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RESEARCH ARTICLE / ARAŞTIRMA MAKALESİ

Metformin Administration to Glucose-restricted Cells Attenuates PKA Signaling in *S. cerevisiae*

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Abstract
Recent research in cancer treatment points to metformin, a drug for type 2 diabetes, as a potential anti-cancer therapeutic, as well as carbon limitation as a dietary measure. A new study, investigating effects of metformin treatment on colorectal cancer cells, pointed to the fact that response to metformin treatment depended on extracellular glucose concentration. That is why in the current study, effects of both carbon limitation and metformin treatment are explored via transcriptomics analyses. It is demonstrated that cells grown in glucose-limited and metformin treated medium had the highest variance according to transcriptional profiles, compared to individual treatments. Metformin administration, when combined with glucose restriction, downregulates proliferative pathways such as transcription initiation and ribosome biogenesis while upregulates energy derivation and autophagic mechanisms. Enrichment analyses point to an attenuated cAMP-PKA signaling pathway in the cells grown in combined treatment medium. It is proposed that combined treatment exerts its beneficial effect on this pathway, since cAMP-PKA signaling may be a potential target for pharmacological treatment of tumors.
Keywords: Systems Biology, *S. cerevisiae*, Metformin, Glucose Restriction, Transcriptomics, Cancer

Öz
Kanser tedavisindeki son araştırmalar, tip 2 diyabet tedavisi için kullanılan metforminin potansiyel bir kanser önleyici terapötik olduğunu ve ayrıca bir diyet önlemi olarak karbon sınırlamasının önemini işaret etmektedir. Metformin tedavisinin kolorektal kanser hücreleri üzerindeki etkilerini araştıran yeni bir çalışma, metformin tedavisine verilen yanıtın hücre dışı glikoz konsantrasyonuna bağlı olduğu gerçeğini ortaya koymuştur. Bu nedenle bu çalışmada hem karbon sınırlamasının hem de metformin tedavisinin etkileri transkriptomik analizlerle araştırılmıştır. Glikoz-sınırlı ve metformin ile tedavi görmüş ortamda büyütülen hücrelerin, bireysel tedavilere kıyasla transkripsiyonel profillere göre en yüksek varyansa sahip olduğu gösterilmiştir. Metformin tedavisi, glikoz kısıtlaması ile birleştirildiğinde, transkripsiyon başlangıcı ve ribozom biyogenezi gibi proliferatif yolakları baskılarken, enerji ortaya çıkışı ve otofajik mekanizmaları tetiklemiştir. Gen ontolojisi zenginleştirme analizleri, kombine tedavi ortamında büyütülen hücrelerde zayıflamış bir cAMP-PKA sinyal yolağına işaret etmektedir. cAMP-PKA sinyal yolağı, tümörlerin farmakolojik tedavisi için potansiyel bir hedef olabileceğinden, kombine tedavinin yararlı etkisini bu yolak üzerinden gösterdiği düşünülmektedir.
Anahtar kelimeler: Sistem Biyolojisi, *S. cerevisiae*, Metformin, Glikoz Kısıtlaması, Transkriptomiks, Kanser

1. INTRODUCTION

Cancer is a multi-faceted disorganization of the cell, involving alterations in numerous signaling pathways which result in abnormal cellular growth and a potential to invade other parts in the body. Recently, revisiting of an old theory emerged as one of the prospective anti-cancer treatments: nutrient limitation. First stated by Otto Warburg in 1920s [1], tumor cells should be deprived of glucose to fight cancer, based on the observation that they rely on an excess amount of extracellular glucose to sustain growth. Indeed, recent work showed that administration of glucose restriction and chemotherapy increases the therapeutic effect as well as reduces side-effects that healthy cells endure [2-3].

Metformin is an FDA approved drug, a biguanide used to treat type 2 diabetes. Its mechanism of action relies on reducing blood glucose concentrations, without causing overt hypoglycemia [4]. Several clinical studies have indicated that type 2 diabetic patients treated with metformin might have a lower cancer risk [5] compared to those who do not follow such a regimen. Numerous research on metformin treatment in cancer has been conducted since but a definite result was not obtained. Of note, several studies have pointed to the fact that metformin treatment affected aggressiveness in solid cancers [6].

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In a recent study, the two players mentioned here were combined and the role of extracellular glucose on the response to metformin treatment was investigated for colorectal cancer cells. It was demonstrated that the cell lines show inhibitory growth after metformin treatment under physiological (low) glucose conditions, but not in high glucose conditions [7]. This result underlines the importance of experimental design in cancer research: glucose concentrations in the experiments should be considered carefully when evaluating the sensitivity of cancer cells to biguanides or other therapeutic agents.

Saccharomyces cerevisiae, the budding yeast, has long been used as a model organism for human diseases and cancer, both for the broader investigation of cellular machineries leading to cancer biogenesis [8] and for studying the response to anti-cancer agents [9]. The ease of manipulation of yeast cells, coupled with their genetic and metabolic similarity to tumor cells such as preferring fermentation over respiration, even when glucose is abundant, renders yeast an attractive model organism for cancer studies. In fact, deeper understanding of vital processes playing roles in tumorigenesis such as DNA damage response [10], autophagy [11], and lipid metabolism [12] are elaborately studied in yeast first. Moreover, several anti-cancer drug screening experiments as well as mechanism of action studies are also conducted in yeast [12-14], broadening our understanding of cancer therapy.

In this study, the yeast *S. cerevisiae* is used as a model organism to explore the effects of metformin treatment and glucose restriction at the transcriptome level. According to the results, simultaneous administration of both interventions captures the highest variance in the transcription data and seems to result in attenuation of cAMP-PKA signaling pathway.

2. MATERIALS AND METHODS

2.1. Yeast Strains and Growth Conditions

Saccharomyces cerevisiae strain adopted for the cultures was *ΔHO* derived from BY4742 background, Matα; *his3Δ1; leu2Δ0; lys2Δ0; ura3Δ0*; YDL227c::kanMX4 obtained from EUROSCARF deletion collection. The overnight grown cultures in SDC were diluted to an OD_{600} value of 0.1 before the main inoculation. All experiments were carried out in biological triplicates in micro-aerated flasks, with a working volume of 1:5. Temperature was set to 30 ^{0}C and rpm to 180 for the batch-wise grown cultures. At the mid-exponential phase (OD_{600}~0.6), the main culture was split to three aliquots and treatments are performed. Prior to transfer to the treatment media, the cells of the three aliquots were centrifuged at 6000 rpm for 5 min and washed with water. Treatment media comprised of fresh SDC (2% glucose) for the control case, C; SDC + 0.25% glucose for the carbon limited case, CR; SDC + 1.66% metformin for the metformin treated case, M; and SDC + 0.25% glucose + 1.66% metformin for simultaneous treatment case, CR+M. Samples for RNA extraction were collected 2 hours after the transfer to the treatment media.

2.2. Microarray Analysis

Extracted RNA was quantitatively assessed by UV-vis spectrophotometer (NanoDrop ND-1000, Thermo Fisher Scientific Inc., U.S.A) while qualitatively examined with Bioanalyzer 2100 for RNA integrity number (RIN, using RNA6000 Nanokit (Agilent Technologies, USA). Samples with a RIN value > 7 were processed. The Affymetrix GeneChip®Expression Analysis Technical Manual was followed for the microarray analysis wet-lab steps. dChip software [15] was adopted for the outlier detection. Raw cell files without outliers were then processed in R 4.1.3. via "affy" and "affycoretools" packages of Bioconductor [16], with Robust Multichip Average (rma) normalization. Log_2 transformed expression values for the genes were used for further statistical analyses. Data were submitted to Arrayexpress platform under the accession numbers E-MTAB-6847 and E-MTAB-3001.

2.3. Statistical Analyses

Principal Component Analysis (PCA) was performed with log_2 transformed expression values in MATLAB R2013. Data were processed with one way analysis of variance (ANOVA, α=0.01) in MATLAB R2013 to determine significantly expressed genes. GO term and KEGG pathway analyses of the differentially expressed gene groups were performed with the web-based "gprofiler" tool [17]. GeneCluster 2.0 software was adopted to cluster the data with self-organizing maps algorithm, using the default values [18].

2.4. ReporterFeatures Analysis

Corrected p-values calculated by ANOVA and transcription factor-gene interaction data downloaded from YEASTRACT (February 2023) [19] were the two input files to ReporterFeatures software [20], with default settings.

3. RESULTS AND DISCUSSION

3.1. Transcriptomics Analyses Reveal that the Highest Impact on Cellular Reprogramming is Encountered when Metformin was Administrated to Carbon-limited Cells

When the normalized and log-transformed gene expression data belonging to glucose restriction, metformin administration and simultaneous application of both treatments are scrutinized, it is

demonstrated that principal component 1 captures the highest variance in the data (94%), separating metformin administrated carbon limited cells from the remaining points on the principal component analysis (PCA) plot (Figure 1).

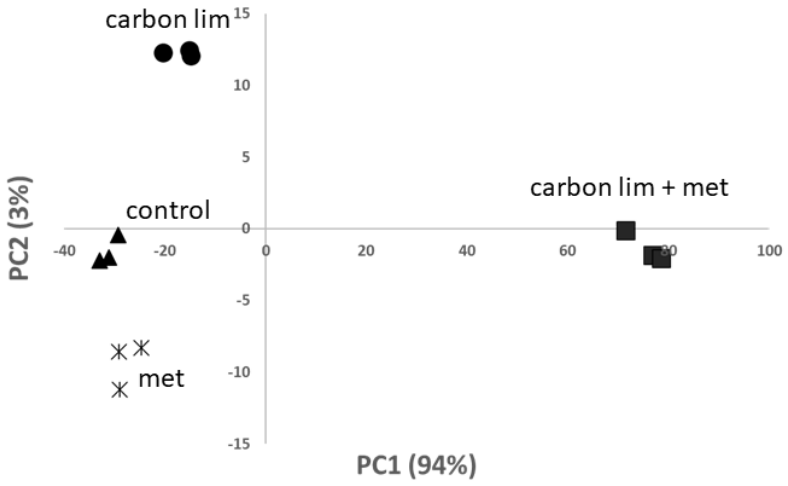

Figure 1. PCA plot of transcriptomics data. Triangles, circles, stars and squares represent data of the cells grown in control, carbon-limited, metformin-administered and both carbon-limited and metformin-administered media, respectively.

The dominance of the simultaneous treatments' effects on the variance of the data is also supported by the ANOVA results. This statistical analysis identified a total number of 332 genes which were differentially expressed in metformin-treated case and 326 for carbon-limited case, according to FDR corrected p-values ($p \leq 0.01$). However, none of those genes passed the fold change threshold of 1.5. Instead, 1185 genes were significantly expressed according to the simultaneous effect of both carbon limitation and metformin treatment, 605 upregulated and 580 downregulated, each passing the fold change threshold of 1.5 (Figure 2).

This result may be of importance when the current anti-cancer therapeutic strategy of "combining nutrient limitation and drug administration" [21] is taken into consideration. It is demonstrated in this study (Figure 1) that this kind of combination creates an effect greater than that of the individual responses, at least at the transcriptional level.

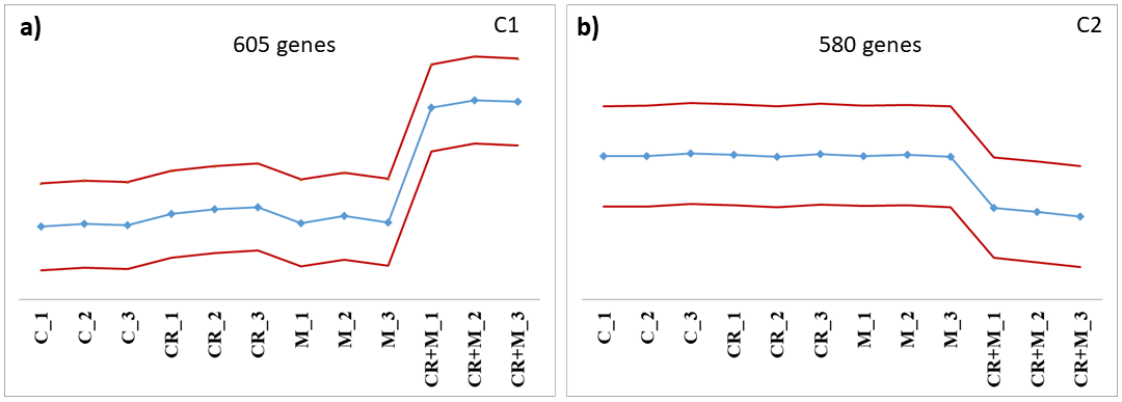

Figure 2. Self-organizing maps of the transcriptome profiles of interaction specific genes around a 1 x 2 arrangement. C, CR, M and CR+M denote control, carbon limited, metformin administrated, and simultaneous treatment cases respectively. a) significantly upregulated genes whereas b) significantly downregulated genes. The cluster number and number of genes in each cluster are indicated in the top right corner and in the top center of each cell. The blue and red curves represent the centroids and standard deviation around the centroids, respectively.

3.2. Cells Grown in Carbon-limited and Metformin Treated Medium Upregulate Genes in the Energy Derivation and Oxidation Reduction Processes while Repress Transcriptional and Translational Machineries

The GO Process term enrichment of the 605 genes of cluster C1 on Figure 2 resulted in numerous processes related to energy derivation, ranging from aerobic respiration to fatty acid oxidation (Table 1). It seems that yeast cells adapt to this environment *via* inducing catabolic pathways to produce energy, i.e., stimulation of aerobic respiration and fatty acid oxidation (FAO) (Table 1) and suppressing energy requiring machineries such as transcription and translation (Table 2), since nutrients are scarce. At a first glance, this is exactly the expected healthy cellular response from cells grown in glucose-limited medium, as proven to take place in our previous study [22], contrary to the behavior of cancer cells: tumor cells generally exhibit induced energy derivation pathways along with proliferative ones, such as increased ribosome synthesis [23].

Addition of metformin to the carbon limited medium however, gives rise to other terms such as "autophagy of mitochondrion" (Table 1). In our previous study, we concluded that metformin hampered copper-dependent cytochrome c oxidase (complex IV) activity, mildly uncoupled the completion of electron transport during respiration and thus stimulated a pseudo-hypoxic response, even in aerated culture [22]. In fact, the dose-dependent effect of metformin on cellular respiration has already been demonstrated also in mammalian cells [24]. Thus, it may be hypothesized that the simultaneous treatment helps to eliminate damaged mitochondria through upregulating mitophagy. For cells which are grown in glucose restricted medium, aerobic respiration is already expected to be upregulated due to the attenuation of the Crabtree effect [25] which is defined as the repression of respiration because of elevated glucose concentrations. Crabtree effect, as well as Warburg effect, is one of the hallmarks of cancer cells [26], thus alleviation of this effect might be one of the beneficial effects of the current study's interventions. FAO is also expected to increase when glucose is scarce, however it is a double-edged sword for cancer therapy. Recently, an important role of FAO in chemoresistance has been shown, stating that elevated FAO results in increased phospholipid synthesis, which in its turn elevates phospholipids in mitochondrial membranes to induce mitochondrial fitness of tumor cells [27]. However, no increase of *de novo* synthesis and/or uptake of exogeneous lipids is detected in this study, at least at the transcriptional level. Moreover, mitophagy is one of the induced processes, hinting that the simultaneous treatment does not increase mitochondrial fitness but eliminate damaged mitochondria.

"Glutamine family amino acid catabolic process", "methylglyoxal catabolic process to lactate", "cellular response to oxidative stress", "NADPH regeneration" and "cellular oxidant detoxification" are among the enriched terms pertinent to upregulated genes, hinting cells of this combined medium cope with the ROS encountered by aerobic respiration/hampering of aerobic respiration and fatty acid oxidation also by properly regulating genes of the oxidative stress response, apart from mitophagy. In fact, methylglyoxal is a debated tumor-promoting factor, hence its catabolism to lactate may be one of the beneficial effects of the combined medium [28-29].

Table 1. Enriched GO process terms of the genes of cluster C1 (Figure 2)

GO BP Term	Term No	p-val
aerobic respiration	GO:0009060	7.12E-21
cellular response to oxidative stress	GO:0034599	5.10E-07
glycogen biosynthetic process	GO:0005978	1.41E-05
respiratory chain complex IV assembly	GO:0008535	4.91E-05
cellular oxidant detoxification	GO:0098869	3.06E-04
glutamine family amino acid catabolic process	GO:0009065	3.97E-04
mitochondrial translation	GO:0032543	5.52E-04
fatty acid beta-oxidation	GO:0006635	1.05E-03
methylglyoxal catabolic process to lactate	GO:0061727	1.81E-03
autophagy of mitochondrion	GO:0000422	5.27E-03
long-chain fatty acid import into peroxisome	GO:0015910	9.42E-03
protein kinase A signaling	GO:0010737	1.09E-02
NADPH regeneration	GO:0006740	1.60E-02

Downregulated genes are enriched mainly in translation and transcription related terms (Table 2). "Ribosome biosynthesis" as well as "transcription initiation at RNA polymerase I promoter" terms are the main terms of Table 2, demonstrating the cellular reprogramming encountered in carbon limitation and metformin treatment. The reduction in protein synthesis machinery is generally attributed to deficiencies in TOR signaling [30]. Although the repression of TOR signaling in response to carbon limitation is currently under debate, simultaneous administration of metformin and carbon limitation seems to alter this signaling machinery, possibly in part by the upregulated "glutamine amino acid catabolic process term" (Table 1). In fact, metformin has already been proposed to inhibit TORC1 signaling in mammalian cells [31]. The last one of the enriched terms of significantly downregulated 580 genes is the "methylation" term. Hyper or hypomethylation of DNA in cancer has long been studied [32], hinting that one of the beneficial effects of simultaneous administration of both interventions, is to suppress this mechanism.

Table 2. Enriched GO process terms of the genes of cluster C2 (Figure 2)

GO BP Term	Term No	p-val
ribosome biogenesis	GO:0042254	1.49E-135
methylation	GO:0032259	1.16E-38
transcription initiation at RNA polymerase I promoter	GO:0006361	6.37E-09
cellular amino acid biosynthetic process	GO:0008652	6.46E-04
peptidyl-diphthamide biosynthetic process from peptidyl-histidine	GO:0017183	4.04E-03
pyrimidine nucleoside monophosphate biosynthetic process	GO:0009130	4.82E-02

3.3. Metformin Administration to Carbon-limited Cells Attenuates PKA Signaling

Another upregulated term in Table 1 is "Protein kinase A (PKA) signaling", with a p-value of 1.09E-02. When the 4 genes responsible for this enrichment result are investigated in depth, namely *TPK1*, *TPK2*, *GPA2*, and *YAK1*, it was seen that nearly all key members of PKA pathway were upregulated in cells grown in simultaneous treatment medium, except *TPK3* (Figure 3). This result is in strict contradiction with our previous study, where *TPK1* and *TPK2*, the two catalytic subunits of cAMP-dependent protein kinase of yeast, were found to be among the significantly downregulated genes unique to carbon limited case, with fold change values of 1.54 and 1.30. Thus, metformin administration reverses this behavior.

The cAMP-PKA signaling in *S. cerevisiae* is a major controller of numerous essential cellular processes associated with fermentative growth, the entrance into stationary phase, stress responses and developmental pathways [33]. cAMP synthesis in yeast is induced in response to two stimuli: extracellular fermentable sugars, which is not the case in this study, and intracellular acidification [34]. *CYR1* encoding the adenylate cyclase which required for cAMP production is found to be upregulated nearly 1.3 fold in cells grown in simultaneous treatment medium. This leads to the hypothesis of increased intracellular acidification in those cells since extracellular glucose is limited. Although extracellular acidification by metformin treatment is demonstrated in *S. cerevisiae* [22], other studies point to a role for metformin also in the acidification of lysosomal/endosomal compartments in rat microglia [35]. Establishment of such acidification may be of potential for anti-cancer treatment, since acid stress triggers apoptosis [36].

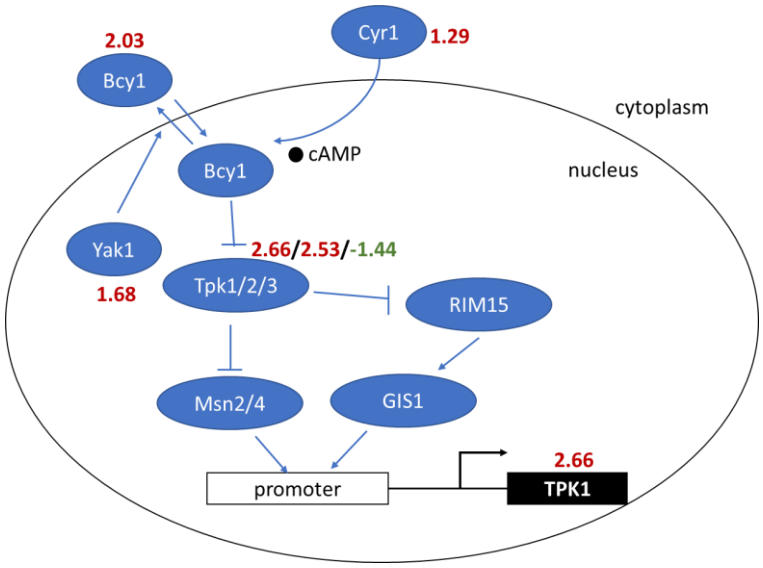

Figure 3. Negative regulation of *TPK1* by active cAMP-PKA signaling. Red denotes upregulation in gene expression while green is for downregulation.

TPK1, *TPK2*, *TPK3*, and *BCY1* promoters, are all known to be activated under low or null protein kinase A activity [37], and they are all upregulated in this study (Figure 3). Current study demonstrates that, at the transcriptional level, the increase in *CYR1* levels does not induce the subsequent PKA activation in cells grown in carbon limited and metformin administered medium, leading to an attenuated PKA activity, which in its turn upregulates genes involved in its own pathway.

Although cAMP signaling in cancer cells is affected by the type of cell and its surroundings, oncogenic activation of PKA signaling is found to be in many tumor types [38]. Thus, another beneficial effect of combined treatment may be the attenuation of this pathway.

3.4. Cells Grown in Combined Treatment Medium are Under the Effect of Msn2p/Msn4p Transcription Factors

Adoption of the Reporter Features software, which is used for the identification of key biological features around which transcriptional changes are significantly concentrated, enabled the identification of the transcription factors which are responsible for the organization of the current transcriptome data. Msn2p and Msn4p are among the top ten regulated transcription factors, with Z-scores of 10.62 and 9.85 respectively (Figure 4). Msn2p and Msn4p are regulators of the stress response in *S. cerevisiae*, under the negative effect of PKA signaling. Hence, this result also supports a low PKA signaling activity in cells grown in the combined medium.

Although functional orthologues are not identified in mammalians, Msn2p and Msn4p are involved in response to various stress factors in *S. cerevisiae*. Genes of Figure 4, significantly upregulated in combined medium, do not yield an enrichment result. However, deeper investigation shows that most of them are upregulated in response to osmotic shock, a pathway under Hog1p control in yeast. Hog1p is the yeast orthologue of mammalian MAPK p38, which is involved in the inflammatory and stress responses. Results of the current study may imply an effect of combined medium on MAPK signaling, an outcome in line with metformin exerting an apoptosis-mediated effect through activating the JNK/p38 MAPK pathway in lung tumor cells [39].

In summary, glucose restriction and metformin treatment, when applied together, have a greater effect than individual treatments at the transcriptional level. The enrichment results of the differentially expressed genes point to repression of ribosome and cellular amino acid biosyntheses, while terms related to energy generation such as fatty acid oxidation and aerobic respiration are induced. Another transcriptionally induced term is “PKA signaling”, and it is known that this pathway is activated under low or null protein kinase A activity, hinting a low PKA activity in the cells grown in combined medium. This result is further supported by the determination of key transcription factors (TFs): Msn2p and Msn4p which are under the negative control of PKA signaling are among the top ten key TFs and they positively upregulate genes situated at the downstream of MAPK signaling pathway.

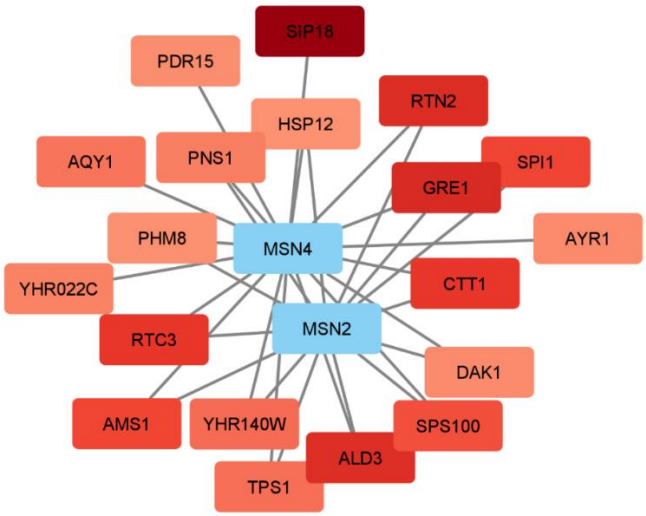

Figure 4. Differentially upregulated genes under the positive control of Msn2p-Msn4p

4. CONCLUSIONS

Recently, carbon limitation, i.e., glucose restriction has taken a greater interest in treating tumor cells, especially as a subsidiary intervention combined with chemotherapeutic agents. In this study, I tried to capture the cellular reprogramming in terms of transcriptional responses, when glucose limitation is combined with the promising anti-cancer agent metformin administration. Results of the current study points to downregulation of terms pertinent to ribosome biogenesis and proliferation. Another key result of the study is the low cAMP-PKA signaling activity in the cells of the simultaneous treatment medium. Drug administration, when combined with nutrient limitation, may downregulate PKA signaling cascade to properly respond to cellular stress and induce autophagic machinery. Another shadow player seems to be the MAPK machinery, demonstrated by the genes responsive to Msn2/4p, although further experimentation is needed for a definite result. Experiments which target MAPK and/or cAMP-PKA pathway in combination with dietary restriction and metformin administration will provide valuable insights for anti-tumor therapy.

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