



## Effect of Eukaryotic Initiation Factor 4G (eIF4G) on Glyceraldehyde-3-phosphate Dehydrogenase 1 (TDH1) Depletion in *Saccharomyces cerevisiae* Model System

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### ABSTRACT

During viral infection, the cleavage of eukaryotic initiation factor 4G (eIF4G) results in cap-dependent protein synthesis shut-off in host cells. To gain information how the level of intracellular eIF4G affects on protein synthesis during infection, *Saccharomyces cerevisiae* strain BY4742 (*tetO<sub>7</sub>-eIF4G1*) was used as a study model. To reduce the intracellular level of eIF4G, 200 ng/mL of doxycycline was added. The yeast growth rate was reduced 49.9%. Global protein expression during decrease the level of eIF4G was investigated by 2-dimensional gel electrophoresis. Thirty-five protein spots which showed different expression were chosen for protein identification by ESI-Quad-ToF MS. It was found that glyceraldehyde-3-phosphate dehydrogenase 1 (TDH1) was down regulated. TDH1 is a protein in central carbon metabolism which catalyzes glyceraldehyde-3-phosphate to 1, 3-biphosphoglycerate before breaks down to pyruvate. Using STRING database, TDH1 showed interaction with other translation initiation factors such as CDC33, PGK1 and other glyceraldehyde-3-phosphate dehydrogenases (TDH2 and TDH3).

**Keywords:** Eukaryotic initiation Factor 4G, Proteomics, Glyceraldehyde-3-phosphate dehydrogenase 1

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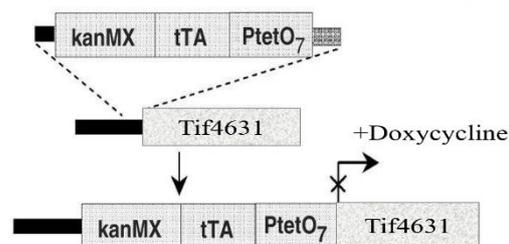
## Introduction

In recent years, the incidence of viral infection in human has increased especially the viruses in group of *Picornaviridae* such as enterovirus poliovirus (PV), coxsackie B virus (CVB) and rhinovirus (HRV) that cause polio, foot and mouth diseases and avian influenza. Picornaviruses can produce proteases that specially cleave eukaryotic translation initiation factor 4G (eIF4G). The eIF4G protein is a component of cap-binding protein complex eIF4F that contains binding sites for many translation initiation factors which are required in cap-dependent translation. Proteolysis of eIF4G leads to the decreasing of eIF4G intracellular level and the inhibition of cap-dependent translation and host cell protein synthesis. However, the positive-strand RNA picornaviruses contains a *cis*-acting internal ribosome entry site (IRES) that supports the recruitment of a cellular 40S ribosomal subunit in cap-independent translation for viral protein synthesis (Breyneet *al.*, 2008; Kobayashi *et al.*, 2012).

Currently, the effect of viral infection on global protein synthesis has not been fully investigated. It is unclear about how the reduction of eIF4G intracellular level in infected cell affects the global protein synthesis. Therefore, this study will investigate the effect of eIF4G on global protein synthesis in *Saccharomyces cerevisiae* model system after the decreasing of eIF4G intracellular level under the control of tetracycline-regulatable cassettes.

## Objective of the study

The aim of this study was to investigate the effect of eIF4G on total protein translation using *S. cerevisiae* model.



**Figure 1** Strategy for promoter substitution upstream of eIF4G-encoding gene (Sangthonget *al.*, 2007).

## Materials and Methods

### Yeast stains, media and growth conditions

*S. cerevisiae* BY4742 and PTC210 in this work were obtained from Prof. John McCarthy, the University of Warwick, United Kingdom. The genotypes of BY4742 and PTC210 were shown in Table 1. PTC210 contains eIF4G gene under the control of tetracycline regulatable promoter (*tetO<sub>7</sub>*-eIF4G1) using kanamycin as a selectable marker. Yeast cells were usually grown in YPD medium (1% yeast extract, 2% meat peptone, 2% glucose). A PTC210 colony was selected by adding G418, a kanamycin derivative, at the final concentration of 300 µg/mL into YPD agar and incubated at 30°C for 24-48 h. Doxycycline was added at various final concentrations indicated in each experiment. The cell culture was incubated at 30°C with 160 rpm agitation.

**Table 1** The genotypes of yeast strain BY4742 and PTC210

Yeast strains	Genotypes
BY4742	<i>MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0</i>
PTC210	<i>MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 tif4632Δ TIF4631 (-306,-1)::KanMX4-tTA-tetO<sub>7</sub></i>

### **Optimal concentration of doxycycline in regulated intracellular eIF4G level**

PTC210 preculture was incubated for 18-24 h and diluted to new YPD medium at optical density measured at a wavelength of 600 nm ( $OD_{600}$ ) in range of 0.100-0.150. Culture was incubated until  $OD_{600}$  was about 1.0 and diluted again to new YPD medium at  $OD_{600}$  in the range of 0.100-0.150. Doxycycline was added to final concentrations of 0, 12.5, 25.0, 50.0, 100, 200 and 300 ng/mL, respectively. Growth rate was studied by measuring  $OD_{600}$  every 3 h for 24 h. The optimal doxycycline concentration was obtained by reducing PTC210 growth about 50% compared to the growth in YPD medium without doxycycline.

### **Protein extraction and two-dimensional gel electrophoresis (2-DE) analysis**

Yeast cells in mid-log phase which were grown in YPD medium with and without doxycycline ( $OD_{600} = 1.0$ ) were employed in this experiment. Protein extraction was performed using glass beads in lysis buffer (7 M urea, 2 M thiourea, 4.0% (w/v) CHAPS, 40 mM DTT) and 1 mM PMSF. Protein concentration was determined by Bradford assay. Then protein samples were separated in 12.0% sodium dodecyl sulfate - polyacrylamide gel electrophoresis (SDS-PAGE) to monitor the suitability of protein extraction method. The effect of intracellular eIF4G level decreasing on global protein synthesis was studied by two-dimensional gel electrophoresis (2-DE) analysis. Protein samples were separated by isoelectric focusing (IEF) in 13 cm immobilized pH gradient (IPG) strip (pH 3-10, nonlinear) and followed by 12.0% SDS-PAGE.

### **Global protein image analysis**

2-DE gels were scanned and analyzed with ImageMaster™ 2D Platinum version 5.0 software. For

images analysis, triplicate gels from each experiment were compared.

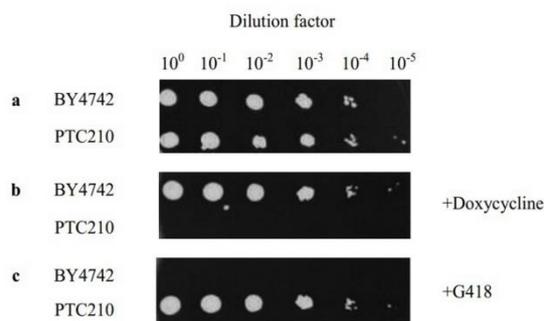
### **LC-MS/MS analysis**

The interested protein spots were cut from 2-DE gels and were analyzed using liquid chromatography-tandem mass spectrometry (LC-MS/MS) at Genome Institute, National Center for Genetic Engineering and Biotechnology (BIOTEC), Thailand. Firstly, the protein samples were cleaved by trypsin and separated by high performance liquid chromatography (HPLC). Then, the protein mixtures were analyzed by mass spectrophotometer. The protein fragments were converted to ions by ESI technique and analyzed by quadrupole-time of flight (Quad-TOF) analyzer. The protein mass finger print (PMF) data were searched for its peptide sequence using Mascot program using “*Saccharomyces cerevisiae*” as taxonomy. The fixed and modifications parameters were “Carbamidomethyl (C)” and “Oxidation (M)”, respectively. “Monoisotopic” and “ $\pm 1.2$  Da” were used as mass values and peptide mass tolerance.

### **Results**

PTC210 was the yeast strain that *tif4632* gene (encoding eIF4G2) was deleted and substituted tetracycline (*tet*)-regulatable cassette in natural promoter at position 1 to 306 bp upstream of eIF4G1-coding gene as shown in Figure 1 (Brook *et al.*, 2009). The *tet*-regulatable promoter system is commonly used in genetic studies in many eukaryotic organisms. The cassette contains a kanamycin selectable marker (*KanMX4*), a tetracycline-responsive transactivator (tTA) protein and a tetracycline operator (*tetO*) promoter (Wishart *et al.*, 2005). In the absence of tetracycline or its derivative, doxycycline, tTA binds to

*tetO* promoter allowing activation of eIF4G-coding gene transcription. Whereas, the transcription from *tetO* promoter is repressed in the presence of the antibiotic that binds to tTA protein, resulting in a conformation change and transcription activation activity decreasing (Sangthong *et al.*, 2007). In 1998, Belli and co-workers indicated that the concentration of doxycycline at least 2 µg/ml in growth medium is sufficient to confer repression (Belliet *et al.*, 1998).

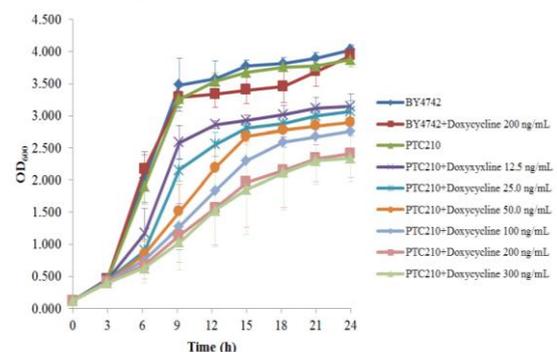


**Figure 2** The growth of yeast strain BY4742 and PTC210 on YPD plates (a), YPD plate with 2 µg/ml doxycycline (b) and YPD plate with 300 µg/ml G418 (c). Yeast cells were diluted in ten-fold serial dilution.

To determine the effect of doxycycline on yeast cell growth, we designed to culture both of wild type and PTC210 yeast strains on YPD agar in the presence and absence of doxycycline and G418 at the concentration of 2 and 300 µg/ml, respectively. The result indicated that the growth of BY4742 was similar to PTC210 in the absence of doxycycline and G418 condition (Figure 2a). BY4742 growth was observed in the presence of doxycycline condition whereas PTC210 growth was repressed (Figure 2b). In addition, PTC210 growth was only observed in the presence of G418 condition (Figure 2c).

The studies of BY4742 and PTC210 growth were performed by cultivation in the presence and

absence of various doxycycline concentration conditions and measuring OD<sub>600</sub> in every 3 h for 24 h. Growth rates of both strains are shown in Figure 3. The result revealed that the growth rates of BY4742 and PTC210 in the absence of doxycycline condition were similar. Doubling time of BY4742 and PTC210 growth in the absence of doxycycline condition was 2.11 and 2.15 h, respectively (Table 2). In addition, the doubling time of BY4742 growth in the presence of doxycycline condition was 2.15 hours, whereas PTC210 growth was decreased when increasing of doxycycline concentrations. However, the optimal doxycycline concentration was obtained by reducing PTC210 growth about 50% compared to the growth in the absence of doxycycline condition. It was found that the growth rate of PTC210 was reduced to 49.88%, when doxycycline 200 ng/ml was added. The doubling time was increased from 2.15 to 4.33 hours. Thus, 200 ng/ml of doxycycline was used to determine the effect of eIF4G on global protein expression.



**Figure 3** Growth rate of BY4742 and PTC210 in YPD medium with and without doxycycline at concentration of 12.5, 25.0, 50.0, 100, 200 and 300 ng/ml.

Yeast strain BY4742 and PTC210 were cultured in YPD medium with doxycycline 200 ng/ml (BY4742+doxy and PTC210+doxy) and without doxycycline (BY4742-doxy and PTC210-doxy). The

yeast cells were harvested at mid-log phase. It was found that the optimal time of cell harvesting were 6, 6.5 and 8.5 hours after BY4742<sup>-doxy</sup>, BY4742<sup>+doxy</sup>, PTC210<sup>-doxy</sup>, and PTC210<sup>+doxy</sup> cultivation, respectively.

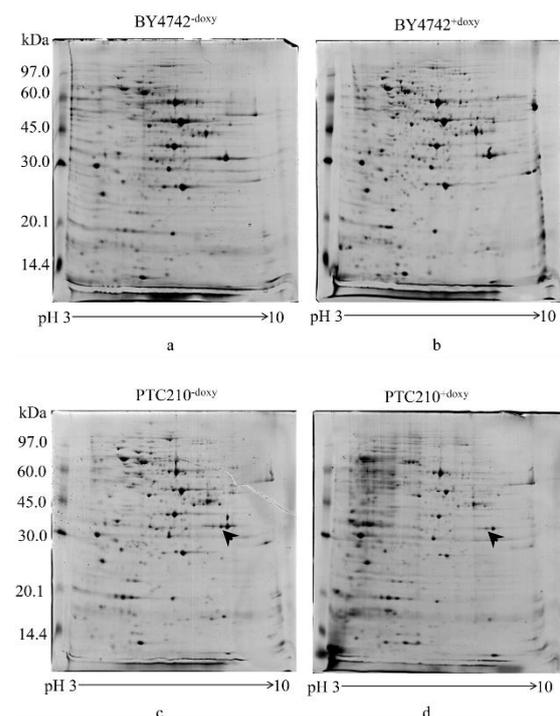
**Table 2** Doubling time and growth rate reducing percent of BY4742 and PTC210 in YPD medium with and without doxycycline at various concentrations.

Yeast strains	Concentration of doxycycline (ng/ml)	Doubling time (h)	Growth rate reduction (%)
BY4742	0	2.11	-
	300	2.15	1.93
PTC210	0	2.15	-
	12.5	2.45	12.28
	25.0	2.70	20.47
	50.0	3.46	36.57
	100.0	4.08	45.92
	200.0	4.33	49.88
	300.0	4.95	55.92

The total proteins were extracted using glass bead and protein concentration was measured by Bradford assay. It was revealed that the extracted protein concentration from BY4742<sup>-doxy</sup>, BY4742<sup>+doxy</sup>, PTC210<sup>-doxy</sup>, and PTC210<sup>+doxy</sup> were 3.99, 4.70, 4.86 and 2.69 mg/ml, respectively. The protein was then analyzed by 12.0% SDS-PAGE and the result revealed that the protein molecular weights ranged between lower than 14.4 kDa and higher than 97.0 kDa.

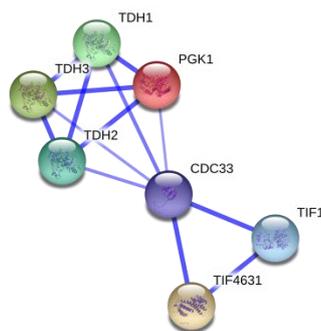
2-DE is a powerful and widely used for the analysis of complex protein mixtures extracted from cells. This technique separates protein according to two independent properties in two discrete steps. The first-dimension step is isoelectric focusing (IEF) which separates protein according to their isoelectric points

(pI). The second-dimension step is SDS-PAGE which separates protein according to their molecular weights (MW)(Gorget *al.*, 2004).In this research, the proteins were separated by IEF in a 13 cm IPG strip (pH 3-10 nonlinear) following by 12.0%SDS-PAGE. The result indicated that the protein patterns of BY4742<sup>-doxy</sup> and PTC210<sup>-doxy</sup> was similar to BY4742<sup>+doxy</sup> and PTC210<sup>+doxy</sup>, respectively and the protein spots about 1,500 spots were observed (Figure 4). The majority of the protein spots were located between pH 4 and 8 with molecular weights between 20.1 and 97.0 kDa. In addition, it was found that there were 35 different protein spots when the proteins spots of BY4742<sup>-doxy</sup> and PTC210<sup>-doxy</sup> were compared with BY4742<sup>+doxy</sup> and PTC210<sup>+doxy</sup>, respectively.



**Figure 4** 2-DE analysis of the extract proteins from BY4742<sup>-doxy</sup> (a), BY4742<sup>+doxy</sup> cells (b), PTC210<sup>-doxy</sup> (c) and PTC210<sup>+doxy</sup> (d). Black arrows were ID14519 which was identified as TDH1.

After 2-DE gels analysis, thirty-five differently expressed protein spots were analyzed by LC-MS/MS. The protein spots ID14519 was identified as glyceraldehyde-3-phosphate dehydrogenase 1 or TDH1. Using yeast model in this study, TDH1 was down-regulated due to intracellular eIF4G reduction. TDH1 is an enzyme in central carbon metabolism pathway which catalyzes the conversion of glyceraldehyde-3-phosphate to 1, 3-bisphosphoglycerate before break down to pyruvate (Delgado *et al.*, 2001; Dickinson and Schweizer, 2004). Then, STRING program was investigated protein interaction between TDH1, TIF4631 and other proteins in database. The results are shown in Figure 5.



**Figure 5** Protein interaction analysis of TDH1, TDH2, TDH3, PGK1, TIF1, CDC33 and TIF4631. Stronger associations are represented by thicker blue lines.

### Discussion and Conclusion

Due to the global protein analysis during viral infection that may effect to eIF4G and protein synthesis has not been fully investigated, it is unclear how the reduction of eIF4G intracellular level in infected host cell affects on global protein synthesis. In this study, the effects in the decreasing of intracellular eIF4G level on global protein synthesis were investigated using the model system of *S.*

*cerevisiae* BY4742 and PTC210. The intracellular eIF4G level was regulated using a tetracycline regulatable cassette. The global protein changes profiling was investigated using 2-DE and LC-MS/MS.

The activity of *tet*-regulatory promoter cassette in PTC210 strain was studied by serial 10-fold dilution of PTC210 yeast cells on YPD agar plates with and without G418 and doxycycline. The results indicated that the growth of PTC210 strain was regulated under *tet*-regulatory cassette due to the growth observation in the presence of G418 and the growth repression in the presence of doxycycline, whereas the growth of BY4742 was similar to PTC210 in the absence of both doxycycline and G418 condition. BY4742 growth was only observed in the presence of doxycycline condition. In addition, the growth rate of PTC210 was reduced 49.88% when doxycycline at the concentration of 200 ng/mL was added.

The changes on global protein synthesis were studied by 2-DE when the intracellular eIF4G level was decreased under the control of *tet*-regulatable cassette. About 1500 spots of the protein spots were observed. Thirty-five differently expressed protein spots were analyzed by LC-MS/MS. Using MASCOT, the protein spots ID14519 was identified as glyceraldehyde-3-phosphate dehydrogenase 1 (GADPH 1) or TDH1 which was down-regulated due to the intracellular eIF4G reduction. Using STRING program, TDH1 was found interaction with TDH2 (GADPH 2), TDH3 (GADPH 3), PGK1 (phosphoglycerate kinase 1), TIF1 (ATP-dependent RNA helicase eIF4A), CDC33 (mRNA cap binding protein eIF4E) and TIF4631 (eIF4GI).

TDH1, TDH2, TDH3 and PGK1 are enzymes which involved in carbon metabolism pathway and were co-expressed in yeast cells (Varela *et al.*, 2012). In addition, TIF4631, TIF1 and CDC33 were eukaryotic initiation factors which involved in eIF4F complex formation of translation initiation (Brook *et al.*, 2009; Castello *et al.*, 2011). This indicated that the decreasing of intracellular eIF4GI level affected the reduction of eIF4F complex formation resulting in the decreasing of mRNA translation and the TDH1 expression in cap-dependent translation. In addition, the low-expression of TDH1 might be due to the decreasing in carbon metabolism, where the energy producing occurred for cell utilization, leading to the decreasing in yeast growth.

According to the yeast model, it supported the trend in host cell death due to viral infection and the eIF4GI cleavage. When the host cell was infected by virus producing protease which cleaved eIF4GI, the host cell protein synthesis was inhibited especially in cap-dependent translation pathway. This incidence led to the decreasing in cellular activity and the death of infected host cell. This research suggested that the decreasing of cellular activities in infected host cells might be due to the low-expression of TDH1, the protein involved in carbon metabolism. Whereas, all enterovirus mRNAs contain IRES that promote cap-independent translation initiation to sustain ongoing the viral protein synthesis while the host cell protein synthesis is inhibited (Breyne *et al.*, 2008; Kobayashi *et al.*, 2012).

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