Self-organisation of amino acid regulation in yeast

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Abstract

When *Saccharomyces cerevisiae* are grown continuously, respiration autosynchronises resulting in stable oscillatory dynamics, where small molecules mediate communication. Transcriptome-wide and metabolome-wide studies indicate that the oscillation functions to temporally separate catabolic and anabolic processes. Consistent with this, the production of amino acids have distinct phase relationships with the oscillation cycle where amino acids are produced in conjunction with reductive phase. In addition, oscillation is highly sensitive to amino acid and Rapamycin perturbation. These data indicate a role for the master amino acid regulator Gcn4p in the regulation of oscillatory dynamics, where the Gcn4p is activated by non-aminoacylated tRNAs. Therefore we explore the role of non-aminoacylated tRNAs in oscillatory regulation by constructing a regulatory model of amino acid regulation then testing this experimentally to provide a mechanistic understanding of amino acid feedback on gene regulation. We will also discuss the context of our work in the emergence of coherent behavior in biochemical networks.

1 Introduction

Cell adapts with varying metabolic requirements by changing its metabolite profile along with transcriptome and proteome. Metabolite mediated regulation is mostly exerted by small molecules binding and changing functionalities of transcription factors, translational regulators, enzymes and RNA molecules [4, 13, 2]. These metabolite mediated regulations result in the self-organisation of metabolic structure of the cell [7]. However, compare to our knowledge of protein-protein interactions and protein-DNA interaction, biochemical mechanisms that link the metabolite to transcriptome and proteome are poorly understood [4]. Therefore, to investigate the links between metabolite to transcriptome and proteome, we have used continuously grown *Saccharomyces cerevisiae* as model organism.

Continuously grown cultures of yeast cells show robust oscillations in respiration. The oscillations can be observed by monitoring the residual oxygen levels in the medium [14]. This synchronization of oscillation is the result of inter-cellular communication and is also correlated to the NAD(P)H and ATP oscillations (redox and energy metabolism). Analysis of gene expression level has shown specific patterns in expression during the period of oscillation [11]. This further suggests that metabolism related inter-cellular communication influences gene expression. Previous studies have suggested that

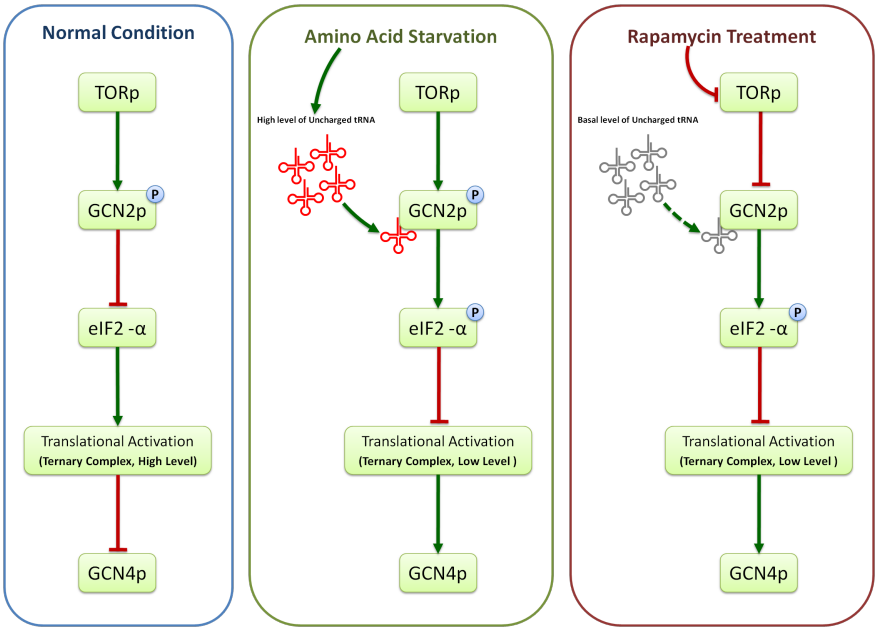

Figure 1: Post transcriptional activations of Gcn4p
a. In non-amino acid starvation condition TORp mediated pathway keeps the Gcn2p phosphorylated and leaving eIF2-alpha unphosphorylated. Unphosphorylated eIF2-alpha forms matured ternary complex which further inhibit the activation of Gcn4p. b. During amino acid starvation, high level of uncharged tRNAs accumulate in the cell and form complex with Gcn2p. This uncharged tRNAs-Gcn2p complex phosphorylate the eIF2-alpha and hinders the matured ternary complex formation and consequently activates Gcn4p. c. During rapamycin perturbation, TORp mediated pathway preventing Gcn2p to be phosphorylated. Unphosphorylated Gcn2p then phosphorylates the eIF2-alpha and resulting the activation of Gcn4p.

this oscillation plays a pivotal role in the temporal separation of catabolism, anabolism and cell cycle mechanisms [11]. Consistent with this, the production of amino acids have distinct phase relationships with the oscillation cycle where amino acids are produced in conjunction with reductive phase. In addition, amino acid biosynthesis is tightly regulated and the system is hyper-sensitive to nM concentrations of Rapamycin during the oscillation. This suggests that the function of general amino acid control system is tightly linked with the oscillatory response. Therefore we investigate the amino acid feedback on gene regulation in oscillatory regulation by constructing a regulatory model of amino acid regulation then testing this experimentally to provide a mechanistic understanding of this feedback system.

Previous studies have shown that genes involved in amino acid biosynthesis are mostly controlled by a master transcriptional regulator called Gcn4p [6]. The Gcn4p is post transcriptionally activated by non-aminoacylated tRNAs through a Gcn2p and eIF2-alpha (Eukaryotic initiation factor 2) mediated pathway [12, 10] (figure 1). When amino acid levels are high in the cell, the Gcn2p remains phosphorylated and leaving the eIF2-alpha unphosphorylated. In unphosphorylated state, eIF2-alpha forms matured ternary complex [12, 6]. Presence of high number ternary complex hinders the translation of Gcn4p by initiating translation from any of the four upstream ORF (open reading frame) of GCN4 mRNA [10]. However, it has been shown that increase in the concentration of non-aminoacylated tRNA result in the formation of complex with Gcn2p [10, 12]. This non-aminoacylated tRNA and

Gcn2p complex then phosphorylates the eIF2-alpha which reduces the formation of matured ternary complex [6, 10]. Reduction in the number of matured ternary complex increases the probability of ribosomal scanning to skip four uORFs of the GCN4 mRNA and reaching the actual start codon then initializing the translation by 50 percentage [6].

Based on the above mentioned observations, we assume that during amino acids starvation condition non-aminoacylated tRNAs accumulate due to the reduction in the concentration of intracellular amino acids. Accumulation of non-aminoacylated tRNAs then post transcriptionally activates Gcn4p and there by amino acid biosynthesis genes. Therefor charging and unchanging (aminoacylation and non-aminoacylation) of the tRNAs could be the signal which links the amino acids to the transcriptome. In this progress report I have discussed about the studies and observations we have done to verify this regulatory model of amino acid regulation.

2 Methods

The IFO 0233 strain of yeast cells are used for all of the following experiments. The yeast cells are grown in precise continues culture conditions in fermenters as described in reference[14, 11]. The time-series experiments were conducted on yeast cells showing respiratory oscillation (50 minutes oscillation cycle). From this continues cell culture 40 time points sampling were performed with five minutes interval.

2.1 RNA Extraction

The RNA extraction method used in this study was modified from the original method described in the following reference[8]. In order to extract the total RNA, 500µl of yeast cells were quenched with 1ml of ethanol and kept in -80°C for overnight. The samples were then pelleted by centrifuging at 10,000g for 2min in 4°C followed by re-suspending the pellets in 250µl of sodium acetate buffer (0.3 M sodium acetate pH 4.5-5.0; 10mM Na2EDTA). Then one volume of phenol (250µl) equilibrate with sodium acetate; and zirconium beads (mixture of 0.1 and 0.5 mm approximately 300µl volume) were added into the samples. The cells were then disrupted by bead-beating 12 times for 10 seconds with 30 second interval in between. The samples were then centrifuge at 12000g for 15 min in 4°C and moved the aqueous phases into new tubes. An aliquot of 125µl of sodium acetate buffer was added into into the phenol phase for back extraction. The back extracted aquas phases ware combine with the previous aqueous phases. The aquas phases ware then mixed with 2.5 volumes of ice-cold ethanol and kept overnight at -20°C. The RNA was pelleted by centrifuge at 12000g for 30 min in 4°C. The pellets were washed three times in 70% ethanol and dry by keeping the lid open. The pellets were then dissolved in 50µl of 10mM sodium acetate (pH4.5-5.0) followed by quick froze and stored in -80°C. The RNA concentration was determined by optical density measured using GenQuant pro (amersham pharmacia biotech) apparatus.

2.2 Metabolite Extraction

In order to extract the metabolites, 1ml of yeast cells were quenched with 1ml Methanol (-70°C) and pellet was obtained by centrifuging at 20400g in -9°C. The pellet was re-suspend well in 500µl of -70°C methanol containing internal standards (CSA, MES and 3-AP). This sample was mixed with chloroform and distilled water in a 1:1:0.4 ratio, and sonicated at -4°C followed by centrifugation at 20400g for 10 mins in -9°C. The supernatant was then centrifuged for 2 hours at 20000g (-9°C) in 5kDa cutoff tubes to remove the proteins. The filtrant was lyophilised for CE-MS analysis. This method was performed in five different ways by following the parameters given in Table 1.

Method	Quenching	Standards	Chloroform	Sonication (minutes)	Freeze-thaw in LN_2(times)	Lyophilization
1	Yes	500µl (40µM)	Yes	5, 10, 20	No	Yes
2	No	500µl (40µM)	Yes	5, 10, 20	No	Yes
3	Yes	500µl (40µM)	[1]No	No	0, 10, 10	Yes
4	Yes	100µl (200µM)	No	No	3, 5, 10	No
5	Yes	100µl (200µM)	No	No	3, 5, 10	No

Table 1: Parameters used for the metabolite extraction.
Each method carried out in triplicates. In method 5, the samples were filtered through 0.1 and 0.22 cutoff tubes after the freeze-thaw step.
[1] Chloroform is added in one sample among the triplicates. [2]liquid nitrogen.

2.3 RT-PCR

The RT-PCR reactions were preformed on Opticon DNA Engine (MJ research) instrument according to the instructions given on Express Sybr Green kit. The sequences of GCN4 primer pairs used are 5' AACAGGATACCCCTTCGAACC 3' (left) and 5' AACGGTCTTGGCATCAGGTG 3'(right).

2.4 Acid urea polyacrylamide gel

Approximately 20µg of RNA were run on 6.5% acid urea polyacrylamide gel having the dimension of 0.5mm x 20 cm x 40 cm. Gel mixture was prepared with the final concentration of 6.5% Long Ranger, 0.1 M sodium acetate pH5.0 and 8M urea. The ingredients were dissolved under stirring and adjusted the volume to 80ml followed by degassing for 5-10min. Freshly prepared ammonium persulfate (0.7% w/v) and TEMED (0.15% v/v) were added just before casting the gel followed by allowing to polymerize for ~20 min. After electrophoresis the gel was fixed in fixing buffer (5% acetic acid, 5% methanol and 90% distilled water) for 10 minutes followed by staining with GelRed solution. The gel image was obtained by scanning on Typhoon 9400 scanner.

3 Results

Previous studies have shown that Gcn4p is the master regulator of amino acid bio synthesis [6, 12]. In order to get a focused view on the role of Gcn4p in amino acid biosynthesis, we have constructed an enzyme-metabolite [9], protein-protein [1], protein-DNA [5] interaction map of yeast cell (figure 2). This map has clearly shown that most of the edges from Gcn4p are connected to the amino acid biosynthesis genes which again confirms the master regulator role of Gcn4p in amino acid biosynthesis.

Prior to all of the following experiments, the total RNA concentration were obtained (see methods section) and plotted against the dissolved oxygen concentration during the respiratory oscillation, to observe the total RNA turn over (figure 3). Form these analysis we observed that the total RNA concentration shows approximately .6 fold difference during the respiratory oscillation. Interestingly we have also observed a pattern in the change of RNA concentration during respiratory oscillation by precise analysis of RNA samples (data not shown).

To investigate the role of charged and uncharged tRNAs ratio in the activation of Gcn4p with varying intracellular amino acid concentrations during respiratory oscillation, we have run time series RNA samples which extracted form yeast cells showing respiratory oscillation on acid urea polyacrylamide gel (see methods for details) (figure 4). The ratio between charged and uncharged tRNA concentrations were calculated based on the densitometry analysis results. These ratios were then

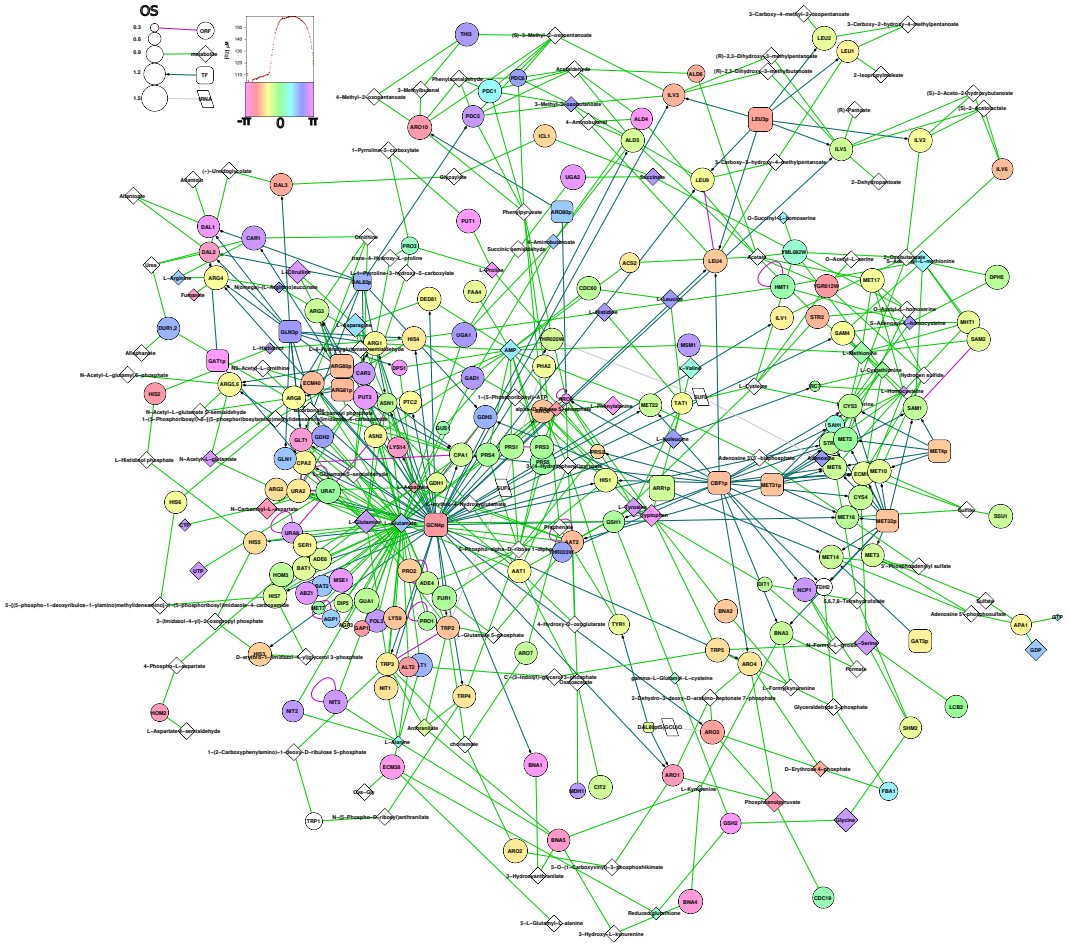

Figure 2: The amino acid biosynthesis map

The amino acid biosynthesis model constructed by integrating enzyme-metabolite [9], protein-protein [1], protein-DNA interactions[5]. Production or expression of each component in the model are mapped with the phase angles of respiratory oscillation (color gradient from -pi to pi) where pi is the minimum first derivative of the dissolved oxygen trace. The oscillation strength of each component is represented as the diameter of the symbol.

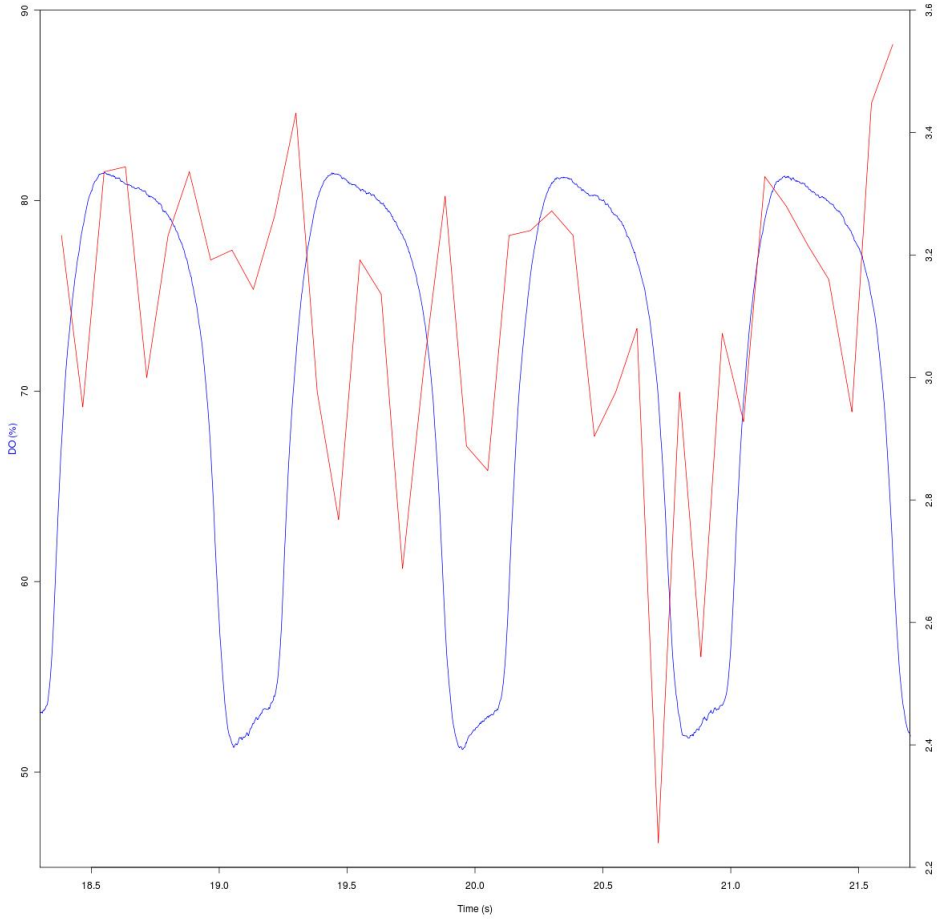

Figure 3: Total RNA turn over during the respiratory oscillation
The blue line indicates the dissolved oxygen (DO) level in the media during respiratory oscillation. The red line indicates total RNA concentration. The right side y axis is the total RNA concentration in µg/µl.

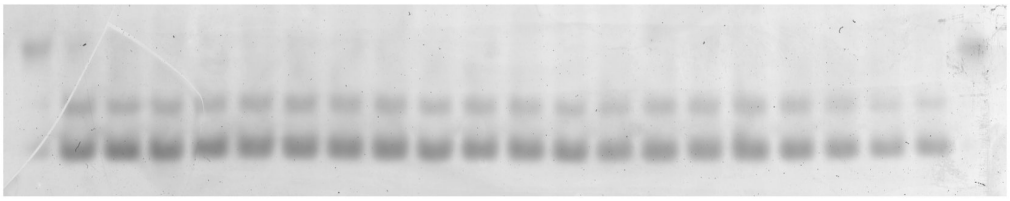

Figure 4: 20 time point of charged and uncharged tRNAs
A continuously grown culture of *Saccharomyces cerevisiae* showing oscillatory dynamics was sampled at 5 min intervals (40 samples). Total RNA was extracted and run on acid urea-PAGE gel on 20 consecutive samples. The upper bands are the aminoacylated tRNAs and lower bands are the non-aminoacylated tRNAs.

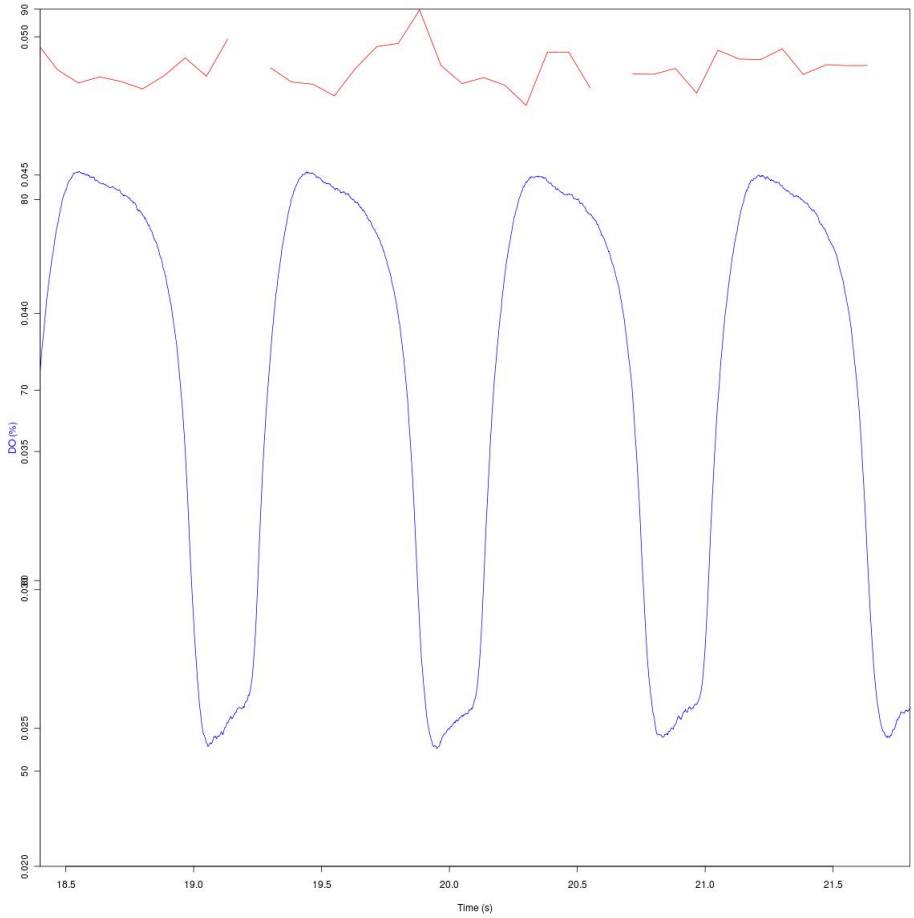

Figure 5: Transcript levels of GCN4 during respiratory oscillation
The blue line indicates the dissolved oxygen (DO) level in the media during respiratory oscillation. The red line indicates GCN4 mRNA concentration.

plotted against dissolved oxygen concentration. Interestingly we have observed that the charged and uncharged tRNA ratios oscillates during the respiratory cycle. The ratio is high during reductive phase and lower during oxidative phase.

It has been shown that Gcn4p activation is mostly exerted on post transcriptional level [3, 6] where the mRNAs are rather stable during the respiration cycle[11]. Therefore to observe the transcript levels of GCN4 during respiratory oscillation we have performed RT-PCR analysis on 40 time point total RNA samples with GCN4 primers (see methods for details) (figure 5). As expected the GCN4 transcripts have appeared to be maintaining stable levels during respiratory oscillation, which shows that GCN4 transcription has no or very less impact from respiratory oscillation.

4 Future work and discussion

Based on the above mentioned results and analyses we have made a hypothetical model of self organization of amino acid regulation in yeast (figure 6). According to our model, when intracellular amino acid concentrations are low uncharged tRNA will get accumulated. Accumulation of uncharged tRNAs will then activates the Gcn4p translation, which further leads to the activation of amino acid biosynthesis genes. Activation of these genes will produce the enzymes which catalyze the amino acid production and resulting increase in the intracellular amino acid concentration. Increase in the intracellular amino acid concentration subsequently hinders the Gnc4p activation by increasing the concentration of charged tRNAs.

Our preliminary analysis on charged and uncharged tRNAs during respiratory oscillation has shown that the charged-uncharged tRNA ratios have an oscillatory pattern which is in-phase with the respiratory oscillation. This oscillation in charged-uncharged tRNA ratios and the steady state of GCN4 transcripts during the respiratory oscillation are supporting our hypothesis that Gcn4p activation could be mediated by the charged-uncharged tRNA ratios. However, in order to strengthen this argument we are planning to measure the Gcn4p concentration and Gcn2p phosphorylation rates during respiratory oscillation. In addition, quantifying the aminoacyl tRNA synthetase and correlating it with the tRNA charging ratio might broaden the view on this process. As the final step we are planning to integrate the above mentioned results and correlate with the amino acid concentration levels during the respiratory cycle (the amino acid concentrations were obtained from CE-MS analysis of time series samples; data not shown), thus closing one (of many) feedback cycles underlying the dynamics of yeast redox oscillation.

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