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Transcriptional response to dietary restriction in *Drosophila melanogaster*

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Abstract

Dietary restriction (DR) extends lifespan in a wide variety of organisms. Although several genes and pathways associated with this longevity response have been identified, the specific mechanism through which DR extends lifespan is not fully understood. We have recently developed a novel methodology to screen for transcriptional changes in response to acutely imposed DR upon adult *Drosophila melanogaster* and identified groups of genes that switch their transcriptional patterns from a normal diet pattern to a restricted diet pattern, or 'switching genes'. In this current report we extend our transcriptional data analysis with Gene Set Enrichment Analysis to generate a pathway-centered perspective. The pattern of temporal behavior in response to the diet switch is strikingly similar within and across pathways associated with mRNA processing and protein translation. Furthermore, most genes within these pathways display an initial spike in activity within 6 to 8 hours from the diet switch, followed by a coordinated, partial down-regulation after 24 hours. We propose this represents a stereotypical response to DR, which ultimately leads to a mild but widespread inhibition of transcriptional and translational activity. Inhibition of the protein synthesis pathway has been observed in DR in other studies and has been shown to extend lifespan in several model organisms.

Keywords

Life span; dietary restriction; dietary switch; gene expression; pathway analysis; *Drosophila melanogaster*

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1. Introduction

Diet plays a central role in organism development, fitness, and in the progression of disease. Its importance is demonstrated by the fact that dietary restriction (DR) is one of the most robust interventions known to extend lifespan in many model organisms, and to delay the onset of chronic diseases (Colman et al., 2009; Houthoofd et al., 2003; Krauchunas et al., 2012; Lin et al., 2004; Mair et al., 2003; Mattison et al., 2012; Mattson, 2005). The mechanisms through which DR extends lifespan have been extensively investigated, but a clear and consistent picture of how this is achieved has not emerged yet. DR is likely to promote longevity through several complementary pathways, as demonstrated by the fact that most single gene mutations found to be required to promote longevity upon DR only contribute to a fraction of the full DR response. We have recently completed a study to identify genes more closely associated to the DR longevity response based on whole-genome transcriptomics of adult flies switched from a normal diet to a restricted diet. This intervention dramatically reduces mortality to the same level observed with a chronic DR diet within 24 to 72 hours from the diet switch (Mair et al., 2003; Mair et al., 2004; Whitaker et al., 2014). In our initial work we applied standard t-tests to identify 144 switching genes – i.e. gene whose level of expression rapidly changes to the same level observed in a cohort continuously kept on the restricted diet. These genes were primarily involved in carbohydrate and lipid metabolism. Here we apply a Gene Set Enrichment Analysis (GSEA) to identify affected pathways that would normally be missed by using a gene-centric approach. GSEA detects pathways characterized by small but coordinated transcriptional changes of many genes which, taken individually would not reach statistical significance, but as a whole constitute a significant change in the pathway metabolic flux. We identify a large number of pathways showing such coordinated responses that further extend our understanding of the molecular mechanisms underlying the effect of DR beyond the few metabolic pathways detected in our previous work.

2. Materials and methods

2.1. Transcriptional profiling of fruit flies

We analyze a microarray dataset generated to study the effect of a switch in diet on the *Drosophila melanogaster* transcriptome (accession number GSE47631). Canton-S fruit flies were reared at 25°C in vials at a density of 25 males and 25 females per vial, and randomly assigned on either Control Food (CF, 150 g/L sucrose, 150 g/L yeast, and 20 g/L agar; 240 vials total) or Restricted Food (RF, 50 g/L sucrose, 50 g/L yeast, and 20 g/L agar; 120 vials total). At adult age 40 days, 120 vials of the CF flies were switched from control food to restricted food, this group thereafter referred to as Switch food cohort (SF). Female flies from each cohort were collected 2, 4, 6, 8, 12, 18, 24, 32, 40, 48, 56, 72 hours after the diet switch. RNA was isolated from head and thorax of at least 75 fruit flies using Trizol (Invitrogen) and further purified using RNeasy columns (QIAGEN). 5 μg total RNA was used with Affymetrix One Cycle DNA conversion Kit (Cat # 900431) and all steps were carried out according to the Affymetrix manual. Briefly, first RNA was converted to double stranded cDNA followed by a clean-up step using spin columns. The double stranded cDNA was amplified in an in-vitro transcription reaction overnight at 37 °C using Affymetrix IVT

labeling kit (cat # 900449), resulting in biotin labeled cRNA. After clean-up of the labeled cRNA with spin columns, 15 μg of cRNA were fragmented using metal induced hydrolysis. 10 μg of the fragmented RNA were hybridized to Drosophila 2.0 arrays overnight at 45 °C, 60 rpm. The array was stained using Affymetrix Hybridization-Wash-Stain kit and Fluidics Script FS450_0002 on the Affymetrix 450 fluidics station and finally, the arrays were scanned using an Affymetrix 3000 G7 scanner. The Robust Multichip Analysis (RMA) Bioconductor package was used to generate expression scores in the log2 scale for each gene. qPCR was used to validate a subset of genes and time-points and we observed a consistent correlation between qPCR and microarray trends (Whitaker et al., 2014).

2.2 Gene set enrichment analysis (GSEA)

Pathway enrichment was tested by utilizing the command line version of the Gene set enrichment analysis (GSEA) software (Mootha et al., 2003; Subramanian et al., 2005). For early time points we computed the log2 fold-changes of RF vs. CF, SF vs. CF, an RF vs. SF and averaged them across al time between 2 and 24 hours from the diet switch. For late time points we repeated the same process for all times between 24 and 72 hours from the diet switch. We then ranked the average log2 fold-changes and used them as input the GSEA software with the pre-ranked option turned on. We repeated this process for each pairwise comparison. Pathways were selected as significantly enriched if the GSA reported False Discovery Rate (FDR) was smaller than 0.25. The relaxed threshold was used for selecting gene sets that were then clustered via hierarchical clustering with Pearson correlation metric using the GENE-E software package (http://www.broadinstitute.org/cancer/software/GENE-E/).

3. Results

Dietary restriction significantly extends lifespan in *Drosophila melanogaster* by reducing age-specific mortality. When a cohort of flies is switched from control food to restricted food, mortality rate rapidly drops to the same level as that observed in the cohort continuously kept on restricted food. This phenomenon has been independently observed in different strains of fruit flies at different ages, and is independent of changes in reproductive activity (Good and Tatar, 2001; Mair et al., 2003; Whitaker et al., 2014). Because the drop in mortality occurs rapidly – typically within 48 to 72 hours from the diet switch – all physiological changes responsible for the change in mortality must occur within this short time window. Hence we profiled RNA expression using microarrays at variable time intervals between 2 hours and 72 hours following the diet switch in all three cohorts (see Methods): two cohorts of flies continuously fed on control food (CF) and restricted food (RF) respectively, and a cohort of flies fed CF for 40 days and then switched to RF (Figure 1).

In our previous study we analyzed this dataset to detect individual transcripts whose level of expression changed significantly in response to the diet switch by applying a standard differential expression analysis (t-test) at the single gene level to each pairwise comparison between the three cohorts. This selective approach identified 144 switching genes (Whitaker et al., 2014), i.e. genes whose level of expression in both the SF and RF cohorts was

significantly different from that in the CF cohorts, but no significant difference was detected between the SF and RF cohorts. Among these 144 genes we identified seven specific enriched pathways: “pyruvate metabolism”, the “pentose phosphate pathway”, “fructose and mannose metabolism”, “glycolysis/gluconeogenesis”, “fatty acid metabolism”, “galactose metabolism” were enriched for switching genes down-regulated in DR, while the “folate biosynthesis” pathway was enriched for switching genes up-regulated in DR. While informative, this gene-centric approach has limited resolution to discover affected pathways, because it limits the search based on the small set of genes with relatively large changes. Accordingly, to identify pathways with small coordinated expression changes in many genes here we apply Gene Set Enrichment Analysis (GSEA) (Mootha et al., 2003; Subramanian et al., 2005). This method relies on the statistics of the position of each gene in a pathway within the rank distribution of all genes on the microarray, where ranks are computed according to the fold-change between two conditions.

We applied GSEA to perform pathway analysis in the three cohorts in the early (between 2 and 24 hours) and late (between 24 hours and 72 hours) time points after the diet switch. 102 pathways annotated in the Kyoto Encyclopedia of Genes and Genomes (KEGG) (Ogata et al., 1999) showed a significant change in at least one pair-wise comparisons between the three cohorts, in either early or late time points (Figure 2). We then defined as switching any pathway where at late time points both the RF and SW cohorts showed a significant change in the same direction with respect to the CF cohort (i.e. they were either both up-regulated or both down-regulated). The direction of change was defined as the sign of the GSEA normalized enrichment score, which measure the overall trend of the expression changes for genes in a given pathway. Of the 102 selected pathway, 23 showed switching behavior (Table 1): 4 displayed coordinated gene up-regulation in both chronic and transient DR, and 19 showed coordinated down-regulation in both conditions. Among these, only "galactose metabolism" showed switching behavior in both the early and late time points. Many pathways showed either no significant GSEA enrichment or opposite trends at the early time points. Pathways marked with a vertical green bar at the bottom of Figure 2 ("early spiking") showed up-regulation in the SF cohort with respect to the CF cohort at the early time points, and down-regulation at the late time points. Among these, 9 pathways demonstrated switching behavior: "ribosome biogenesis in eukaryotes", "RNA transport", "RNA degradation", "spliceosome", "RNA polymerase", "Aminoacyl-tRNA biosyntesis", "mRNA surveillance pathway", "ubiquitin mediated proteolysis", and "progesterone-mediated oocyte maturation". Two additional switching pathways, the "dorso-ventral axis of formation", and the “JAK-STAT signaling pathway" likewise displayed a similar pattern of expression, although hierarchical clustering did not group them within the same sub-cluster as the previously mentioned pathways.

Figures 3–5 detail the early-spiking behavior within the "ribosome biogenesis in eukaryotes" pathway. Figure 3 shows the heatmap and hierarchical clustering of the log2 fold-changes in the level of expression of all the genes in the "ribosome biogenesis in eukaryotes" pathway between the SF and CF cohorts. Most genes in this pathway follow a similar trend of up-regulation in the early time points following the diet switch, with a prominent peak between 6 and 8 hours, and a small but significant level of down-regulation in the late time points (Figure 4). In Figure 5 we mapped all genes on the microarray onto the "ribosome

biogenesis in eukaryotes" pathway (green boxes) and marked in red all genes within the boxed cluster of Figure 4. It is clear that much of the pathway is characterized by the spiking behavior previously described. Similar behaviors are displayed by other pathways such as "RNA transport" (Figure S1-3) and "RNA degradation" (Figure S4-5).

4. Discussion

When adult Drosophila are switched from an abundant to a restricted diet, most (83%) of the biochemical process pathways that dynamically switch their integrated level of gene expression do so by down-regulating their associated mRNA. Only four pathways were significantly up-regulated and included "ubiquinine and other terpenoid-quinone biosynthesis", "riboflavin metabolism" "ether lipid metabolism", and "folate metabolism". While our previous analysis (Whitaker et al., 2014), based on the sample of 144 DR-switching genes identified switching behavior in the “folate metabolism” pathway, our newly identified pathways were overlooked because too few individual genes showed large enough changes to meet the requirements of the gene-centric analysis. Among the 6 down-regulated pathways identified with a gene-centric analysis, only two pathways, “galactose metabolism” and “fatty acid metabolism”, showed a clear switching pattern in our GSEA. All of the remaining pathways, “pyruvate metabolism”, “pentose phosphate”, “fructose and mannose metabolism”, and “glycolysis/gluconeogenesis” showed at least a consistent down-regulation in the SF versus the CF cohorts, at both early and late time points.

GSEA identified 19 down-regulated switching pathways, which includes a repertoire that goes beyond the metabolic pathways identified previously. A common temporal pattern among these down-regulated switching pathways involves “early-spiking”, whereby genes in the SF cohorts are rapidly up-regulated within the first 6 to 8 hours of the diet switch and then down-regulated thereafter. Remarkably, eight of the nine "early-spiking" switching pathways observed in this GSEA are associated with transcriptional and translational activities. Four pathways – “RNA polymerase”, “splicosome”, “RNA degradation”, and “mRNA surveillance”, which ensures fidelity and quality of mRNA molecules - are associated with mRNA transcriptional and post-transcriptional regulation. The remaining four pathways – “ribosome biogenesis”, “RNA transport”, biosynthesis of a specific tRNA, aminoacyl-tRNA, and “ubiquitin-mediated proteolysis” - are associated with protein translation and degradation. Overall, these trends suggest there is a global initial spike in both transcriptional and translational activities, followed by a global down-regulation of such pathways after 24 hours from the diet switch. This pattern of behavior is shared, at least in part, by the JAK-STAT pathway which in Drosophila integrates responses to environmental cues and stress (Amoyel and Bach, 2012). The striking similarity of the patters of expression within and between the early-spiking pathways hints at a possible shared regulatory mechanism responsible for mediating the coordinated inhibition of both transcriptional and translational functions in response to DR. We propose that this stereotypic behavior is a response to DR-induced stress that ultimately leads to reduced transcriptional and translational activity.

Protein synthesis is likely to play a prominent role in the response to nutritional changes and in longevity. When nutritional resources are low or the animal is subject to stresses such as

hypoxia or DNA damage, TOR activity is repressed to inhibit cell growth and proliferation. Inhibition of TOR activity has been observed in DR animals (Stanfel et al., 2009), and gene-by-environment analyses with invertebrates have been used to verify that TOR mediates at least part of the longevity response of DR (Hansen et al., 2008; Hansen et al., 2007; Kaeberlein et al., 2005; Kapahi et al., 2004). Direct inhibition of TOR signaling has been shown to extend life span in yeast (Kaeberlein et al., 2005), worms (Vellai et al., 2003), flies (Kapahi et al., 2004), and mammals (Harrison et al., 2009). Increased life span can also be achieved by modulating the expression of genes downstream of the TOR pathway such as genes involved in ribosomal biogenesis and translation initiation factors. Steffen et al. showed that deletion of genes encoding proteins within the 60S subunit or its processing factors extend lifespan in *S. cerevisiae*, and an analogous effect can be achieved by using a small molecule inhibitor of 60S subunit biogenesis (Steffen et al., 2008). Teleman et al. (Teleman et al., 2008) have recently proposed a model in which Myc, a transcriptional regulator whose inappropriate expression is correlated with a wide array of cancers, mediates TOR's regulation on ribosomal biogenesis. However the TOR pathway was not identified as significantly affected by the diet switch neither in our GSEA analysis nor in the gene centric analysis in (Whitaker et al., 2014). We interpret this as an indication that the early effects of DR on the TOR pathway genes are posttranscriptional and do not translate into changes in mRNA levels for the first 72 hours from the diet switch.

Our study demonstrates that the combination of DR, high resolution time-course expression profiling and gene set enrichment analysis can identify coordinated pathway changes that would be missed by a gene centric approach, and generates candidate mechanisms and hypotheses that can be functionally tested in genetic experiments.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Research Highlights

- Gene set enrichment analysis of pathways affected by diet switch in Drosophila
- Several pathways display early spiking behavior followed by partial down-regulation
- Most of these pathways are associated with mRNA processing and protein translation

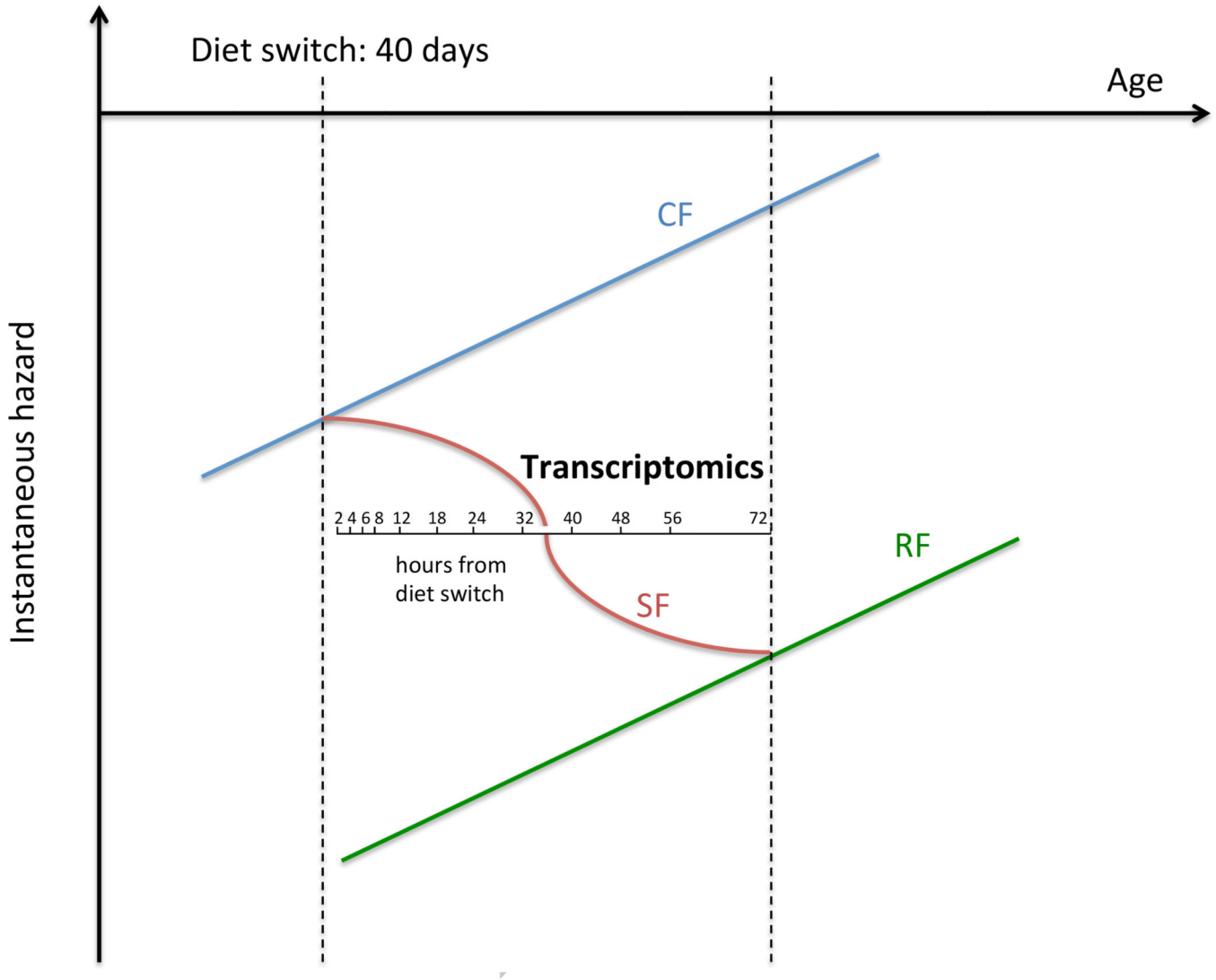

Figure 1. Experimental setup

Representation of the typical drop in age specific mortality in response to the diet switch. Transcriptional profiling was conducted on the three cohorts (CF = control food; RF = restricted food; SF = switched food) at variable time intervals between 2 and 72 hours after the diet switch.

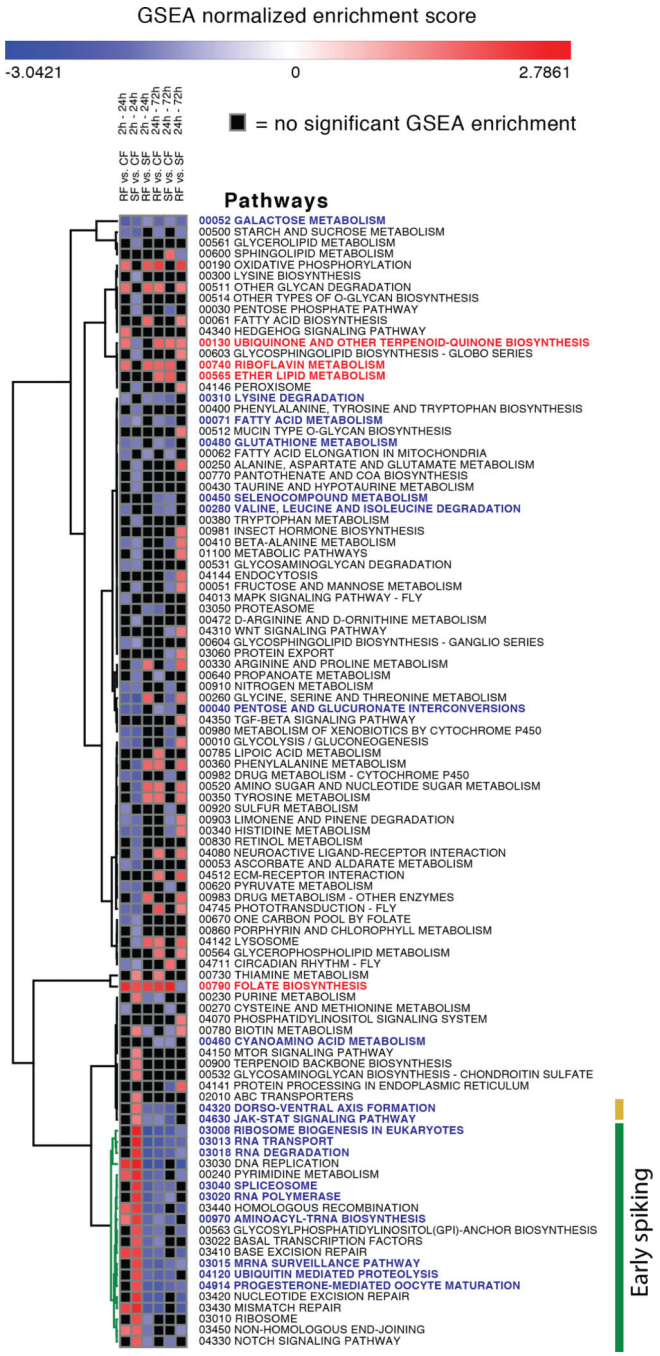

Figure 2. Heatmap of pathway expression changes in response to diet switch

KEGG pathways with a significant change (FDR < 0.05) in at least one of the pairwise comparisons between the three cohorts (CF = control food; RF = restricted food; SF = switched food) at either early time points (between 2 and 24 hours after the diet switch) or late time points (between 24 and 72 hours after the diet switch). Black squares correspond to comparisons not significant according to GSEA (FDR ≥ 0.05). Colored squares are color-coded to represent the GSEA normalized enrichment score, which indicates the direction and degree of overall change in the genes within each pathway. Pathways colored in red and

blue showed a significant switch and were respectively up- and down-regulated upon dietary restriction.

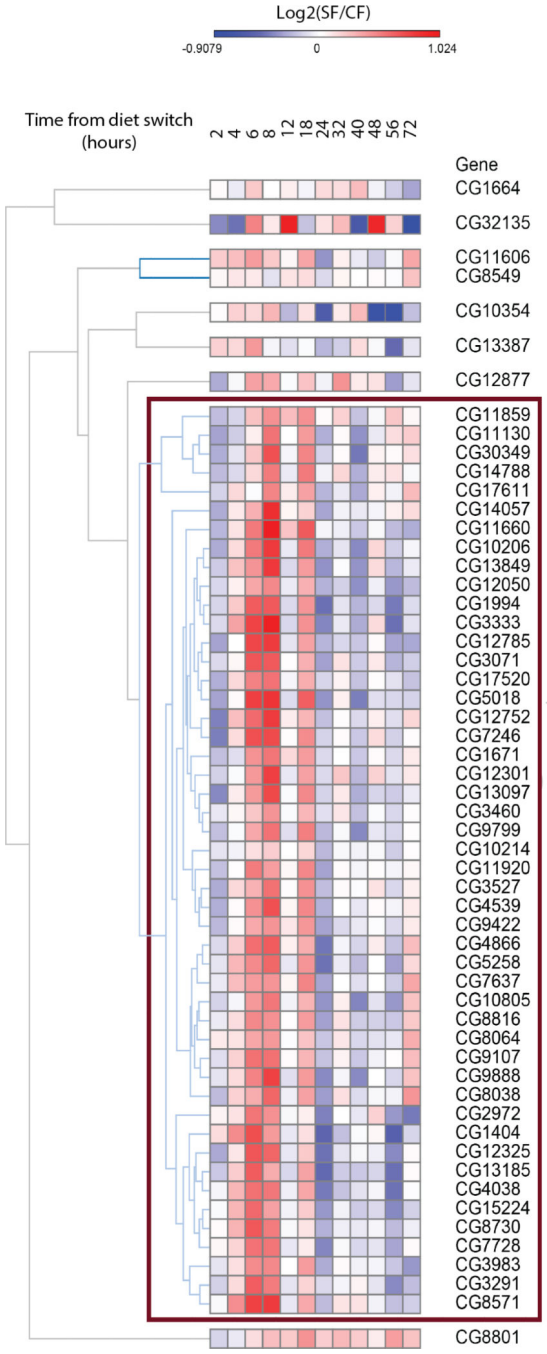

Figure 3. Clustering of genes in the "ribosome biogenesis in eukaryotes" pathway

Heatmap and hierarchical clustering of the log2 fold-changes in the level of expression of all the genes in the ribosome biogenesis pathway between the SF and CF cohorts.

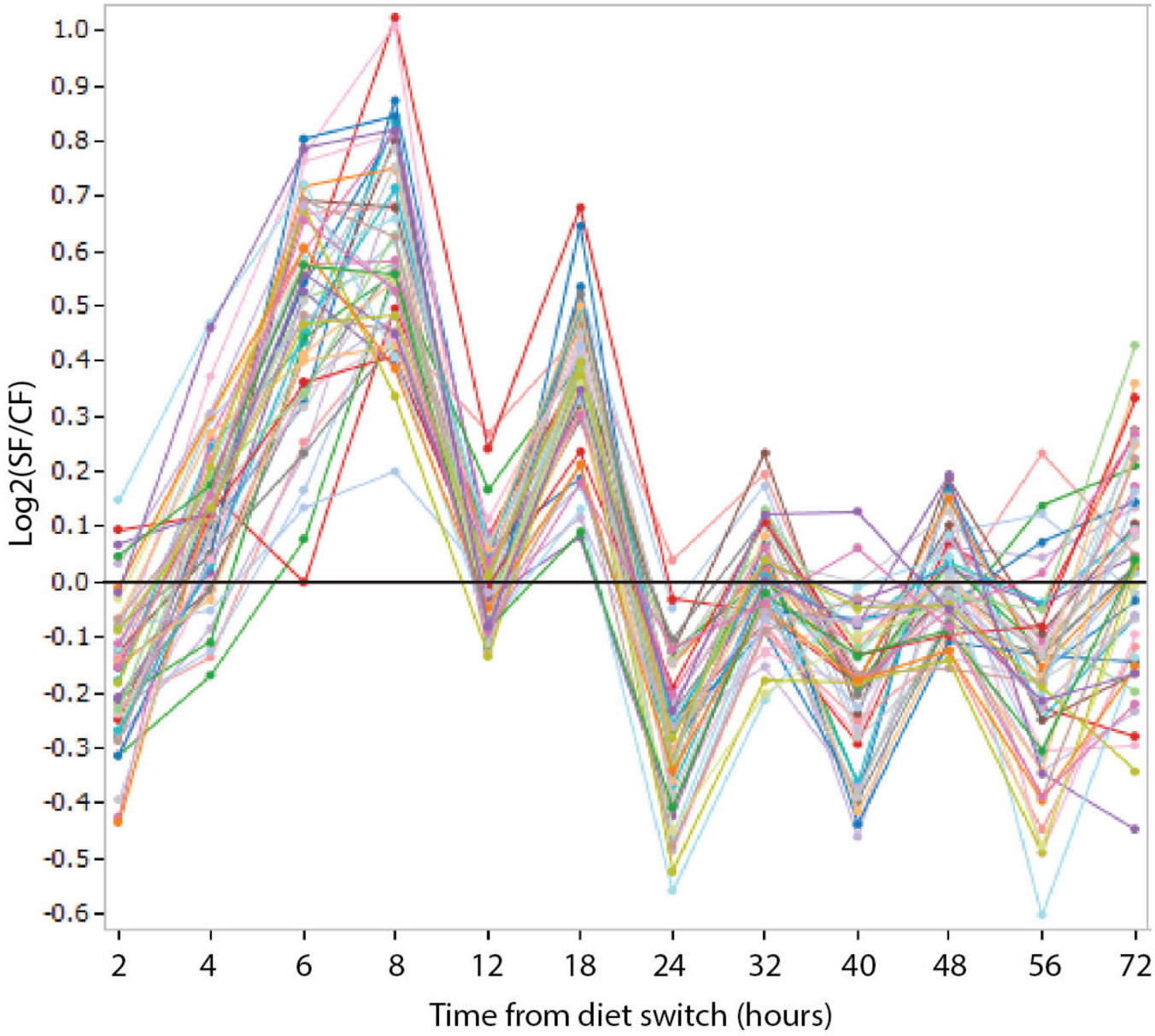

Figure 4. "Ribosome biogenesis in eukaryotes" trend over time

Log2 fold-changes in the level of gene expression between the SF and CF cohorts for genes within the boxed region of Figure 3. The vast majority of genes in this pathway follows a similar trend of up-regulation in the early time points following the diet switch and a small but significant level of down-regulation in the late time points.

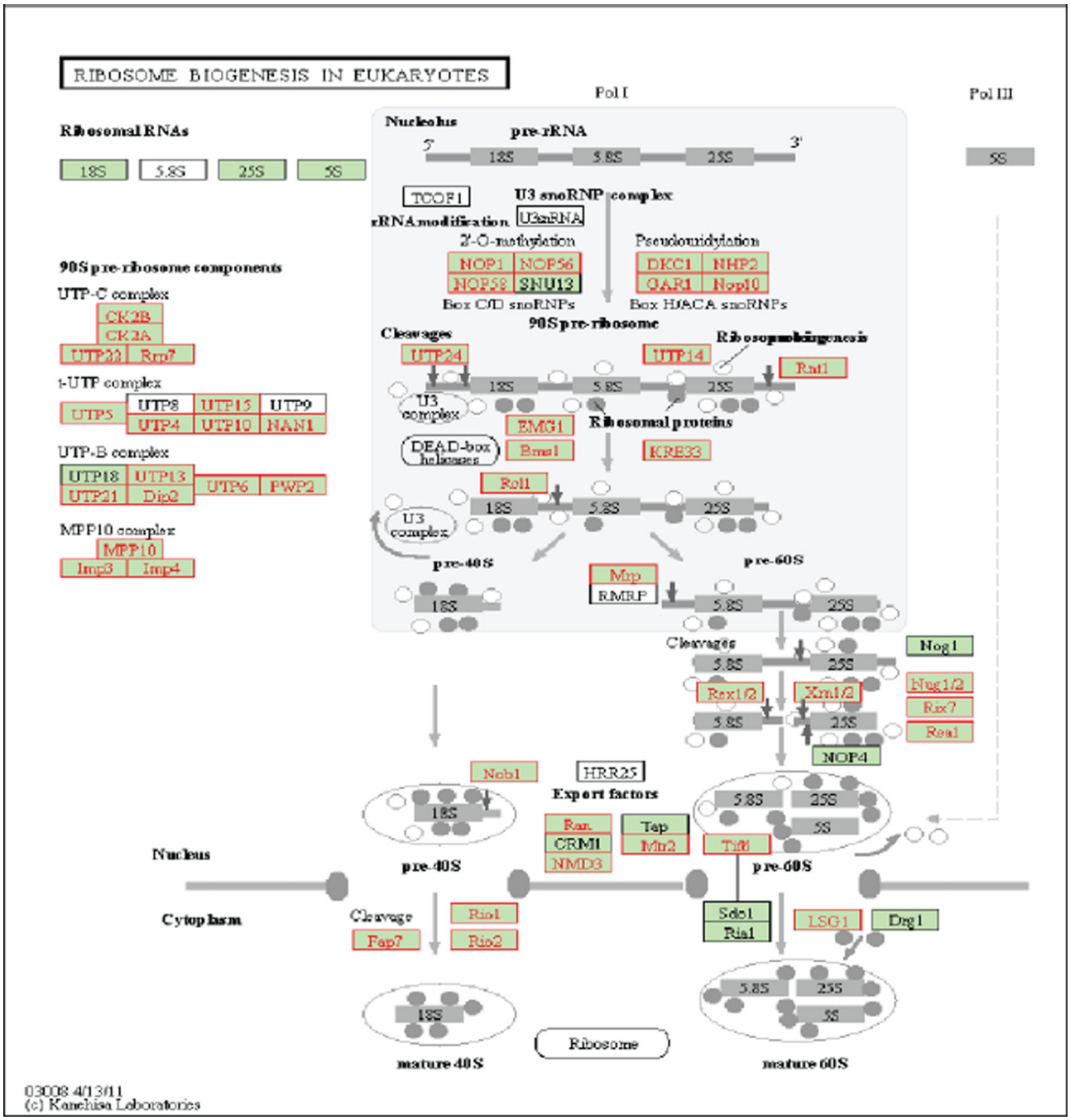

Figure 5. The "Ribosome biogenesis in eukaryotes" pathway

Genes in the ribosome biogenesis pathway on the microarray in the context of the corresponding KEGG pathway representation are marked in green. Genes marked in red showed the typical temporal trend shown in Figure 4.

Table 1

List of switching pathways

KEGG ID	Pathway
Positive GSEA normalized enrichment score	
00130	UBIQUINONE AND OTHER TERPENOID-QUINONE BIOSYNTHESIS
00740	RIBOFLAVIN METABOLISM
00565	ETHER LIPID METABOLISM
00790	FOLATE METABOLISM
Negative GSEA normalized enrichment score	
00052	GALACTOSE METABOLISM
00310	LYSINE DEGRADATION
00071	FATTY ACID METABOLISM
00480	GLUTATHIONE METABOLISM
00450	SELENOCOMPOUND METABOLISM
00280	VALINE, LEUCINE AND ISOLEUCINE DEGRADATION
00040	PENTOSE AND GLUCURONATE INTERCONVERSIONS
00460	CYANOAMINO ACID METABOLISM
04320	DORSO-VENTRAL AXIS FORMATION
04630	JAK-STAT SIGNALING PATHWAY
03008	RIBOSOME BIOGENESIS IN EUKARYOTES
03013	RNA TRANSPORT
03018	RNA DEGRADATION
03040	SPLICEOSOME
03020	RNA POLYMERASE
00970	AMINOACYL-TRNA BIOSYNTHESIS
03015	MRNA SURVEILLANCE PATHWAY
04120	UBIQUITIN MEDIATED PROTEOLYSIS
04914	PROGESTERONE-MEDIATED OOCYTE MATURATION