Understanding the Self-organisation of amino acid regulation in yeast

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Abstract

When *Saccharomyces cerevisiae* are grown continuously, cellular processes auto-synchronise resulting in stable oscillatory dynamics. Respiration is the most readily measured oscillatory parameter, however, 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 has distinct phase relationships with the oscillation cycle. Intracellular amino acids show different oscillation patterns during the respiration cycle, for example, the concentration of cysteine peaks prior to aspartate, glutamate, leucine and valine whereas threonine and isoleucine concentrations peak later during an oscillation cycle. Furthermore the oscillation is highly sensitive to amino acid and Rapamycin perturbation. Taken together 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. Here we show that the ratio between aminoacylated and non-aminoacylated tRNAs oscillates in phase with respiration cycle, indicating the observed Gcn4p dynamics result from translational activation by the cyclic amino-acid synthesis and aminoacylation of tRNAs. Closer examination using Northern blotting of tRNAs representing single amino acids will use to explore the role of non-aminoacylated tRNAs in oscillatory regulation and the concentration response of individual amino acids thus revealing the dynamic activation of amino acid biosynthesis via the Gcn4 gene network during the respiration cycle.

1 Introduction

Cell adapts with varying metabolic requirements by changing its metabolite profile along with transcriptome and proteome. The *lac* operon, explained by Jacob and Monod, is a classic example of metabolite mediate genetic regulation, in which the bacterial cells adapt their transcription there by the enzyme production to the nutrition supply[2]. Metabolite mediated regulations are mostly exerted by small molecules binding and changing functionalities of transcription factors, translational regulators, enzymes and RNA molecules [5, 16, 4]. These regulations then feedback to the metabolite state and result in the self-organisation of metabolic structure of the cell [9]. 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 [5]. Therefore, to investigate the links between metabolite to transcriptome and proteome, we have used continuously grown culture of synchronised *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 [18]. This synchronization of oscillation is the result of inter-cellular communication, mostly mediated by small molecules, and is also correlated to the NAD(P)H and ATP oscillations (redox and energy metabolism). Analyses of gene expression levels have shown specific patterns in expression during the period of oscillation [14]. This further suggests that metabolism related inter-cellular communication influences gene expression. Previous studies have suggested that this oscillation plays a pivotal role in the temporal separation of catabolism, anabolism and cell cycle mechanisms [14]. 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 intracellular amino acids show different oscillation patterns during the respiration cycle, for example, the concentration of cysteine peaks prior to aspartate, glutamate, leucine and valine whereas threonine and isoleucine concentrations peak later during an oscillation cycle. Furthermore the system is hypersensitive 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 dynamics 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 [8]. The Gcn4p is post transcriptionally activated by non-aminoacylated tRNAs through a Gcn2p and eIF2-alpha (Eukaryotic initiation factor 2) mediated pathway [15, 13] (figure 1). When intra-cellular amino acid levels are high, the Gcn2p remains phosphorylated and leaving the eIF2-alpha unphosphorylated. In unphosphorylated state, eIF2-alpha forms matured ternary complexes [15, 8]. Presence of high number ternary complexes hinders the translation of Gcn4p by initiating translation from any of the four upstream ORF (open reading frame) of GCN4 mRNA instead of the actual starting codon [13]. However, it has been shown that increase in the concentration of non-aminoacylated tRNA result in the formation of complex with Gcn2p [13, 15]. This non-aminoacylated tRNA and Gcn2p complex then phosphorylates the eIF2-alpha which reduces the formation of matured ternary complexes [8, 13]. Reduction in the number of matured ternary complexes 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 [8].

Based on the above mentioned observations, we assume that during amino acids starvation or low intra-cellular amino acid concentration conditions 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. The activation of amino acid biosynthesis genes increases the concentration of the enzymes which catalyze the production of amino acids and there by increase the concentration of intra-cellular amino acids. Which suggests that charging and unchanging (aminoacylation and non-aminoacylation) of the tRNAs could be the signal which links the amino acids to the transcriptome. To understand this mechanism, we investigate the ratio between aminoacylated and non-aminoacylated tRNAs during the respiration cycle, where the Gcn4p dynamics and cyclic amino acid synthesis take place.

As the first step of the experimentation, we have decided to extract a time series of total RNA, proteome and metabolome from continuously grown yeast cells showing respiratory oscillation to quantify the concentrations of aminoacylated and non-aminoacylated tRNAs, Gcn4p and amino acids,

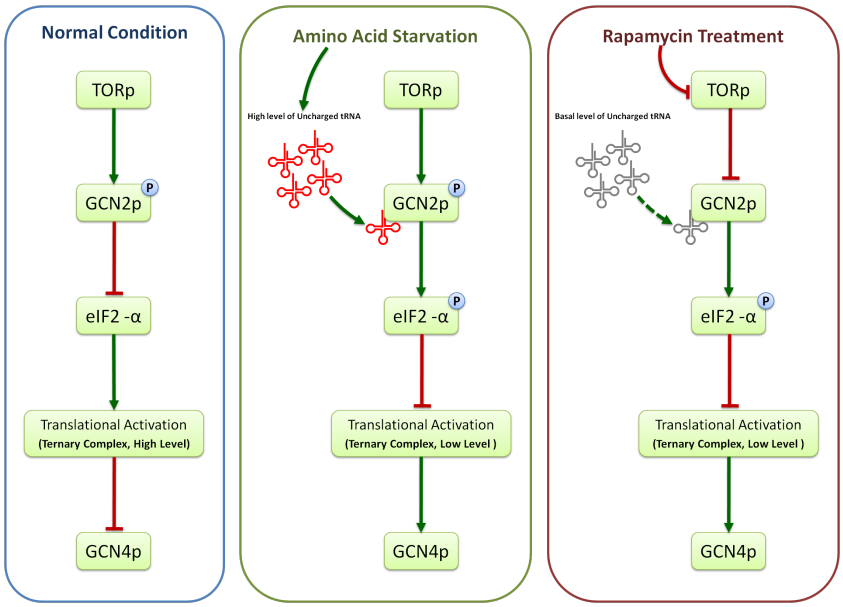

Figure 1: Post transcriptional activations of Gcn4p
a. In non-amino acid starvation condition TORp mediated pathway keeps the Gcn2p phosphorylated and leaving eIF2-alpha unphosphorylated. Unphosphorylated eIF2-alpha forms matured ternary complex which further inhibit the activation of Gcn4p. b. During 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.

respectively. However most of the methods available at that point were not suitable for time course experimentation on continuously grown yeast cells. Therefore we have developed optimized methods for time course extraction of DNA, RNA, proteins and metabolites from yeast cells grown in continues cultures.

2 Progress Report

In the first section of the progress report I have discussed about the results from the our newly developed methods to extract DNA, RNA, protein and metabolites. The second section details the studies and observations we have done to verify the feedback regulatory model of amino acid biosynthesis.

2.1 Yeast DNA, RNA, Protein and Metabolite Extraction Methods Optimized for Continues Culture

In terms of the requirements for the time course experiments, most of the conventional DNA, RNA, protein and metabolite extraction methods are suffering from the lack of reliability, consistency, precision and speed. When yeast cells grow on continues culture, due to the slow growth rate, the cell walls tend to be harder compare to the cells grown in batch cultures. Therefore the cell disruption methods which are optimized for the batch culture are generally not suitable for the continues culture. It sounds logical that the consistency and concentration of the yield of intra-cellular components are directly proportional to the cell disruption rate, which further highlight the importance to have an efficient cell disruption method which is consistent through out the time-series.

Conventional enzymatic cell wall digestion methods are usually suitable for small number of samples however, which can be expensive for a time course experiments having many samples. As most of the time course experiments require precise quantification of intra-cellular macro molecules and metabolites, it is important to minimize the false-positive and false-negative results due to the experimental errors. Therefore it is crucial to treat all the time course samples in identical way. In this aspect, in addition to the longer experimentation time, lengthier experimental methods have higher probability for errors than shorter procedures.

Taking care of the above mentioned bottlenecks of conventional experimental methods, we have developed, inexpensive, shorter, efficient and consistent methods, which are optimized for time course extraction of DNA, RNA, proteins and metabolites from yeast cells grown in continues cultures. From the our results we have found that the use of acid washed zirconium beads for the mechanical disruption of cells have shown consistent and efficient cell disruption rates over the time course samples of yeast cells grown on continues culture. In addition we have significantly improved the cost effectiveness, speed, consistency and reproducibility by reducing the use of expensive reagents and length of the experimental procedures.

2.1.1 Methods

2.1.2 Strain and culture conditions

- Preculture and strain

In this study we have used IFO 0233 diploid strain of *S. cerevisiae*. The colonies were maintained at 4°C on YEPD agar petri plates with a composition of 2% (mass/volume) yeast extract (Bacto), 2% dextrose (Wako), 1% peptone (Bacto) and 2% agar (Bacto). Preculture was prepared by inoculating yeast cells into 10 ml of reduced YEPD broth (1% yeast extract, 0.5% peptone and 2% dextrose) and incubating at 30°C in an orbital incubator for 48 hours.

- Medium composition

The basic media used was composed of 5 g/l of $(NH_4)_2SO_4$, 2 g/l of KH_2PO_4, 0.5 g/l $MgSO_4 \cdot 7H_2O$, 0.1 g/l of $CaCl_2 \cdot 2H_2O$, 0.02 g/l of $FeSO_4 \cdot 7H_2O$, 0.01 g/l of $ZnSO_4 \cdot 7H_2O$, 0.005 g/l of $CuSO_4 \cdot 5H_2O$, 0.001 g/l of $MnCl_2 \cdot 4H_2O$, 1 ml/l of 70% H_2SO_4, 1 g/l of Bacto yeast extract, 0.2 ml/l of Sigma Antifoam A and either supplemented with 20 ml/l ethanol[10] or 20 g/l of D-glucose[18] as a carbon source.

- Growth condition

The continues culture of yeast cells were grown in a modified version of jar fermentor (Eyela, Japan) at 30°C (± 0.02°C), with a pH of 3.4 (± 0.03), aerated at 0.150 l/min (± 0.02 L/min), with a working volume of 650 ml and agitated at 750 rpm (± 3 rpm). The reactor pressure was maintained below 101700 Pa and the dilution rate of the media was kept at 0.087 h-1 (± 0.004 h-1). The fermentor control and devices were instrumented as previously described by Douglas Murray et. al[14]. Prior to initiate the continues culture the batch culture was starved in fermentors for 6 hours. The culture was self synchronized and shown stable autonomous respiratory oscillation after 16 hours from the beginning of the continues culture.

- Sampling and quenching

Unless otherwise stated, in this study we have used auto synchronized continues culture grown on glucose media. The cell culture was sampled from the fermenter through a 30 cm long metal tube attached to the fermenter. One end of this tube is immersed into the cell culture and the other end is connected to a three way valve out side the fermenter. The cell cultures were taken by vacuum aspiration using a disposable plastic syringe through the three way valve. A 10 ml syringe is sufficient enough to sample up to 7 ml of culture. While sampling, to clean the sampling tube and remove the residual cell culture which might left in the tube, about 200 µl of cell culture was taken using the syringe 15 seconds prior to the desired sampling time and discarded. The syringe then connected back to the three way valve immediately and sampled the required volume on desired time. To degas the cell culture after sampling, the syringe was detached from the valve and close the open end of the syringe using the thump (must wear hand gloves) and pull out the piston carefully. To quench the cells, 500 µl of cell culture was transferred using a pipette from the syringe into 1 ml of ethanol kept in -80°C dry ice ethanol bath within 10 seconds.

2.1.3 DNA Extraction - Phenol-TAE extraction method

Yeast cell culture with the volume of 500 µl was sampled from the fermenter and quenched as described above. The fixed samples were stored at -80°C for at least six hours. The cells were pelleted by centrifuging on 10,000 x g for 2 minutes at 4°C and discarded the supernatant, followed by re-suspending the pellets in 250 µl of TAE buffer (Tris base 40 mM; Na2EDTA 1 mM; acetic acid 1.142 µl/ml; pH 8). Subsequently 2.5 µl of RNase (RNase, Dnase-free, Roche Applied Science) was added and mixed well by vortexing. Triplicates of the cell culture-RNase mixture were then incubated in a shaking mixer (BIOER, Mixing Block, MB-101) at 37°C for 40, 80, 120 and 160 minutes respectively. After the incubation one volume of TE-saturated phenol equilibrate with the volume of TAE buffer (250 µl) were added followed by adding a mixture of 0.1 and 0.5 mm acid washed zirconium beads with approximately 150 µl volumes of each. To disrupt the cells, the mixture were then bead-beat 12 times for 10 seconds with 30 seconds intervals in between and 2 minutes intervals after every 3 times of bead-beating. The bead-beating has performed on Tomy, Micro Smash™ MS-100 while the inbuilt freezer has kept turned on.

After bead-beating, the samples were centrifuged at 12,000 x g for 15 minutes on 4°C and moved the aqueous phase into new tubes. To increase the DNA yield a back extraction has conducted

by adding 125 µl of TAE buffer into the phenol phase and mixed well by vortexing then followed the centrifugation step as described above. The aqueous phases were then combined and added 2.5 volumes of ice-cold ethanol and sodium chloride with a final concentration of 0.1 M then kept overnight at -20°C for precipitation. The samples were then centrifuged for 15 minutes at 12,000 x g on 4°C and discarded the supernatant. The pellets were then washed two to three times using 500 µl of 70% ethanol and dried by keeping the lid open on room temperature for 10 minutes. The pellets were dissolved in 50 µl of double distilled water followed by quick froze and store on -20°C. For comparison same experiment has been repeated by treating the DNA samples with RNase after the extraction while the cells were kept untreated. After extraction, the DNAs were dissolved in 250 µl of double distilled water followed by adding 2.5 µl of RNase and repeated the time-series incubation as described above. After the RNase treatment, to remove the RNase from the DNA samples, one volume (250 µl) TE-saturated phenol-chloroform mixture (1:1) were added and mixed gently for 2 minutes. The DNAs were then recovered by following the same procedures as described above (from the centrifugation step after the bead-beating).

- Optical density measurement

The optical density of the samples were measured using SpectraMax, Plus instrument at 230 nm, 260 nm, 280 nm and 320 nm wavelengths. The data has been obtained using SoftMax PRO software by following its "blank subtraction using plate blank" method. The samples used for OD measurements were prepared with a dilution factor 50 and a final volume of 100 µl. The concentration of nucleotides were calculated using the following formula.

- Quantification of genomic DNA

The qPCR to quantify the genomic DNA was conducted on a DNA Engine, Opticon, MJ Research instrument. In this study we have used Lys20 primer. The 20 µl of PCR reaction mixture contained 1 µl of DNA sample, 9.1 µl of SYBR® Premix Ex Taq™ II (Takara), 9.1 µl of nuclease free water and 0.8 µl of forward and reverse primer mix (1:1). The cycling conditions are as follows; incubation at 95°C for 5 minutes followed by 40 cycles of, 10 seconds at 95°C, 20 seconds at 55°C and 20 seconds at 72°C. The output data was acquired and processed using Opticon Monitor (v1.8) software.

- Purification of genomic DNA

The genomic DNA was purified to remove small RNA fragments using Mbiotech, SpinClean™ Genomic DNA Purification Kit by following the manufacturers instruction.

2.1.4 RNA Extraction – Phenol- sodium acetate method

The phenol- sodium acetate RNA extraction method has been developed by modifying the method by Caroline Köhrer and Uttam L. Rajbhandary[11]. The total RNAs were extracted in similar way as phenol-TAE extraction, with the modification of using sodium acetate buffer (0.3 M sodium acetate pH 4.5-5.0; 10mM Na_2EDTA) instead of TAE buffer and removing the RNasing step. After extraction, the pellets were dissolved in 50µl of 10mM sodium acetate (pH4.5-5.0) followed by quick froze and stored in -80°C.

- Optical density measurement

The optical density of the samples were measured using SpectraMax, Plus instrument at 230 nm, 260 nm, 280 nm and 320 nm wavelengths. The data has been obtained using SoftMax PRO software by following its "blank subtraction using plate blank" method. The samples used for OD measurements were prepared with a dilution factor 100 and a final volume of 100 µl.

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.

2.1.5 Metabolite Extraction - Bead beating method

Yeast cell culture with the volume of 500 µl was sampled from the fermenter and quenched as described above but with 500µl of methanol containing 4mM NEM instead of 100% ethanol. The fixed samples were stored at -80°C for at least six hours. The cells were pelleted by centrifuging on 20,000 x g (flash) at -9°C and discarded the supernatant, followed by re-suspending the pellets in 300µl of -70°C methanol containing internal standard (200µM of MES, Methionine sulfone, Trimesic acid each). In this stage samples can be stored at -80°C for long term. Then the samples are mixed with one volume of chloroform and 0.4 volume of Milli Q water (double distilled water) and mixed by vortexing. Subsequently mixture of 0.1 and 0.5 mm acid washed zirconium beads with approximately 150 µl volumes of each were added and flushed each sample tubes with nitrogen. To disrupt the cells, the mixture were then bead-beat 12 times for 10 seconds with 30 seconds intervals in between and 2 minutes intervals after every 3 times of bead-beating. The bead-beating has performed on Tomy, Micro Smash™ MS-100 while the inbuilt freezer has kept turned on.

After bead-beating, the samples were centrifuged at 12,000 x g for 10 minutes on -9°C and moved the aqueous phase into new tubes. To remove the proteins the supernatant were centrifuge for 6 hours in -9°C at 20,000 x g in 5kDa cutoff filters. The filtrant were then lyophilised (approximately 3.5 hours) for CE-MS analysis and stored the pellets at -80°C. For comparison we also have extracted metabolite in the following ways.

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. The results are still under processing and not included in this manuscript.

2.2 Results

2.2.1 DNA extraction

We have extracted DNA from yeast cells using three different methods for comparison. First, we extracted DNA using our original method, in which we treated the cells with RNase prior to the cell disruption to remove the intra-cellular RNAs. We pre-treated the cells with RNase to avoid the time consuming phenol-chloroform extraction and washing steps after the DNA extraction. A time-

series of RNAs treatment has conducted to determine an optimum time where maximum removal of RNA occurs. After treating with RNase the cells were disrupted by bead-beating with acid washed zirconium beads in the presence of TE-saturated phenol and TAE buffer. The phenol dissolves the proteins including RNase and helps to increase the cell lysis[17]. The DNAs dissolved in TAE buffer were then recovered by ethanol precipitation. We have conducted over night precipitation of DNAs in 2.5 volumes of 100% ethanol at -20°C. However precipitation using one volume of isopropanol or 2.5 volumes of 100% ethanol in room temperature for 5 to 10 minutes may also recover DNAs with lower efficiency, which is suitable for non-quantification purposes. The DNA pellets were then washed with 70% ethanol for the complete removal of residual phenol. To compare the efficiency of RNase pre-treatment (before DNA extraction) with post-treatment (after DNA extraction) to remove the intra-cellular RNAs , we have repeated the same experiment with post-treating instead of pre-treating with RNase. In this experiment also, we have used the same time-series as previous method to determine the optimum RNase treatment time. However additional phenol-chloroform extraction and washing steps has conducted to remove the RNase from the DNA samples. Finally, to compare the quality and extraction efficiency of our method, the DNAs were extracted from same volume of cell cultures using bust 'n grab method[6]. After extracting the DNAs, a time-series of RNase treatment has been conducted same as above.

- Analysis of nucleotide extraction efficiency, purity and RNasing efficiency

The extracted DNA pellets were dissolved in 50 µl of double distilled water and the optical density of all samples were measured. From the optical density measurements the quantity of nucleic acid in µg/µl units (260nm) , the quality of extraction (260nm / 280nm) and presence of salts (260nm / 230nm) were calculated (results are not shown). The bust 'n grab method has shown comparatively poor nucleotide concentrations which is probably resulted from the inefficient cell disruption by the freeze-thaw technique. Among RNase pre-treated and post-treated samples, the pre-treated control (incubated 160 minutes without RNase) has shown low nucleotide concentration than post-treated control. We reasoned that this is probably because of the degradation of RNA by the intra-cellular RNase. This additional source RNase also explains the progressive nucleotide concentration decrease in the time-series of pre-teared samples where post-treated samples remain rather constant.

- DNA quantification by PCR

To relatively compare the DNA isolation efficiency between methods and understand the consistency within the methods we have quantified the DNA by conducting qPCR on all DNA samples using Lys20 primer. The abundance of DNA was calculated from the cycle threshold (Ct) values (data not shown). Compare to phenol-TAE extraction, the amount of DNA extracted using bust 'n grab method was very low. Among phenol-TAE extraction methods, RNase post-treatment method has gained lower DNA yield than pre-treatment method. We reasoned that, this decrease in DNA concentration was probably due to the lose of DNA during the additional phenol-chloroform extraction and washing steps after RNasing. Therefore among the three methods RNase pre-treatment method has shown higher DNA yield.

- DNA purification

To understand the total DNA (genomic and mitochondrial DNA) extraction efficiency of RNase pre-treatment method, we have compared the total volume of extracted nucleotides with calculated volume of total DNA. We have estimated the volume of DNA in each samples from the cell count. From our previous studies we have shown that 1 ml of culture has approximately 5 x 108 yeast cells under our continues culture conditions, in which 70% of cells are on G1 phase where remaining 30% cells are on G2 phase. Using the following equation we have estimated that each 500 µl for culture (2.5 x 108 cells) used in this study contains approximately 8.5 µg of DNA. However the concentration

of nucleotides extracted using RNase pre-treatment method was more than 3 fold higher than this estimated volume of DNA. Considering the absence of any strong signals (bands) other than the DNA bands on the agarose gel (data not shown), we reasoned that this high concentration of nucleotides probably due to the carry over of small RNA fragments resulted from the RNase treatment and these fragments may went through the gel because of the small size. Therefore we have purified the DNA (two time-points, 80 and 160 minutes) to remove this small fragments using SpinClean™ Genomic DNA Purification Kit. The concentration of DNA after purification were calculated from the OD measurements (data not shown). However the average of the DNA concentration was lower than the estimated DNA concentration. We suspected that this may be due to the lose of DNA during purification using the filter. Therefore we have conducted qPCR on the purified and original (non-purified) DNA samples from two time-points and quantified that 0.61 and 0.48 fold reduction of DNA concentration in respective purified samples. Considering this DNA lose during purification, extracted DNA concentrations were close to the estimated volume of DNA (data not shown).

2.2.2 RNA extraction

We have extracted total RNA using our phenol-sodium acetate method and compared the results with three other methods. In this phenol-sodium acetate method we have used sodium acetate buffer pH 4.5-5.0 for dissolving RNAs. Maintaining the lower pH is critical as higher pH (above pH 7) causes lose of aminoacylation of tRNAs and DNA contamination. The RNAs were extracted from a time series of 40 samples with 4 minutes interval. The sampling and quenching has done as described previously, and the total RNAs were extracted at once. In order to compare the quality and extraction efficiency of our method, the RNAs were extracted from same volume of cell cultures using Brown method[3]. The results are still under processing and not included in this manuscript.

2.3 Understanding the Self-organisation of amino acid regulation in yeast

2.3.1 Methods

2.3.2 RNA extraction

- Time series RNA extraction

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.

- Optical density measurement

The RNA concentration was determined by optical density measured using GenQuant pro (amersham pharmacia biotech) apparatus.

- 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.

2.4 Results

Previous studies have shown that Gcn4p is the master regulator of amino acid bio synthesis [8, 15]. In order to get a focused view on the role of Gcn4p in amino acid biosynthesis, we have constructed an enzyme-metabolite [12], protein-protein [1], protein-DNA [7] 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 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.

3 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 5). 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.

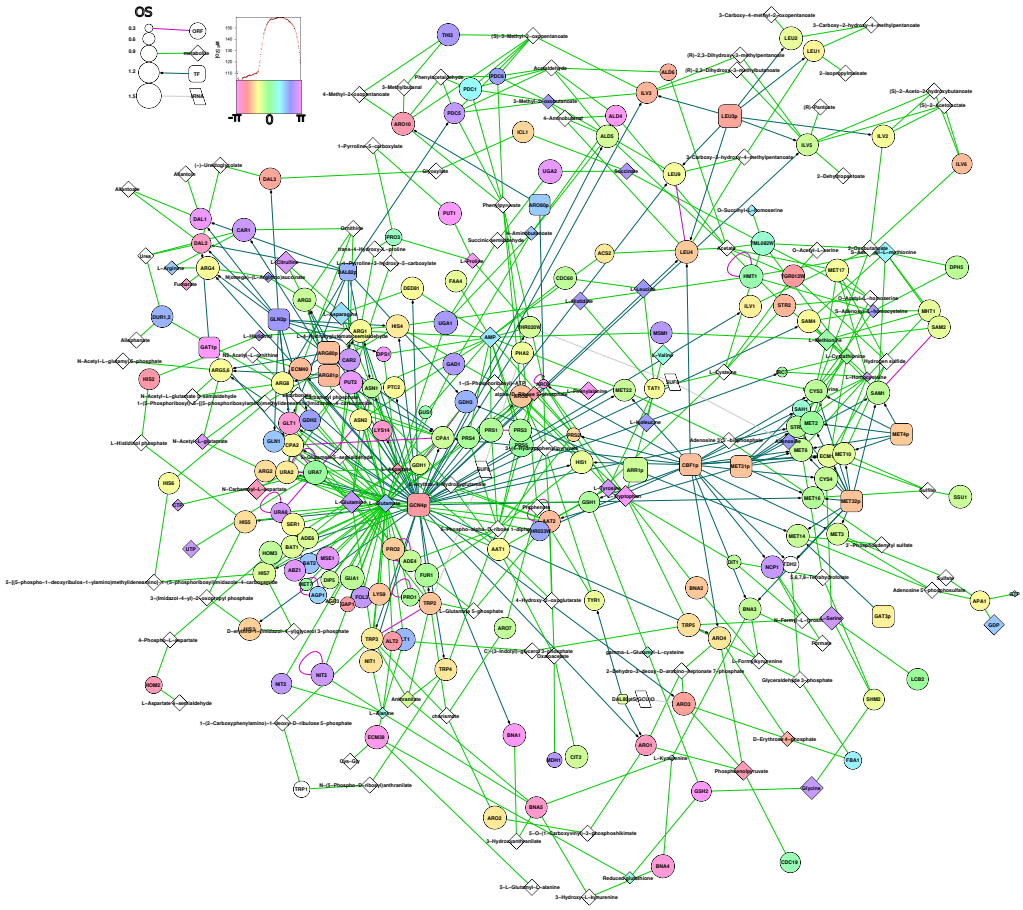

Figure 2: The amino acid biosynthesis map

The amino acid biosynthesis model constructed by integrating enzyme-metabolite [12], protein-protein [1], protein-DNA interactions[7]. 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.

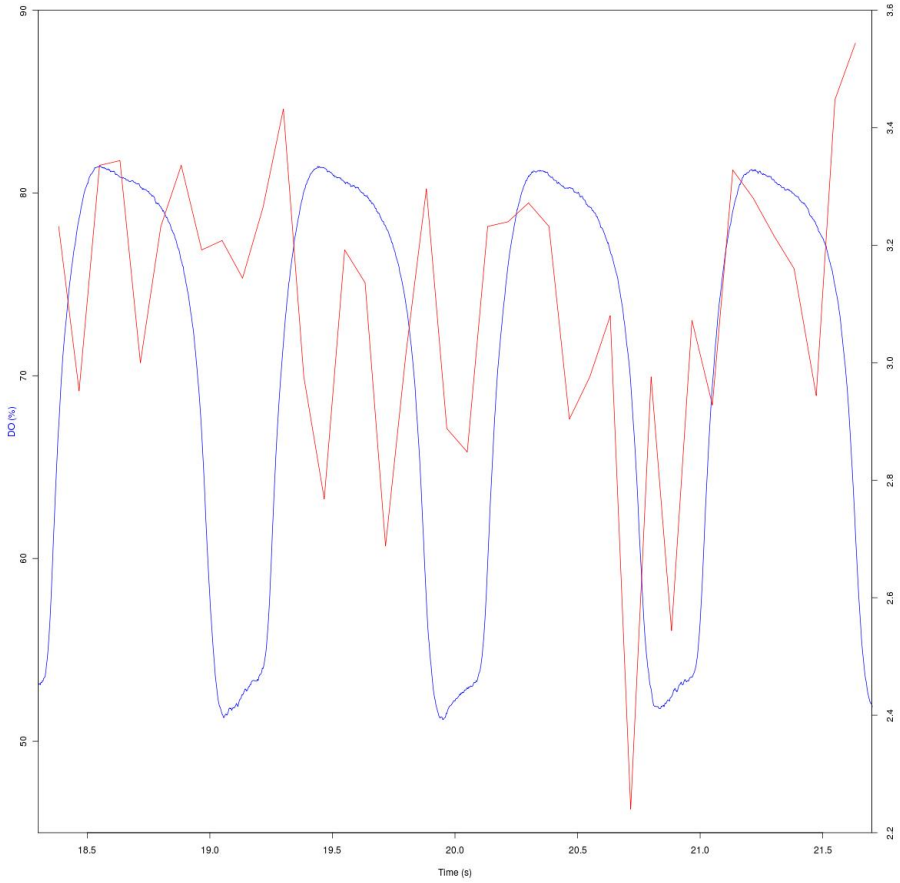

Figure 3: Total RNA turn over during the respiratory oscillation
The blue line indicates the dissolved oxygen (DO) level in the media during respiratory oscillation. The red line indicates total RNA concentration. The right side y axis is the total RNA concentration in µg/µl.

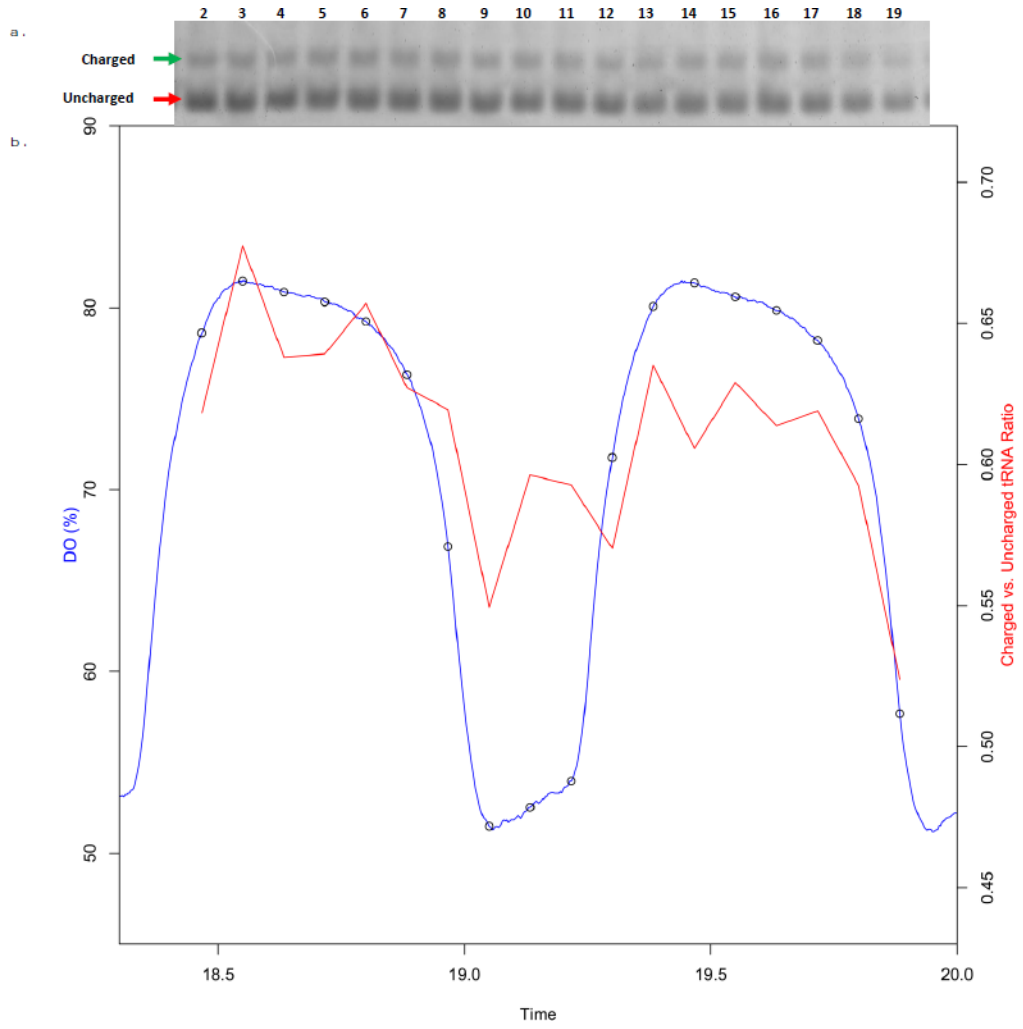

Figure 4: 20 time point of charged and uncharged tRNAs
a. Continuously grown culture of *Saccharomyces cerevisiae* showing oscillatory dynamics was sampled at 5 min intervals (40 samples). Total RNA was extracted 5 and run on acid urea-PAGE gels 5 on 18 consecutive samples. The upper bands (green arrow) are the aminoacylated tRNAs and lower bands (red arrow) are the non-aminoacylated tRNAs.
b. The blue line indicates the dissolved oxygen (DO) level in the media during respiratory oscillation. The red line indicates the charged vs. uncharged tRNA gel band density ratio. The black circles indicate the time points in which the samples were taken for total RNA extraction.

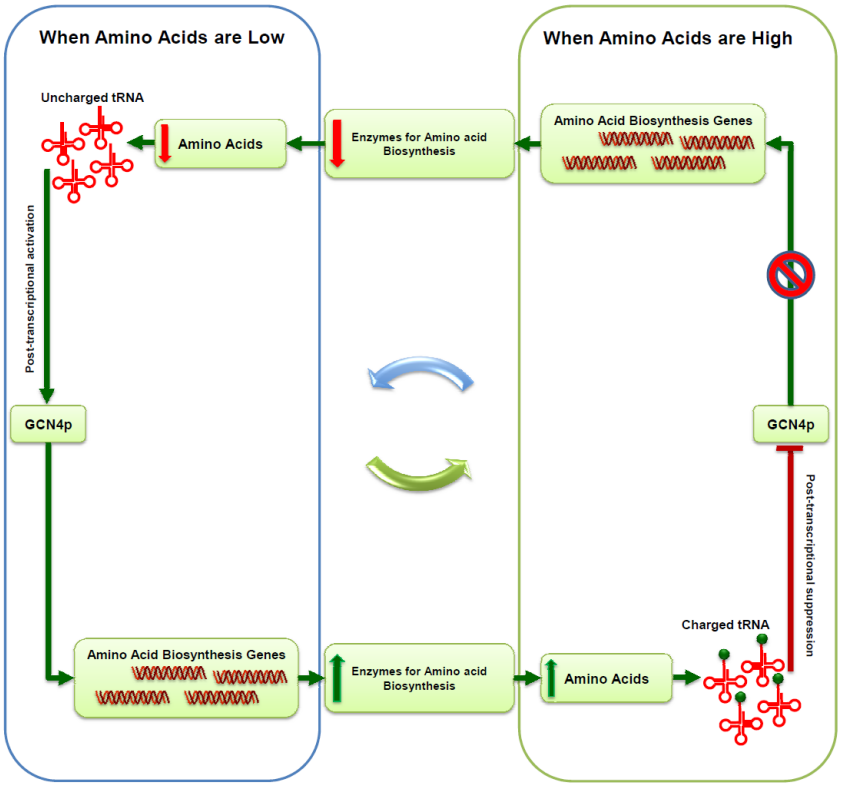

Figure 5: Hypothetical model of self organization of amino acid regulation in yeast

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