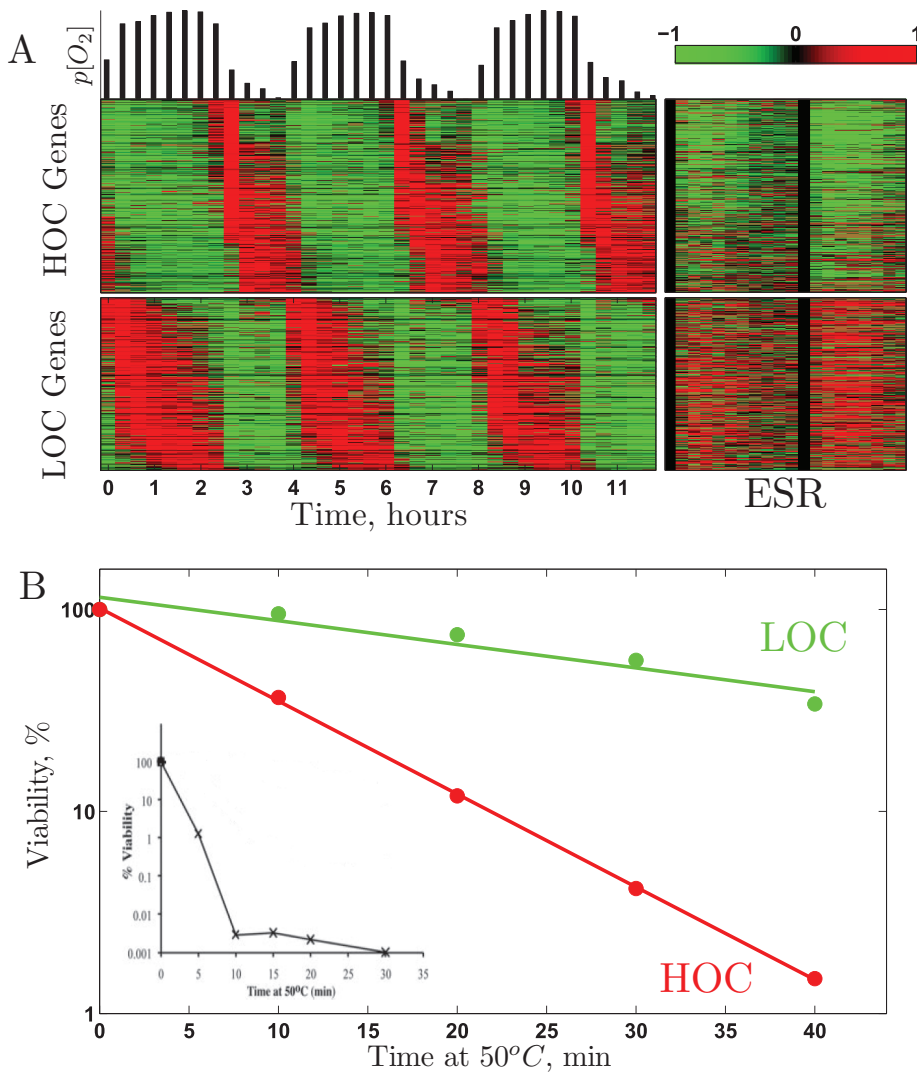


FIGURE 1: Correlation between metabolic cycling, transcriptional ESR, and sensitivity to lethal heat shock. (A) Left, phase-ordered metabolic genes expressed periodically in a budding yeast culture that was YMC synchronized (Tu *et al.*, 2005). The bars on the top correspond to dissolved oxygen in the medium. Right, the corresponding expression levels of the genes, measured by Gasch *et al.* (2000) during stress (H_2O_2 , heat). The gene expression data in this and all other figures are displayed on a \log_2 scale (Eisen *et al.*, 1998). (B) Percentage of the cells surviving after 50°C heat shock with duration indicated on the x-axis. Samples were taken from the HOC phase and from the LOC phase of a metabolically synchronized culture growing at $\mu = 0.10 \text{ h}^{-1}$. The counting error is <10%; see *Materials and Methods*. The inset shows the same type of heat shock applied to a phosphate-limited asynchronous culture growing at $\mu = 0.3 \text{ h}^{-1}$ (Lu *et al.*, 2009).

expression profiles of the same genes during the ESR (Figure 1A). The result indicates a remarkable correlation: HOC genes are down-regulated during the ESR, whereas LOC genes are up-regulated during the ESR. The simplest mechanism that can account for this correlation is that stress causes HOC-phase cells to transition into the LOC phase, increasing the fraction of cells in LOC, and thus increases the population-average levels of genes peaking in LOC. Because the fraction of cells in the HOC phase decreases correspondingly, so do the population-average levels of genes peaking in HOC. If continued mass accumulation requires the HOC phase, then these transitions will cause growth to slow or stop until HOC phases can be reinitiated.

with metabolically dependent stress sensitivity. This conclusion is fortified by the biphasic survival curve (inset in Figure 1B) measured in rapidly growing asynchronous culture of haploid yeast (Lu *et al.*, 2009). Such biphasic behavior shows two distinct rates of stress resistance that very likely correspond to the different rates of stress resistance of LOC and HOC cells that we measured in a metabolically synchronized culture (Figure 1B). The absolute rates of cell death measured by Lu *et al.* (2009) are somewhat higher than the ones we measured, possibly because of a strain difference: We used a diploid strain with *S288c* background, whereas Lu *et al.* (2009) used a haploid strain with *CEN.PK* background.

Cells in LOC are more resistant to lethal heat shock than are cells in HOC

If stress can induce HOC-to-LOC transition, a potential mechanism accounting for the increased stress resistance after stress and at slow growth could be that cells in LOC are more resistant to lethal stress than are cells in HOC; since slowly growing and stressed cultures have a larger fraction of cells in LOC, they would be naturally more resistant to lethal stress. To test this prediction directly, we metabolically synchronized a glucose-limited, continuous culture of the diploid strain *DBY12007* with *S288c* background. Samples were taken during the LOC and the HOC phases of the culture and subjected to lethal heat shocks at 50°C (see *Materials and Methods*). The fraction of cells surviving the heat shock after 40 min is ~10-fold higher for the LOC samples (green) compared with the HOC samples (red; Figure 1B). This difference confirms the expectation that cells in LOC are much more resistant to stress than cells in HOC.

This result has to be considered in the context of the subpopulations in a metabolically synchronized culture. Whereas a subpopulation of the culture is in G1 phase and consumes oxygen at a high rate, denoted as the HOC phase, another subpopulation is in S phase replicating DNA (Slavov and Botstein, 2011; Slavov *et al.*, 2011). The 10-fold higher death rate in HOC relative to LOC cannot be explained by stress sensitivity of S-phase cells alone, since the fraction of S-phase cells in the conditions of metabolic synchrony in our experiment is ~30% (Slavov and Botstein, 2011). Even instantaneous death of the 30% of the cells replicating DNA in the HOC sample would result in a 1.3-fold, not 10-fold, difference.

Given that LOC cells are more resistant to stress than HOC cells and that the fraction of LOC and HOC cells changes with growth rate, the stress sensitivity of asynchronous cultures can be explained in terms of their composition of single cells

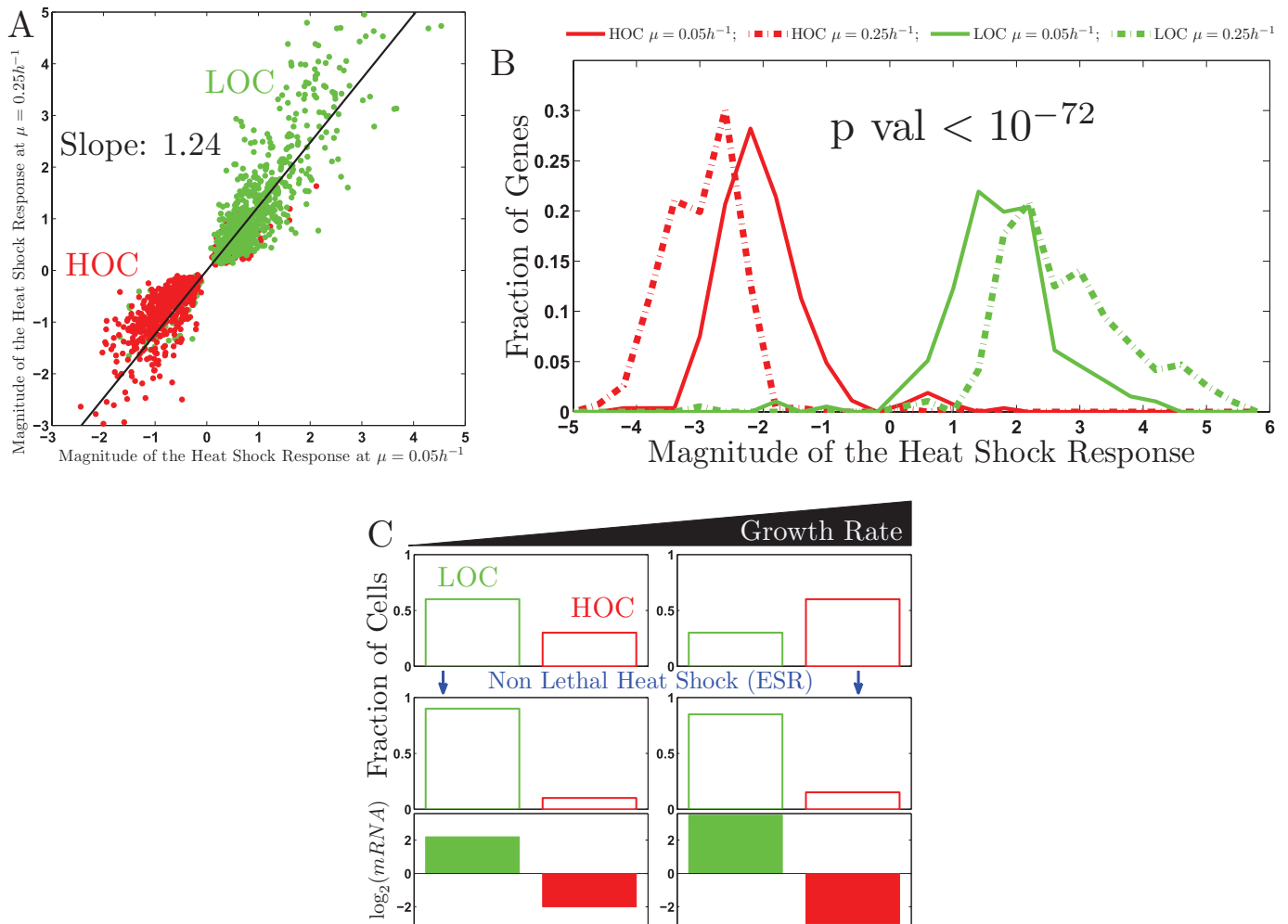


FIGURE 2: The magnitude of the ESR increases with the growth rate. (A) Comparison of the magnitude of the transcriptional heat shock response at growth rates $\mu = 0.05$ and $\mu = 0.25 h^{-1}$ for all YMC-periodic genes peaking in expression either during the LOC or during the HOC phase. (B) Distribution of fold change in the levels of genes from A whose expression levels change more than fourfold in at least one of the cultures after the heat shock. The p value is computed from a rank sum test. (C) Mechanism that can account for the observed increase in the magnitude of the ESR with growth rate. The gene expression data and the magnitude of the heat-shock response are displayed on a \log_2 scale.

The magnitude of the environmental stress response increases with growth rate

If a substantial part of the ESR is due to stress-induced transition of cells from HOC into LOC, then the magnitude of the transcriptional ESR has to be proportional to the fraction of cells that make the transition. As the fraction of cells in HOC increases with the growth rate (Slavov and Botstein, 2011), so should the fraction of cells making the transition, and thus the magnitude of the transcriptional ESR. This expectation can be tested with the measured heat-shock transcriptional responses of continuous budding yeast cultures growing at different growth rates (Lu *et al.*, 2009). Consistent with our expectation, Lu *et al.* (2009) found that ~200 genes increase in expression following a heat shock to a degree depending on the growth rate. Using their data, we tested our expectation further by testing whether their observation can be extended and generalized to genes decreasing in expression after a heat shock and ultimately to the expected positive correlation between the growth rate and the magnitude of the transcriptional stress response for all LOC and HOC genes. We compared the fold changes in the expression levels of all YMC-periodic genes (from Figure 1A) that either increase or

decrease at growth rates $\mu = 0.05$ and $0.25 h^{-1}$ and found that heat shock induces or represses mRNA levels to a greater degree in the faster-growing culture ($\mu = 0.25 h^{-1}$; Figure 2A). The magnitude of the heat shock response increases by ~24% for all YMC-periodic genes, as quantified by the linear fit to the data, and is particularly pronounced for the subset of genes whose expression levels change more than fourfold, in at least one of the cultures, after the heat shock, as shown by the distributions of their heat shock-induced fold changes (Figure 2B). These results (Figure 2, A and B) are consistent with the expected correlation: as growth rate increases, so does the magnitude of the ESR.

The simplest mechanism that can account for this observation is the HOC-to-LOC transition (Figure 2C) that was indicated by the correlation between the YMC and the ESR (Figure 1, A and B) and by the increased stress resistance of LOC-phase cells (Figure 1C) that we measured. The changes in the expression of some genes following a heat shock, however, are too large to be explained by the changes in gene expression between LOC and HOC of unperturbed cultures. At slow growth rate, $\mu = 0.05 h^{-1}$, for example, the duration of LOC is several times longer than the duration of HOC

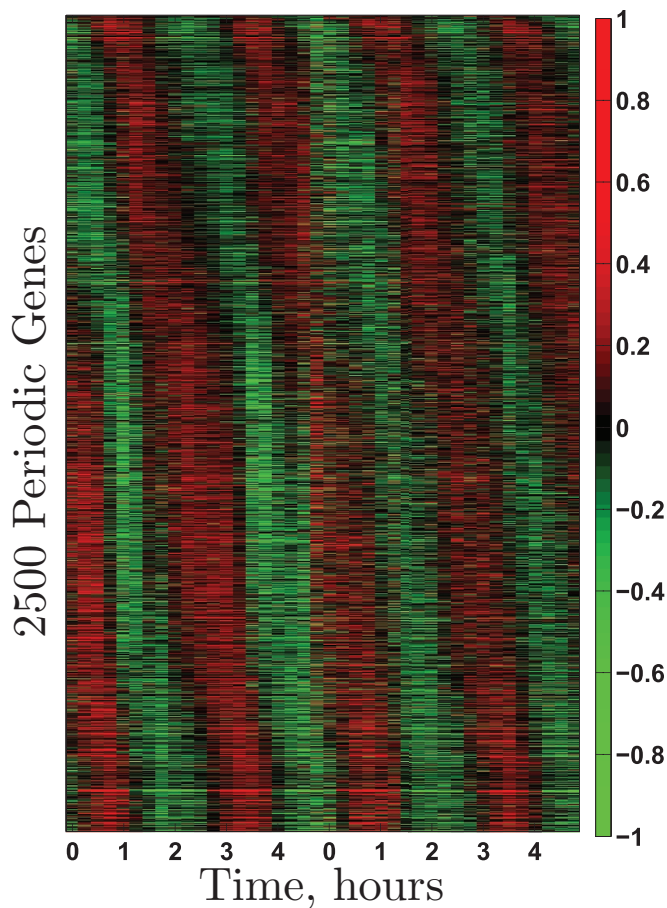


FIGURE 3: CDC periodic genes in fission yeast. Genes expressed periodically in the CDC of fission yeast are arranged by phase of peak expression based on correlation analysis; see *Materials and Methods*. The data were collected from two independent cultures that were CDC synchronized by elutriation (Rustici *et al.*, 2004). The gene expression data are displayed on a \log_2 scale.

(Slavov and Botstein, 2011), indicating that most cells in an asynchronous culture should be in the LOC phase. Therefore a transition of the remaining small fraction of cells from HOC to LOC cannot, alone, account for the twofold to fourfold changes in gene expression observed following a heat shock (Figure 2B). Thus we infer that stress activates signal transduction networks mediating the induction of the LOC phase to a substantially greater degree than what is observed during normal cycling of slow-growing but otherwise unperturbed cultures.

Metabolic genes are expressed periodically in fission yeast and coupled to the ESR

All three groups (Peng *et al.*, 2004; Rustici *et al.*, 2004; Oliva *et al.*, 2005) that measured gene expression in CDC-synchronized fission yeast cultures found hundreds of CDC-related genes expressed periodically during the CDC. In addition to those genes, thousands of other genes are also expressed periodically, albeit with small amplitudes, during the CDC of fission yeast (Oliva *et al.*, 2005; Fan, 2010). To explore whether the periodic expression of these weakly periodic genes is related to metabolic cycling, we sought to identify whether metabolic genes are expressed in different phases. The three groups that measured gene expression during the CDC of fission yeast found that some ribosomal genes are expressed periodically. Apart

from the ribosomal genes, however, no other set of genes with metabolic functions has been reported, despite the observation that many thousands of genes are weakly periodic. We examined whether some of the weakly periodic genes have functions and phases of peak expression that are indicative of metabolic cycling similar to the budding yeast metabolic cycle.

First we identified periodic genes and ordered them by the phase of peak expression (Figure 3) based on correlation analysis (see *Materials and Methods*). Then we grouped the periodic genes into three clusters according to phase of peak expression using K-means clustering. To characterize these clusters and identify overrepresented biological functions in those clusters, each cluster was analyzed with the Gene Ontology (GO) term finder (Boyle *et al.*, 2004). The results for the first cluster (marked in black in Figure 4) showed that it corresponds to G1- and S-phase genes that were identified to cycle with large amplitude by Rustici *et al.* (2004). As these authors observed, the G1 phase is very short in these conditions of fast growth, and the phases of peak expression of the G1- and S-phase genes are very close and hard to identify unambiguously.

More interesting for our analysis are the genes peaking in expression during the G2 phase—the main phase of biomass growth in fission yeast. As noted by Rustici *et al.* (2004), the amplitude of oscillation of those genes is significantly smaller than the amplitude of the G1/S cluster but clearly detectable and statistically significant, given the high-quality data of several CDC-synchronized cultures. Applying the GO term finder to the set of genes peaking in the early G2 phase, we found enrichment not only for protein biosynthesis (ribosomal biogenesis), but also for a wide range of biosynthetic processes listed in Table 1. It is striking that these processes overlap with the processes characteristic of the HOC phase of the budding yeast metabolic cycle. The possibility that the peak of those genes reflects the HOC phase of the fission yeast metabolic cycle is further supported by previous work that found increased oxygen consumption in this early G2 phase of CDC-synchronized cultures of fission yeast (Lloyd *et al.*, 1976). Such biosynthetic peak is fully consistent with the stepwise increase in mRNA and rRNA measured during the CDC of fission yeast (Fraser and Nurse, 1978, 1979; Barnes *et al.*, 1979).

The genes from the other cluster peaking in the G2 phase are related to stress, as noted by Rustici *et al.* (2004). Of interest, those genes peak after, rather than simultaneously with, the biosynthesis genes (Figure 4). This observation is very relevant since many genes peaking in LOC are related to the stress response (Tu *et al.*, 2005; Brauer *et al.*, 2008; Slavov and Botstein, 2011; Slavov *et al.*, 2011). The robustly periodic expression of those genes is thus more likely

GO term	p value
Amino acid biosynthesis	4×10^{-14}
Ribosome biogenesis	4×10^{-12}
Oxoacid metabolism	2×10^{-10}
rRNA processing	1×10^{-8}
Small-molecule biosynthesis	1×10^{-7}

The p values, quantifying the probability of observing the overlap by chance alone, are computed from the hypergeometric distribution and Bonferroni corrected for multiple hypothesis testing (Boyle *et al.*, 2004).

TABLE 1: Overrepresented GO terms within the biosynthesis gene set—the set of genes peaking in the early G2 phase of the fission yeast CDC.

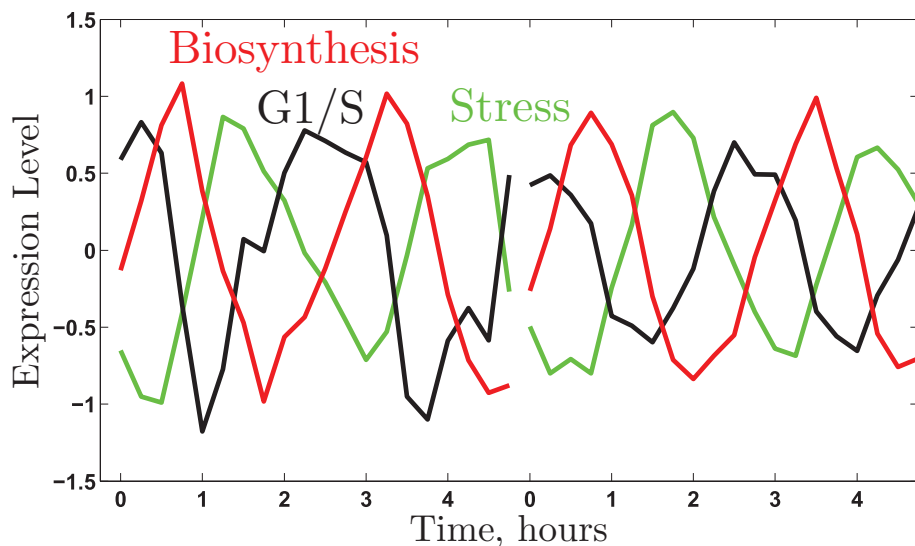


FIGURE 4: Phases of the fission yeast CDC. Mean expression levels of three gene clusters obtained by unsupervised K-means clustering. The gene sets are named after the most significantly enriched GO terms in each cluster. Table 1 lists more significantly enriched GO terms in the biosynthesis cluster. The data were collected from two independent cultures that have been CDC synchronized by elutriation (Rustici *et al.*, 2004) and are converted to z-scores (zero mean and unit variance) and displayed on a \log_2 scale.

related to metabolic cycling than to stress induced by the synchronization. The latter would more likely result in strong initial induction that gradually tapers down rather than in the periodic pattern observed in the data. In contrast to the high oxygen consumption during the peak expression of the second cluster, the oxygen consumption during the peak expression of the third cluster decreases (Lloyd *et al.*, 1976). Therefore we find very close correspondence in the temporal organization of both oxygen consumption and gene expression between the budding yeast metabolic cycle and the analogous physiological and metabolic processes in fission yeast. This close correspondence suggests very strongly the existence of a metabolic cycle in fission yeast.

To test the existence of a metabolic cell growth cycle in fission yeast further, we next considered the coupling between the periodic expression of metabolic genes and the ESR for fission yeast. As shown in Figure 1A, we found for budding yeast that the ESR is strongly correlated with the YMC. For fission yeast, we plotted the analogous data for phase-ordered metabolic genes expressed periodically in CDC-synchronized fission yeast and their corresponding expression levels during the ESR of fission yeast measured by Chen *et al.* (2003) (Figure 5B). The result indicates a remarkable conservation of the relationship between the phase of peak expression of metabolic genes during the growth phases of the CDC and the environmental stress response of these genes. This correlation between YMC and ESR is strong evidence in favor of our hypothesis that fission yeast can cycle metabolically and that a HOC-to-LOC transition might also account for the environmental stress response in *S. pombe*.

Stress and biosynthesis genes are regulated reciprocally in humans

Janes *et al.* (2010) used stochastic profiling to identify coregulated, heterogeneously expressed genes among human epithelial cells in a three-dimensional culture model of mammary acinar morphogenesis. Stochastic profiling is based on DNA microarray measurement of gene expression in repeated, random selections of very small cell

populations and subsequent analysis of the fluctuations to identify transcripts that are heterogeneously coexpressed. Arranging the data from this procedure (Figure 6) shows two large clusters of genes—the genes *within* a cluster are coexpressed with one another in the sampled subsets of cells, which according to the design of stochastic profiling reflects coexpression in single cells; conversely, the genes from the first cluster are reciprocally expressed relative to the genes from the second cluster (Figure 6).

To identify biological functions overrepresented in these gene clusters, we used the GO term finder (Boyle *et al.*, 2004). The results (Table 2) show that the first cluster is strongly enriched for biosynthetic functions, including protein biosynthesis, which can correspond to cells that were profiled in the HOC phase. The second cluster is enriched for genes involved in oxidative stress responses, NF- κ B signaling, development, and differentiation, which can correspond to cells that were profiled in the LOC phase. Thus the data from the stochastic profiling are suggestive of metabolic cycling in human epithelial cells.

In contrast, we could not detect significant periodicity in the expression of metabolic genes in CDC-synchronized human cancer HeLa cells (Whitfield *et al.*, 2002). This lack of evidence for metabolic cycling, however, is more likely due to the transformed phenotype and fast fermentative and dysregulated growth of HeLa cells, rather than to a general absence of metabolic cycling in humans. This result is strongly reminiscent of the observation that most YMC-periodic genes oscillate only weakly, if at all, in fast-growing, fermenting cultures of budding yeast that are CDC synchronized (Spellman *et al.*, 1998).

Gene cluster	GO term	p value
Biosynthesis	Cellular metabolic process	4×10^{-9}
	Cellular respiration	1×10^{-5}
	Primary metabolic process	2×10^{-5}
	Nucleotide metabolic process	3×10^{-4}
	Translation	7×10^{-4}
	Organization or biogenesis	3×10^{-3}
	Generation of precursor metabolites and energy	3×10^{-3}
Stress	Developmental process	8×10^{-11}
	Signaling	3×10^{-6}
	Cell differentiation	8×10^{-6}
	Response to stimulus	3×10^{-5}
	Transport	1×10^{-4}
	Secretion	3×10^{-4}

The p values are computed from the hypergeometric distribution and Bonferroni corrected for multiple hypothesis testing (Boyle *et al.*, 2004).

TABLE 2: Overrepresented GO terms within the sets of reciprocally regulated genes in human acinar epithelial cells.

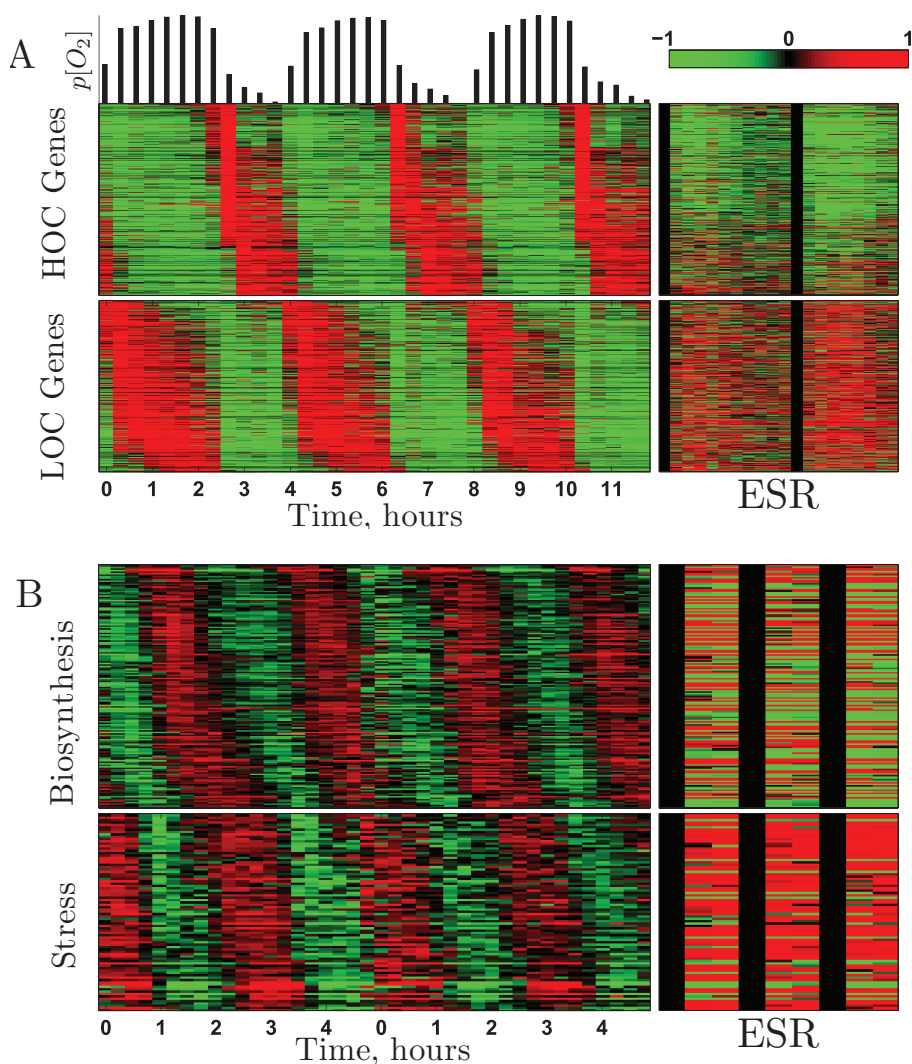


FIGURE 5: Correlation between metabolic cycling and stress response. (A) Left, phase-ordered metabolic genes expressed periodically in a budding yeast culture that was YMC synchronized (Tu *et al.*, 2005). Right, their corresponding expression levels measured by Gasch *et al.* (2000) during stress (H_2O_2 , heat). (B) Left, phase-ordered metabolic genes expressed periodically in two fission yeast cultures that were CDC synchronized by elutriation (Rustici *et al.*, 2004). Right, their corresponding expression levels measured by Chen *et al.* (2003) during stress (H_2O_2 , heat, and cadmium). The labels of the two large clusters (biosynthesis and stress) are derived from the most highly enriched GO terms within each cluster. The genes in both panels are ordered based only on their phase of peak expression in the synchronized cultures, and the gene expression data are displayed on a \log_2 scale.

HOC-to-LOC transition can explain the common stress response of human primary fibroblasts but not of HeLa cells

To test further our hypothesis of metabolic cycling in human cells and its connection to the stress response, we used previously published gene expression data (Murray *et al.*, 2004) to identify genes that increase or decrease in expression when human primary fibroblasts are stressed by a variety of stress factors: heat, dithiothreitol, menadione, and crowding. We selected genes that are consistently down- or up-regulated in at least 22 of the 30 stress conditions and time points in the study. A very large number of genes—7892 DNA clones, corresponding to >6000 unique UniGene IDs—fulfill this criterion and show a strikingly consistent and coherent expression pattern across all stress conditions, albeit with lower fold changes when compared with the 800 genes of the ESR in budding yeast (Gasch *et al.*, 2000). The set of 7892 DNA clones was clustered

hierarchically (Figure 7) and analyzed by submitting the subsets of genes that are induced (2876 unique UniGene IDs) or repressed (3164 unique UniGene IDs) to the GO term finder (Boyle *et al.*, 2004) or GOEAST (Zheng and Wang, 2008). The results indicate a remarkable conservation of biological functions between the ESR of the two yeasts and the primary human fibroblasts. Genes repressed by stress in the primary human fibroblasts are strongly enriched for mitosis (as observed by Murray *et al.*, 2004) and for a variety of biosynthetic functions ($p < 10^{-29}$). Genes induced by stress are strongly enriched ($p < 10^{-6}$) for protein degradation, ubiquitination, cell differentiation, development, response to stimuli and stress, cell localization, and apoptosis. Thus, except for a few functions specific to higher eukaryotes, stress-induced genes have quiescent-like functions characteristic of genes peaking in LOC, and stress-repressed genes have biosynthetic functions characteristic of genes peaking in HOC. This observation suggests that the HOC-to-LOC transition can also explain the stress response of primary human fibroblasts, similar to budding and fission yeast. As we found from the stochastic profiling data, the stress response data suggest metabolic cycling in primary human fibroblasts but not in HeLa cells. HeLa cells have dramatically different transcriptional stress responses (Murray *et al.*, 2004) that cannot be related in the same way to metabolic cycling and explained by HOC-to-LOC transition.

DISCUSSION

The change in the LOC/HOC fraction can provide a mechanistic connection among many phenomenological observations

Our new data, combined with analysis of already published data, support the idea that the periodic expression of genes during the HOC and LOC of the metabolic cycle described by Küenzi and Fiechter (1969), Kwak *et al.* (2003), Klevecz *et al.* (2004), and Tu *et al.* (2005) can form the unifying framework for a quantitative understanding of a number of important phenomena in *S. cerevisiae* and *S. pombe*. Specifically, in budding yeast we found that systematic variation in the timing and duration of the HOC and LOC phases can account for the transcriptional response to differences in the growth rate response observed by Brauer *et al.* (2008) and Slavov and Botstein (2011), the transcriptional response to environmental stress cataloged by Gasch *et al.* (2000), and cross-protection among stress factors (Mitchel and Morrison, 1982, 1983) and between stress and slow growth rate (Elliott and Futcher, 1993; Lu *et al.*, 2009). We could extend our analysis to the distantly related fission yeast by showing that fission yeast genes that are functionally analogous to the genes oscillating in the budding yeast metabolic cycle can

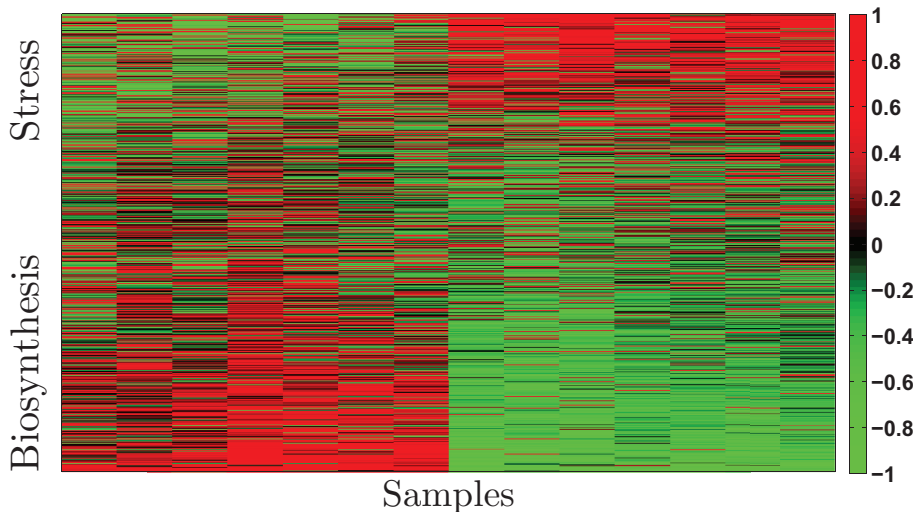


FIGURE 6: Reciprocal regulation of biosynthetic and stress and developmental genes in humans. Clustered gene expression from small sets of stochastically profiled human epithelial cells in a three-dimensional culture model of mammary acinar morphogenesis (Janes *et al.*, 2010). The labels of the clusters (biosynthesis and stress) correspond to the most highly enriched GO terms in each cluster; see Table 2. The gene expression data are displayed on a \log_2 scale.

also be seen to oscillate in fission yeast and that the correlations between the phases of peak expression of these genes and the transcriptional responses to environmental stress are also found in gene expression data from fission yeast.

Of course, it remains true that some transcriptional responses are specific to the type of stress or to the strain, as is observed for smaller subsets of genes (Gasch *et al.*, 2000; Chen *et al.*, 2003; Kvittek *et al.*, 2008; Lu *et al.*, 2009), and, as we verified, the magnitude of the transcriptional changes induced by stress exceeds the magnitude of changes found in the YMC of unperturbed cultures, suggesting that there must be additional signaling mechanisms for stress that enhance the response to the specific type of stress. Compared to other stress types, for example, oxidative stress (hydrogen peroxide or menadione treatments) results in enhanced induction of genes neutralizing reactive oxygen species (Gasch *et al.*, 2000) and in enhanced acquired tolerance to oxidative stress (Berry and Gasch, 2008). Similar stress type-specific responses are observed with other stresses (Gasch *et al.*, 2000; Berry and Gasch, 2008), and it is the common ESR that we suggest can be accounted for by HOC-to-LOC transition.

The presented data and analysis do not exclude the possibility that in addition to the transition from the HOC to the LOC phase, the stress response also involves some graded, rheostat-like changes in the transcriptional and physiological responses. Yet the metabolic cycling in yeast from asynchronous cultures (Silverman *et al.*, 2010; Wyart *et al.*, 2010), taken together with the growth rate dependence of the heat shock response, suggests that the responses of different cells are likely to depend on their phase in the metabolic cycle. Furthermore, the perfect correlation between the stress responses of many hundreds of genes and their expression levels with the metabolic cycle indicates directly large-scale gene expression similarities between the LOC phase and the transcriptional stress response. This transcriptional similarity suggests a functional and physiological connection between the LOC phase and the stress response; this connection and the gene expression similarity remain true regardless of whether the response is switch like, rheostat like, or a combination of both.

Relationship between the metabolic and cell division cycles

Our results regarding the phase of the CDC during which one can observe metabolic cycles using gene expression patterns are different for budding and fission yeast. In budding yeast, we found metabolic cycling in the G0/G1 phase of the cell cycle (Slavov *et al.*, 2011), whereas in fission yeast we observed strong evidence for metabolic cycling in the G2 phase of the fission yeast CDC. This result is readily interpretable as an association of metabolic cycling with the periods of the CDC associated with biomass accumulation. As mentioned, slowly growing budding yeast accumulate mass mainly during G0/G1 phase of the CDC, whereas fission yeast accumulate mass primarily during the G2 phase, even though essentially the same system of cyclin-dependent kinases controls the boundaries between G1 and S and between G2 and M in both yeasts (Beach *et al.*, 1982). In each yeast, these regulators are activated after assessments

concerning cell size (mass) before allowing the cell division cycle to proceed—the difference is mainly at which CDC boundary this assessment is normally made (Hartwell, 1973; Hartwell *et al.*, 1973; Nurse and Bissett, 1981; Beach *et al.*, 1982; reviewed by Reed, 1992; Shiozaki, 2009). We believe that under respiratory conditions, and especially under conditions of relatively slow growth, when the G0/G1 (budding yeast) or the G2 (fission yeast) phases are relatively lengthy, the metabolic cycle occurs as part of the process of biomass accumulation. Indeed, given the evidence we present that human cells might also have a metabolic cycle, we propose that it is a basic property of eukaryotes that mass accumulation and metabolic cycling are linked in this way.

Other differences between budding and fission yeast deserve mention. The number of periodic metabolic genes and the amplitude of oscillation of those genes are smaller in fission yeast than in budding yeast. This difference was expected, given the fast growth rate of the CDC-synchronized fission yeast cultures. In budding yeast, metabolic cycling is more evident at slow growth because the period of mass accumulation (G0/G1; Slavov *et al.*, 2011) occupies a much larger fraction of the total doubling time, and a much larger fraction of the population is therefore undergoing metabolic cycling. If the same is true in fission yeast, then the relatively fast growth rate accounts nicely for the lower amplitudes and the smaller number of oscillating genes detected. Another difference related to the growth rate is that whereas the phase offset between the HOC and the LOC genes in fission yeast is significant, it is smaller than in budding yeast growing at $\mu = 0.1 \text{ h}^{-1}$. This decrease in the phase offset in the case of fission yeast is also attributable to the shorter duration of the growth phase of the CDC with increasing growth rate.

Reciprocal regulation of biosynthesis and stress

For fission yeast, Pancaldi *et al.* (2010) conducted a gene expression meta-analysis of hundreds of conditions and found a reciprocal regulation between two major sets of genes—one containing growth- and metabolism-related genes and another containing stress- and differentiation-related genes. This is an expected outcome if the cells undergo metabolic cycling. For each physiological condition in which cells cycle metabolically, the fraction of cells in LOC is

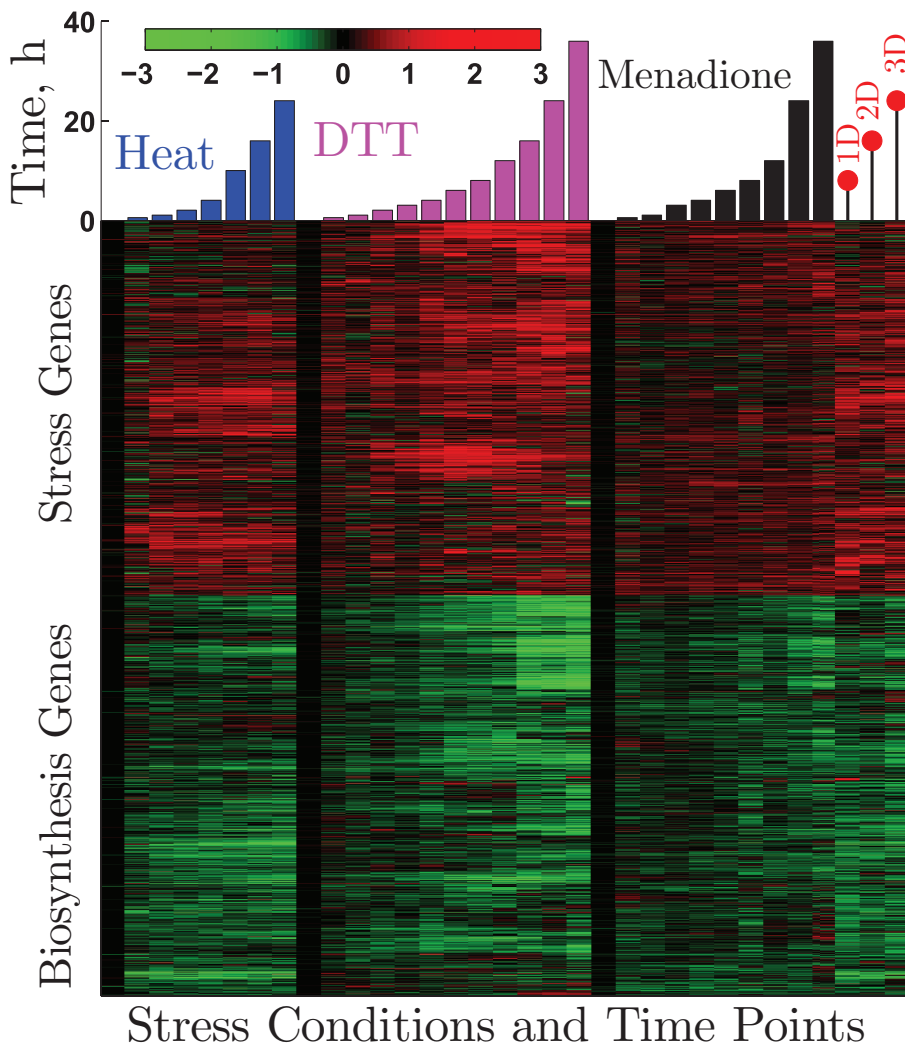


FIGURE 7: The common ESR in human fibroblasts indicates a reciprocal regulation of biosynthetic and stress genes. The expression levels of ~6000 genes either increased or decreased consistently across all stress types applied to primary human fibroblasts (Murray *et al.*, 2004). The labels of the clusters (biosynthesis and stress) correspond to highly enriched GO terms in each cluster. The solid red circles correspond to one-, two-, and three-dimensional crowding (Murray *et al.*, 2004). The gene expression data are displayed on a log₂ scale.

inversely related to the fraction of cells in HOC. For human cells, we previously described (Slavov and Dawson, 2009) a similar reciprocal regulation between cancer and noncancer tissues of ribosomal genes on one hand and stress and development genes on the other hand. The stochastic profiling of small subsets of cells indicates that at least in the case of epithelial cells, such reciprocal regulation is present even among clonal cells growing in the same culture and thus may indeed reflect a cell-autonomous metabolic cycle; cells in each phase of the metabolic cycle express a phase-specific set of genes and are therefore different from cells in the other phase. Another example of reciprocal regulation of biosynthesis and stress genes was reported in somites from zebrafish embryos (Ozbudak *et al.*, 2010). Indeed, such reciprocal regulation is consistent with regulatory interaction between master regulators. Major regulators of growth—protein kinase A and the target of rapamycin (TOR)—sequester in the cytoplasm and inactivate major regulators of stress—the Msn2 and Msn4 transcription factors that induce the expression of many stress response genes (Görner *et al.*, 1998; Smith *et al.*, 1998; Beck and Hall, 1999).

ensure the preservation of the population and may be beneficial in fluctuating environments, as suggested by modeling microbial populations (Thattai and van Oudenaarden, 2004). The oscillations in the nuclear localization of Msn2, a major stress response transcription factor, that are induced by reducing the glucose concentration (Hao and O’Shea, 2011) can also be related to the cell growth cycle and affect the stress resistance accordingly.

The cross-protection among stress factors (Mitchel and Morrison, 1982, 1983) and among stress factors and slow growth (Elliott and Futcher, 1993; Lu *et al.*, 2009) suggest that the functions of many genes induced as part of the general environmental stress response and in the LOC phase provide general protection, even if the mechanisms for inducing the ESR are stress and context dependent (Berry *et al.*, 2011). This inference is supported by our data showing higher heat shock resistance of cell in the LOC phase. Such general protection is likely to be more important at slow growth, when metabolism is fully respiratory (Kaspar von Meyenburg, 1969) and the average age of the proteins is likely to be higher due to lower rates of biomass synthesis and protein dilution by cell division. The connection

Using stress as a stimulus provides a good experimental paradigm for characterizing cellular responses. The transcriptional changes that can be elicited in response to stress, however, are relevant not only to acute external stress but also have physiological functions even in cells that are not exposed to unusual or acute environmental factors. For example, the stress response is linked to longevity in budding yeast (Shama *et al.*, 1998), *Caenorhabditis elegans* (Cypser and Johnson, 2002; Baumeister *et al.*, 2006; Henis-Korenblit *et al.*, 2010), *Drosophila melanogaster* (Vermeulen and Loeschke, 2007), and mammals (Migliaccio *et al.*, 1999). Thus the regulation of the stress response genes by changes in the relative durations of the phases of the cell growth cycle is likely to have substantial impact on cell physiology even in the absence of acute external stress factors. This hypothesis is consistent with the observation that decreased signaling through the target of rapamycin (TOR), a likely master regulator of the cell growth cycle (Slavov and Botstein, 2010; Slavov *et al.*, 2011), can expand lifespan (Kaeberlein *et al.*, 2005; Powers *et al.*, 2006; Boer *et al.*, 2008).

Dependence of stress sensitivity on the phases of the YMC

For budding yeast, we found that stress sensitivity depends on the metabolic state. This finding has important evolutionary implications. It allows for an asynchronous population to grow continuously (since a fraction of the cells are in the HOC phase) while being very stress resistant as a population since a substantial fraction of the cells are in the LOC phase and thus highly resistant to stress (Figure 1). Therefore the different stress sensitivity during the YMC combines overall growth of the population with high stress resistance of at least some cells, which can

between the cell growth cycle and the transcriptional stress response that we describe here suggests that the high-time-resolution gene-expression data from metabolically synchronized cultures (Tu *et al.*, 2005; Slavov *et al.*, 2011) may be used to study the transcriptional regulation of these general mechanisms for cell protection, especially when combined with computational approaches (Slavov, 2010) that minimize assumptions and make testable predications.

Changes in the oxygen consumption, mitochondrial potential, and morphology support the existence of metabolic cycling in humans

Metabolic cycling in human cells is consistent with and can explain recent observations of changes in the oxygen consumption, mitochondrial potential, and morphology of human cells (Schieke *et al.*, 2008; Mitra *et al.*, 2009). Schieke *et al.* (2008) found that human cells in the early G1 phase tend to have lower mitochondrial potential and to consume less oxygen, consistent with these cells being in the LOC phase, compared with cells in the late G1 phase, consistent with these cells being in the HOC phase. Of interest, the oxygen consumption was sensitive to rapamycin, in full accord with our suggestion that TOR regulates the YMC (Slavov *et al.*, 2011). Furthermore, Mitra *et al.* (2009) reported that the increased mitochondrial potential, which likely reflects the HOC phase, is associated with hyperfused mitochondrial morphology during the G1/S transition. This finding is strongly reminiscent of the observation that in continuous YMC cultures, the HOC phase happens immediately before the S phase. All these results are consistent with and can be explained by a cell growth cycle in human cells but do not prove it; rather, these results, taken together, provide a solid basis and motivation for future experiments testing the observed connections and the conservation of metabolic cycling in humans.

Evidence for a single-cell autonomous cell growth cycle and its conservation across eukaryotes

Until recently, it was unclear whether the metabolic cycle is emergent behavior present only in continuous, metabolically synchronized cultures of budding yeast growing in bioreactors or a cycle taking place in single cells from asynchronous cultures. On the basis of mRNA correlations in single cells, we suggested that the metabolic cycle is autonomous and present in single cells from asynchronous cultures (Silverman *et al.*, 2010). This suggestion was bolstered by the correlation between the YMC and the growth rate response of asynchronous cultures (Brauer *et al.*, 2008; Slavov and Botstein, 2011; Slavov *et al.*, 2011), as well as by the correlation between the YMC and the ESR of asynchronous cultures reported here. Our finding of a metabolic cycle in the elutriation CDC-synchronized cultures of fission yeast (Peng *et al.*, 2004; Rustici *et al.*, 2004) provides a third line of evidence that metabolic cycling is intrinsic to single cells. Unlike metabolically synchronized cultures, elutriation-synchronized cultures have only one synchronous population of cells, and there is no indication of cell-to-cell communication or emergent behavior at the level of the population that is not present in single cells. Thus, observing metabolic cycling in the elutriation-synchronized cultures is direct evidence that this cycling takes place in single cells. An advantage of this setup is that we can observe the cycling directly in time rather than infer it indirectly from correlations. The existence of a growth cycle in fission yeast is also supported by the stepwise increase in the RNA biosynthesis measured by Paul Nurse and colleagues (Fraser and Nurse, 1978, 1979; Barnes *et al.*, 1979).

The metabolic cycling observed in fission yeast is remarkably similar to the cycling observed in budding yeast not only in terms of functional groups of the cycling genes, but also in terms of oxygen

consumption and the correlation between the metabolic cycle and the environmental stress response. The conservation of these metabolic and physiological functions and the conservation of their temporal organization suggest that the function and the underlying biology of the metabolic cycling is also conserved across these divergent eukaryotes. These lines of evidence for conservation are related to gene function and physiology rather than to the expression patterns of genes with orthologous DNA sequences. The expression patterns of orthologues is very different even for many of the core genes involved in cell division, with relatively few orthologues having similar patterns of expression (Peng *et al.*, 2004; Rustici *et al.*, 2004), despite the fact that the CDC is highly conserved across divergent eukaryotes. Similarly, many genes expressed periodically with the cell growth cycle are likely to have orthologues with species-dependent expression patterns and some genes may have orthologues with conserved expression patterns as suggested by the prediction of growth rate in divergent species of yeast (Airoldi *et al.*, 2009).

Nurse and Wiemken (1974) presented evidence for increased uptake of arginine (the sole nitrogen source) by CDC-synchronized *Candida utilis* that coincides with the timing of the HOC phase (including protein biosynthesis) of the cell growth cycle of *S. cerevisiae*. This coordination of respiration with the uptake of arginine nicely parallels a similar observation of increased uptake of nitrogen in *S. cerevisiae* that coincides with the increased oxygen consumption (Kaspar von Meyenburg, 1969). Because *C. utilis* is very distantly related to both *S. cerevisiae* and *S. pombe*, this result (Nurse and Wiemken, 1974) provides support for the general conservation of the growth cycle across eukaryotes.

Metabolic cycling across growth rates and in cancer

Our results provide support for the inference that respiratory metabolic cycling is a general phenomenon among eukaryotic cells. In budding yeast, we saw that it is prominent at slow growth and appears to occur mainly during the G0/G1 phase of the cell division cycle, where, for small cells, mass accumulation preliminary to reentry into the CDC occurs. Analogously, in fission yeast we detected it during the G2 phase, where, for small cells, mass accumulation preliminary to reentry into the cell division cycle tends to occur. It seems likely, in either yeast, that metabolic cycling has substantially reduced amplitude or even does not occur in the same way when energy metabolism is entirely fermentative. If one translates these ideas to the data on cycling in human cells, there is an attractive parallel. We found evidence for metabolic cycling in fibroblasts and acinar epithelial cells, some of which might well have been growing slowly or even been entirely quiescent. The patterns of gene expression that we associated with metabolic cycling in primary human fibroblasts and acinar epithelial cells are also reflected in the metabolite patterns of respiring quiescent human fibroblasts (Lemons *et al.*, 2010). In the one cancer cell line (HeLa) that we studied, we found no support for metabolic cycling of gene expression even though Schieke *et al.* (2008) found changes in the oxygen consumption of ~5–10% of the cells in HeLa cultures. Colombo *et al.* (2010) and Tudzarova *et al.* (2011) also described metabolic dynamics taking place during the CDC of HeLa cells and controlled by the ubiquitin ligase APC/C-Cdh1. Thus further studies are necessary to characterize the metabolic differences between normal and cancer cells. We suggest that the difference between the cell growth cycle in cancer and in normal cells may be simply a result of the fact that HeLa growth depends on fermentative energy metabolism, as do very many cancer cells (the Warburg effect; reviewed by Heiden *et al.*, 2009; Jones and Thompson, 2009; Levine and Puzio-Kuter, 2010; Cairns *et al.*, 2011). Our data and analysis support the view

that the general eukaryotic phenomenon of metabolic cycling is most prominent when eukaryotic cells are respiring and growing slowly or are quiescent (Lemons *et al.*, 2010; Slavov and Botstein, 2011; Slavov *et al.*, 2011).

MATERIALS AND METHODS

Thermotolerance during the YMC

To quantify the dependence of stress resistance on the phases of the YMC, we synchronized metabolically a diploid prototrophic strain (DBY12007) with S288c background and wild-type HAP1 using starvation followed by refeeding (Küenzi and Fiechter, 1969; Slavov and Botstein, 2011). The medium was limited on glucose 800 mg/l with composition described by Saldanha *et al.* (2004) and Brauer *et al.* (2008). Chemostats were established in 500-ml fermenter vessels (Sixfors; Infors AG, Bottmingen, Switzerland) containing 300 ml of culture volume, stirred at 400 rpm, and aerated with humidified and filtered air. A 2-ml sample of culture in either LOC or HOC phase was taken and split into five tubes each containing 250 μ l. The first tube (control) was put on ice immediately, whereas the remaining four tubes, each tube corresponding to a time point, were dropped in water bath at 50°C. Tubes were taken from the 50°C water batch at each time point and promptly put on ice.

Each sample was serially diluted 1:10 with liquid yeast extract/peptone/dextrose (YPD; 10 g of Bacto-yeast extract, 20 g of Bacto-peptone, and 20 g of glucose in 1000 ml of water) into nine dilutions spanning nine orders of magnitude. The dilutions were spread on YPD (solid YPD; 10 g of Bacto-yeast extract, 20 g of Bacto-peptone, 20 g of Bacto-agar, and 20 g of glucose in 1000 ml of water) plates to measure viability. Colonies were counted after 36 h of incubation at 30°C. For each time point, a minimum of 100 colonies were counted. The fraction of viable cells in a sample was quantified as the fraction of cells surviving the heat exposure relative to the corresponding control—the cells that were not exposed to heat.

Ordering genes by the phase of peak expression

Among the many possible approaches for finding the phase of peak expression of each gene, we chose the correlation method used by Slavov *et al.* (2011) for its simplicity and versatility. The algorithm has the following steps:

- For each experiment (three cycles), the data for the oxygen consumption (where available) and for the *i*th gene were interpolated at 150 equally spaced time points, resulting in two vectors, **o** and **g_i**; for the oxygen and the gene expression respectively: **o** \in R^{150} and **g_i** \in R^{150} .
- A vector of correlations (**r** \in R^{40}) was computed by sliding the two interpolated vectors (**o** and **g_i**) relative to each other, one element at a time, for the first 40 elements. When oxygen consumption was not available it was replaced by a sentinel gene with robust periodic expression.
- The index (position) of the largest element of **r** was selected to represent the phase (ϕ) of peak expression of the *i*th gene: $\phi = \text{arg max}_j \mathbf{r}(j)$, $j = 1, \dots, 40$.
- The genes were ordered by sorting the phases ϕ of all genes for which the maximum correlation between **o** and **g_i** exceeded 0.6, $r_\phi > 0.6$.

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