Metallomics

Gene expression profiling of selenophosphate synthetase 2 knockdown in Drosophila melanogaster

Journal:	*Metallomics*
Manuscript ID	MT-ART-05-2015-000134.R2
Article Type:	Paper
Date Submitted by the Author:	21-Oct-2015

Complete List of Authors:	Zhang, Yan; Institute for Nutritional Sciences, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Li, Gaopeng; Key Laboratory of Systems Biology, Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Science, Chinese Academy of Sciences, Liu, Liying; Key Laboratory of Food Safety Research, Institute for Nutritional Sciences, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, University of Chinese Academy of Sciences, Li, Ping; Institute for Nutritional Sciences, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences Chen, Luonan; Key Laboratory of Systems Biology, Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Science, Chinese Academy of Sciences, Song, Haiyun; Key Laboratory of Food Safety Research, Institute for Nutritional Sciences, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, University of Chinese Academy of Sciences,

Journal Name

ARTICLE

Gene expression profiling of selenophosphate synthetase 2 knockdown in *Drosophila melanogaster*†

Received 00th January 20xx,
Accepted 00th January 20xx

DOI: 10.1039/x0xx00000x

www.rsc.org/

Gaopeng Li,[abc‡] Liying Liu,[bc‡] Ping Li,[bc] Luonan Chen,[*a] Haiyun Song,[*bc] and Yan Zhang[*bc]

Selenium (Se) is an important trace element for many organisms and is incorporated into selenoproteins as selenocysteine (Sec). In eukaryotes, selenophosphate synthetase SPS2 is essential for Sec biosynthesis. In recent years, genetic disruptions of both Sec biosynthesis genes and selenoprotein genes have been investigated in different animal models, which provide important clues for understanding Se metabolism and function in these organisms. However, a systematic study on the knockdown of SPS2 has not been performed *in vivo*. Herein, we conducted microarray experiments to study the transcriptome of fruit flies with knockdown of SPS2 in larval and adult stages. Several hundred differentially expressed genes were identified in each stage. In spite that the expression levels of other Sec biosynthesis genes and selenoprotein genes were not significantly changed, it is possible that selenoprotein translation might be reduced without impacting mRNA level. Functional enrichment and network-based analyses revealed that although different sets of differentially expressed genes were obtained in each stage, they were both significantly enriched in carbohydrate metabolism and redox processes. Furthermore, PPI-based network clustering analysis implied that several hub genes detected in the top modules, such as Nimrod C1 and regucalcin, could be considered as key regulators that are responsible for the complex responses caused by SPS2 knockdown. Overall, our data provide new insights into the relationship between Se utilization and several fundamental cellular processes as well as diseases.

Introduction

Selenium (Se) is an essential micronutrient for a variety of organisms from bacteria to humans. Selenocysteine (Sec), the predominant biologically active form of Se, is known as the 21st naturally occurring amino acid which is incorporated into selenoproteins by decoding the UGA codon from stop signal to Sec[1–3]. This event needs a complex machinery that includes both common components and differences among the three domains of life[4–7]. In animals, the biosynthesis and insertion of Sec requires an in-frame UGA codon, a Sec insertion sequence (SECIS) element in the 3' untranslated region (UTR) of selenoprotein mRNAs, and several key factors dedicated to Sec incorporation, including selenophosphate synthetase (SPS2), Sec synthase (SecS), Sec-specific elongation factor (eEFSec), SECIS-binding protein 2 (SBP2) and other proteins[7–10]. It has been known for a long time that the major benefits of Se are mediated by selenoproteins, and the complete set of selenoproteins (or selenoproteome) has been investigated in different eukaryotes from unicellular organisms (such as algae) to humans[11–14].

Among all known Sec machinery components, SPS2 is essential for selenoprotein biosynthesis in animals[15]. This enzyme is also a selenoprotein in many Sec-utilizing eukaryotes. The main function of SPS2 is to generate the active Se donor (monoselenophosphate) for Sec biosynthesis, using selenide and ATP as substrates[16]. Orthologs of SPS2 in bacteria (called SelD) have been characterized in many species such as *Escherichia coli*[17]. In addition, a close homolog of SPS2 (named SPS1) is also detected in animals, which is thought to be unrelated to Sec biosynthesis[18,19]. Recent studies showed that SPS1 may regulate intracellular glutamine and vitamin B6 metabolism[20,21]; however, the role of this protein is still unknown.

In recent twenty years, microarray-based techniques have been widely applied to study the differential expression of genes under various laboratory and medical conditions[22–24]. Along with this, bioinformatics and experimental attempts have been made to study the complex mechanisms facilitating such differential gene expression and to identify molecular targets[25–27]. In recent decade, microarray analyses have also been used to study the cellular effect of Se supplementation (with different amount) and knockdowns of several genes

[a] *Key Laboratory of Systems Biology, Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Science, Chinese Academy of Sciences, Shanghai, China. Fax: 86-21-54920120; Tel: 86-21-54920100; E-mail: lnchen@sibs.ac.cn*
[b] *Key Laboratory of Food Safety Research, Institute for Nutritional Sciences, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, University of Chinese Academy of Sciences, Shanghai, China. Fax: 86-21-54920108, 86-21-54920098; Tel: 86-21-54921124, 86-21-54920098; E-mail: yanzhang01@sibs.ac.cn, hysong@sibs.ac.cn*
[c] *Key Laboratory of Food Safety Risk Assessment Ministry of Health, Beijing, China.*
†Electronic Supplementary Information (ESI) available: GO terms enriched by different sets of DEGs at each stage and PPI network at larval and adult stage. See DOI: 10.1039/x0xx00000x
‡These authors contributed equally to this work.

either involved in Sec biosynthesis or encoding selenoproteins using different cell lines or animal models[28–31]. However, a systematic analysis of the knockdown of SPS2 has not been reported *in vivo*.

In this study, we used *Drosophila melanogaster* to study the gene expression profiles of the knockdown of SPS2 in larval and adult stages. Hundreds of differentially expressed genes (DEGs) were identified in one or both stages; however, the expression levels of selenoprotein and other Sec biosynthesis genes did not change significantly. Based on gene functional enrichment and network analyses, we found that SPS2 knockdown could influence a number of genes involved in carbohydrate or vitamin metabolism and cellular redox status. For the first time, our results demonstrate that SPS2 may play an important role in regulating basic metabolic pathways as well as redox homeostasis, probably via some of the key regulators suggested in this study.

Materials and Methods

Fly strains and culture

Fruit flies were raised at 25℃ and 60% relative humidity on cornmeal–yeast food. The SPS2 mutant strain, which contained a P element transposon insertion in the 5th exon of the SPS2 gene and caused a hypomorphic allele, was obtained from the Bloomington stock center (stock # 13684). This mutant strain was back-crossed with w1118 control flies for six generations to equilibrate the genetic background.

RNA extraction and purification

Total RNA was extracted using TRIZOL Reagent (Cat#15596-018, Life technologies, Carlsbad, CA, US) following the manufacturer's instructions and checked for a RIN number to inspect RNA integrity by an Agilent Bioanalyzer 2100 (Agilent technologies, Santa Clara, CA, US). Qualified total RNA was further purified by RNeasy micro kit (Cat#74004, QIAGEN, GmBH, Germany) and RNase-Free DNase Set (Cat#79254, QIAGEN, GmBH, Germany).

RNA amplification and labeling

Total RNA from fruit flies were amplified, labeled and purified by using GeneChip 3' IVT Express Kit (Cat#901229, Affymetrix, Santa Clara, CA, US) following the manufacturer's instructions to obtain biotin labeled cRNA.

Array hybridization and data acquisition

Array hybridization and wash was performed using GeneChip Hybridization, Wash and Stain Kit (Cat#900720, Affymetrix, Santa Clara, CA, US) in Hybridization Oven 645 (Cat#00-0331-220V, Affymetrix, Santa Clara, CA, US) and Fluidics Station 450 (Cat#00-0079, Affymetrix, Santa Clara, CA, US) following the manufacturer's instructions. Slides were scanned the by GeneChip Scanner 3000 (Cat#00-00212, Affymetrix, Santa Clara, CA, US) and Command Console Software 3.1 (Affymetrix, Santa Clara, CA, US) with default settings.

Identification of differentially expressed genes

Raw data were normalized by MAS 5.0 algorithm, Gene Spring Software 11.0 (Agilent technologies, Santa Clara, CA, US). Probes corresponding to more than one Entrez gene IDs were discarded. A gene was considered as DEG between the two groups of samples being compared if (1) p-value <0.05 (t-test); (2) the fold change of gene expression intensities is $\geqslant 2$ or $\leqslant 0.5$; (3) Considering that there are three types of flag/call values of each examined probe (A: absent; P: present; M: marginal), the values of all three replicates of at least one group should be non-A values. For those genes with multiple probes, only the probe with the highest number of P signs was used to represent the expression level of the corresponding gene.

The gene expression values were also transformed into Z-scores based on the average levels of corresponding genes. Hierarchical clustering (based on Pearson's correlation coefficient, PCC) and heat map generation were carried out using the heatmap.2 function from R package gplots.

Gene ontology and pathway enrichment analyses

The R packages GOstats[32] and org.Dm.eg.db from Bioconductor (http://www.bioconductor.org/) was used for gene ontology (GO) enrichment analysis, which uses hypergeometric test for association of categories and genes. In GOstats, the "conditional" option was set to true, and "pvalueCutoff" set to 0.05. We generated false discovery rate (FDR) using Benjamini-Hochberg method for those GO terms containing at least one DEG. The significance cutoff of FDR was set to 0.1.

The Kyoto Encyclopedia of Genes and Genomes (KEGG) database was used for pathway enrichment analysis. Genes involved in all known pathways of *D. melanogaster* were first downloaded from KEGG website (http://www.kegg.jp/kegg/rest/keggapi.html). After examining the presence of those genes in the microarray dataset, a total of 125 pathways with 2368 genes remained. The p-value of the enrichment of DEGs in each pathway was calculated using hypergeometric test, FDR was also calculated (cutoff: 0.1).

Network-based analysis

To investigate protein-protein interaction (PPI) network in different stages, we first retrieved the PPI information of *D. melanogaster* from BioGRID (a database of protein and genetic interactions)[33], which contains 8209 genes (nodes) with 39104 interactions (edges). Then we built a DEG-based PPI network based on the following criteria: (1) all DEGs and edges between DEGs were remained; (2) all edges that do not connect with any of DEGs were removed; (3) for those edges between DEGs and other genes, we calculated PCC of the two genes connected by each edge for both SPS2 knockdown and

control samples. If the difference of the two PCCs (ΔPCC) is greater than an empirical value ($|\Delta PCC| \geq 0.8$ as defined here), both the edge (named differentially expressed edge) and the two corresponding genes were retained.

We used the Markov clustering algorithm (MCL) for network clustering and module division via Cytoscape plugin clusterMaker with default parameters (inflation value =2.5)[34]. We then performed GO analysis and identified hub genes within top modules for each stage.

Validation by quantitative real-time PCR

Eight to ten third instar larvae were quickly frozen in liquid nitrogen and total RNA was extracted with Trizol Reagent (Invitrogen) following standard protocol. 1 μg RNA was used for reverse transcription (ReverTra Ace qPCR RT Kit, TOYOBO). Quantitative PCR was performed with the SYBR Green Master Mix (TOYOBO) on ABI StepOne Real Time PCR System. The following primers were used in the measurement of SPS2 transcripts: 5'-CGGGTGAAGTTGCAAAATCC-3'/5'-ATAGTCCTTGTGCCGCTTGA-3', which are derived from exon 1 and exon 2 of SPS2, respectively. Expression levels of actin and tubulin were used as internal controls for normalization.

Western blot

Protein samples were separated by SDS-PAGE, transferred to PVDF membrane, and immunoblotted with rabbit anti-SPS2[35] and mouse anti-Tubulin (T6199, Sigma-Aldrich) antibodies. HRP-conjugated goat anti-mouse and anti-rabbit secondary antibodies were from Santa Cruz.

Results

General analysis of differentially expressed genes in larval and adult stages

In the current study, SPS2 knockdown flies display no significant phenotype under standard laboratory conditions. Genes expression profiles of 12 fruit fly samples corresponding to larval and adult stages of SPS2 knockdown and control groups (3 replicates for each group) were analyzed using Affymetrix Drosophila genome microarray which contains 12,460 genes. The SPS2 knockdown efficiency (approximately 80-90%, derived from the microarray data) was shown in Fig. 1, which was verified by agarose gel electrophoresis of its reverse transcribed PCR product in flies (Fig. 2A). Western blot analysis revealed that expression of SPS2 protein was also significantly decreased in the SPS2 knockdown fruit flies (Fig. 2B). We checked the expression levels of other Sec biosynthesis genes (SecS, eEFSec and SBP2) and all selenoprotein genes excluding SPS2 (selenoprotein K (SelK) and BthD). No significant change could be found for any of them in the microarray data (Fig. 1) . Considering that the product of SPS2, selenophosphate, is required during the translation of selenoprotein genes and the fact that the function of SPS2 proteins is significantly inhibited, it is possible that the expression levels of selenoproteins in fruit flies might be reduced.

A comparison of DEGs between mutant and control samples in different stages is shown in Fig. 3. First, 409 and 492 DEGs were identified in larval and adult stages, respectively, with an overlap of 125 genes. Further investigation of the up- and down-regulated DEGs revealed that the sizes of the two sets were almost same in the larval stage (203 up- and 206 down-regulated genes, respectively). On the other hand, the majority of DEGs in adult stage were down-regulated (151 up- and 341 down-regulated genes, respectively). The top twenty up- and down-regulated DEGs in each stage are shown in Table 1 and Table 2.

Interestingly, almost all DEGs detected in both stages (119 out of 125 genes) showed consistent patterns (51 and 68 up- and down-regulated genes, respectively). Only 6 DEGs showed opposite expression patterns in different stages. For example, genes for larval serum protein 2 (Lsp2) and a cytochrome P450 family member Cyp6g1 appeared to be up-regulated in the adult stage but down-regulated in the larval stage, whereas 3-hydroxyacyl-CoA dehydrogenase had an opposite trend.

To investigate genes that might have co-regulated expression patterns, we carried out cluster analysis for all detected DEGs. Gene expression profiles were classified by hierarchical clustering approach and displayed in a correlation heat map (Fig. 4). A fraction of the map corresponding to genes that may have expression patterns closely related to SPS2 is also shown. Some of these genes include pigs (pickled eggs), CG10513 (ecdysteroid kinase), CG8768 (TIGR01777 family protein) and CG32850 (RING-finger protein). Further studies are needed to investigate the possible relationship between these genes and SPS2 or Se metabolism.

GO and KEGG pathway analysis

To extrapolate the biological processes that DEGs are involved in, the R package GOstats was used to perform GO term enrichment analysis. Our functional analysis revealed that these DEGs may cover several important biological processes and functions. We identified 2 biological process (BP) terms and 10 molecular function (MF) terms significantly enriched in the larval stage, as well as 1 BP and 9 MF terms in the adult stage, with an overlap of 5 MF terms for both stages (Table 3). Almost all overlapped GO terms are related to electron transport and redox activity (such as GO:0020037, GO:0005506, GO:0009055 and GO:0016705). Besides, several development-related terms (e.g., GO:0040003, GO:0008010, GO:0042302 and GO:0045735) were found to be particularly enriched in larval stage whereas terms related to protein degradation (GO:0006508, GO:0004252 and GO:0008233) and metabolism (GO:0008970 and GO:0004558) in adult stage. These results suggested that SPS2 may play a role in the key cellular processes occurring in different stages.

Considering that DEGs corresponding to the same GO term may have different expression patterns, we further performed

GO analysis for up- and down-regulated genes separately (Table S1 †). Interestingly, we found that most of the GO terms identified above were also significantly enriched by down-regulated genes but not up-regulated genes in both stages. For example, in the adult stage, the only BP and 7 out of 11 MF terms enriched by down-regulated genes were the same as those enriched by all DEGs. On the other hand, no common GO term could be found to be enriched for both up- and down-regulated genes in each stage, implying that genes with different functions might be regulated in different patterns.

To date, only a limited number of genes have been annotated in *D. melanogaster* pathways in the KEGG dataset. Considering that pathways containing too few genes could not yet provide sufficient information for statistical analysis, only those with at least 10 genes were remained in this study. A total of 10 and 11 KEGG pathways were significantly enriched in the larval and adult stages respectively, with an overlap of 8 pathways (Table 4). The majority of these pathways are involved in carbohydrate, vitamins and drug metabolism. For example, in the adult stage, the top three enriched pathways are all carbohydrate-related pathways (dme00500: starch and sucrose metabolism; dme00040: pentose and glucuronate interconversions; dme00052: galactose metabolism). Our results may suggest a strong interaction between SPS2 and these basic metabolic processes.

Considering that the same pathway could be enriched by different genes, we also checked DEGs in each overlapped pathway between the two stages. Our data revealed that each stage contained many specific genes (Table 5). Thus, it appears that although the knockdown of SPS2 may lead to different sets of DEGs in different stages, the general effect might be similar at the pathway level.

Protein-protein interaction network analysis

Interactions between proteins are important for the majority of biological functions[36]. In this study, we used the *D. melanogaster* PPI information derived from BioGRID database to build a DEG-based PPI network (see Materials and Methods). Fig. S1 and S2 † show the whole PPI networks in larval and adult stages.

In the larval stage, the PPI network induced by SPS2 knockdown consisted of 1102 nodes (including 233 isolated nodes) and 815 edges (Fig. S1 †). After clustering analysis, 101 modules containing at least three genes were obtained. To further analyze whether genes in these modules are significantly enriched in functional groups, we carried out GO analysis for large modules containing more than 20 genes (3 modules in total, Fig. 5A). By ranking these modules based on the number of genes they have, we found that Module 1 and Module 3 were both enriched in GO:0005811 (lipid particle), with p-values 7.24E-09 and 7.86E-05, respectively. It has been known for a long time that lipid storage particles or droplets are universal organelles essential for the cellular and organismal energy homeostasis and reactive oxygen species (ROS) production[37,38]. In addition, several redox-related terms (such as GO:0055114, oxidation-reduction process; GO:0016491, oxidoreductase activity) were also enriched in Module 3. In contrast, Only one GO term (GO:0001709, cell fate determination) could be found for Module 2.

In the adult stage, the PPI network contains 1112 nodes (including 295 isolated nodes) and 741 edges (Fig. S2 †). A total of 102 modules containing at least three genes were identified. Within the three modules containing over 20 genes, no or very few GO terms could be enriched for Module 1 and 3, respectively. However, Module 2 was found to be enriched in several redox-related terms (such as GO:0045454, cell redox homeostasis; GO:0016667, oxidoreductase activity, acting on a sulfur group of donors; GO:0016614, oxidoreductase activity, acting on CH-OH group of donors) (Fig. 5B). Thus, our PPI-based network analysis was generally consistent with the above results that SPS2 might play a key role in cellular redox homeostasis.

Identification of key regulators involved in SPS2-related processes

PPI network analysis revealed that several genes involved in different biological process are hub genes in larval and/or adult stages. Thus, they could be considered as key regulators in response to SPS2 knockdown. Through careful analysis of the modules, gene expression levels and literature investigation, we have found some candidate key regulators, such as Nimrod C1 (NimC1), regucalcin and succinyl-CoA synthetase beta subunit (Sucb), which are described in the following part.

NimC1 (Entrez Gene ID: 34816) is a putative phagocytosis receptor, which belongs to the Nimrod gene family[39,40]. The NimC1 molecule is being used as a marker for professional phagocytes, the plasmatocytes, in *D. melanogaster*[41]. However, the relationship between NimC1 and Se metabolism has never been reported. In the SPS2 knockdown flies, the expression level of NimC1 is significantly up-regulated in both stages (Fig. 5A, larval stage: the hub node of Module 2, fold change is 4.35; Fig. 5B, adult stage: the hub of Module 1, fold change is 4.50), implying that knockdown of SPS2 in flies might have a long-term effect to increase the amount of plasmatocytes and activate the fly immune responses.

Regucalcin (Entrez Gene ID: 32165) is a member of the Senescence Marker Protein-30(SMP-30)/Gluconolactonase/LRE-like family. In mammals, it plays a multifunctional role in cell regulation; maintaining of intracellular calcium homeostasis and suppressing of signal transduction, translational protein synthesis, nuclear DNA and RNA synthesis, proliferation, and apoptosis in many cell types[42]. In addition, regucalcin may play a pathophysiological role in metabolic and neuronal diseases[43]. It has been reported that the cold acclimation gene (Dca) involved in cold adaptation in *D. melanogaster* arose by duplication of the ancestral

regucalcin gene[44]. In addition, sequence similarity and expression profile have suggested that Drosophila regucalcin and the anterior fat body protein gene (AFP) of *Sarcophaga peregrina* and *Calliphora vicina* are actually orthologous genes[44]. In this study, the expression level of regucalcin was significantly down-regulated in the adult stage (Fig. 5B; the hub node of Module 2, fold change is 0.36). It has been reported that the expression of regucalcin is decreased in the liver of rats with type I diabetes induced by streptozotocin administration *in vivo*[45]. Overexpression of endogenous regucalcin could stimulate glucose utilization and lipid production in liver cells with glucose supplementation *in vitro*[46]. Considering that Se utilization and homeostasis may play a role in diabetogenesis and some other metabolic disorders[47], the regucalcin could become a potential link between Se metabolism and those metabolic disorders/diseases.

Sucb (Entrez Gene ID: 44001) is one of the two subunits of succinyl-CoA synthetase (or succinate-CoA ligase) that catalyzes the reversible reaction of succinyl-CoA and ADP or GDP to succinate and ATP or GTP, which plays a key role in tricarboxylic acid (TCA) cycle[48,49]. This enzyme is essential for energy production for all animals and its substrate specificity is determined by Sucb[50]. Here, the expression level of Sucb was elevated in larval stage (Fig. 5A; the hub node of Module 1, fold change is 2.09). It has been reported that Se supplementation could revert the activities of several enzymes involved in TCA cycle (such as isocitrate dehydrogenase, succinate dehydrogenase and malate dehydrogenase) that was decreased in hepatoma[51]. Thus, Sucb might be a new mediator between Se utilization and energy metabolism.

The expression levels of some other genes were also found to be significantly different in both stages although they were not considered as hub genes in the PPI network, such as ras-related protein Rala (fold change is 40.37 and 73.12 in larval and adult stages, respectively) and alpha-esterase-2 (fold change is 123.24 and 17.59 in larval and adult stages, respectively). These genes are also good candidates for further understanding SPS2-related cellular processes in fruit fly.

Validation of expression by quantitative PCR

Quantitative PCR (qPCR) was carried out to measure the expression levels of some of the important genes described above, including candidate key regulators (Fig. 6). All tested genes showed the same pattern of expression as that obtained from microarray experiments.

Discussion

It has been known that SPS2, but not SPS1, can synthesize selenophosphate which is the sole donor for Sec biosynthesis from selenite in mammals[15,52]. Previous knockdown of SPS2 gene in NIH3T3 cells (mouse fibroblast cell line) showed that the reduction in the expression of SPS2 could inhibit the expression levels of some selenoproteins such as thioredoxin reductase 1, glutathione peroxidase 1 and selenoprotein T[15]. Compared to mouse that contains 24 selenoproteins[11], only three selenoproteins are present in *D. melanogaster*: SPS2, SelK and BthD[53]. Although it is unclear whether inhibition of SPS2 expression in fruit fly may significantly influence the expression of the other two selenoproteins, it is possible that selenoprotein translation is reduced and this could occur without impacting mRNA levels. In this study, we focused on mRNA expression profiles in response to SPS2 knockdown in flies. It was previously reported that the Drosophila SPS2 loss-of-function mutation could cause aberrant cell proliferation and differentiation patterns in the brain and imaginal discs, suggesting that SPS2 is required for development and cell proliferation[54]. On the other hand, no difference in life span could be observed between wild type and heterozygous flies for SPS2 mutation under normal conditions[55]. Thus, system-level studies on SPS2-related networks would allow us for a better understanding of the complicated function of SPS2 in fruit flies.

In the present study, we carried out a microarray-based analysis to investigate the global impact of SPS2 on fly development and physiology. Although the role of SPS2 is to synthesize selenophosphate (an essential substrate for Sec synthesis), the expression levels of other Sec-related genes and selenoprotein genes did not alter significantly in both larval and adult stages. One possible reason is that SPS2 mutant fly is still able to express SPS2 at low level, which might be enough for maintaining Sec utilization in flies due to few selenoprotein genes.

Different DEGs were identified for larval and adult stages, which may be involved in SPS2-associated cellular processes in different developmental phases. The majority of them appeared to be stage-specific. On the other hand, almost all of the DEGs identified in both stages have consistent expression patterns, implying a long-term and stable regulation of these genes by SPS2. GO term analysis of DEGs in different stages revealed that they were functionally enriched in redox, developmental and/or cellular metabolic processes, especially for down-regulated genes. It appears that down-regulated genes may play a more important role for the general response induced by SPS2 knockdown in flies.

KEGG pathway analysis of DEGs revealed that only a limited number of known fly pathways could be enriched. It is interesting that although the DEGs in different stages are quite different, they could be enriched in several common pathways that are associated with carbohydrate, vitamins and drug metabolism. However, compared to human and mouse, only a few pathways are known for *D. melanogaster* in current KEGG database. In the future, it would be important to identify additional processes or pathways that could be influenced by SPS2.

We also built the DEG-based PPI network to further investigate the global effect of SPS2 knockdown. Using network clustering approaches, we identified important modules for each stage. Analysis of the major function of the top modules confirmed that SPS2 knockdown may significantly influence cellular redox homeostasis, which is basically consistent with previous observation that SPS2 is required for oxidative stress protection[56]. More importantly, PPI network analysis also suggested several candidate key regulators (hub genes) in one or both stages, including NimC1, regucalcin and Sucb. The prediction of the relationship between SPS2 and these genes may provide a new window for understanding the diverse and complex roles of SPS2 in flies.

It should be noted that SPS2 is essential for selenoprotein synthesis and that the majority of selenoproteins are involved in a variety of redox processes[4,12–14]. Although the functions of SelK and BthD in flies are not clear, some of the effects observed in this study (such as redox-related gene enrichment) might be related to these selenoproteins. Further effort is needed to verify such possibility and to investigate new roles of other selenoprotein biosynthesis genes.

Conclusions

In this study, we analyzed gene expression profiles of SPS2-knockdown fruit flies in both larval and adult stages. Several hundred of DEGs were identified for each stage. GO and pathway analyses of DEGs suggested that they are significantly involved in several basic metabolic pathways as well as redox process in both stages. Further DEG-based PPI network analysis suggested important modules and possible key regulators that are involved in SPS2-related cellular responses for each stage, which may provide insights into the relationship between Se metabolism and other important cellular metabolism and processes.

Acknowledgements

We thank Dr. Byeong Jae Lee (Seoul National University, Korea) for providing rabbit anti-SPS2 antibody. This work was supported by the National Natural Science Foundation of China (31171233, 31322039, 31371493), and a grant from Chinese Academy of Sciences (CAS) (2012OHTP10).

References

1 A. Böck, K. Forchhammer, J. Heider, W. Leinfelder, G. Sawers, B. Veprek and F. Zinoni, *Mol. Microbiol.*, 1991, **5**, 515–520.
2 T. C. Stadtman, *Annu. Rev. Biochem.*, 1996, **65**, 83–100.
3 J. Donovan and P. R. Copeland, *Antioxid. Redox Signal.*, 2010, **12**, 881–892.
4 D. L. Hatfield and V. N. Gladyshev, *Mol. Cell. Biol.*, 2002, **22**, 3565–3576.
5 P. R. Hoffmann and M. J. Berry, *Thyroid*, 2005, **15**, 769–775.
6 C. Allmang and A. Krol, *Biochimie*, 2006, **88**, 1561–1571.
7 J. E. Squires and M. J. Berry, *IUBMB Life*, 2008, **60**, 232–235.
8 C. Allmang, L. Wurth and A. Krol, *Biochim. Biophys. Acta*, 2009, **1790**, 1415–1423.
9 J. Donovan and P. R. Copeland, *BMC Evol. Biol.*, 2009, **9**, 229.
10 R. L. Schmidt and M. Simonović, *Croat. Med. J.*, 2012, **53**, 535–550.
11 G. V. Kryukov, S. Castellano, S. V. Novoselov, A. V. Lobanov, O. Zehtab, R. Guigó and V. N. Gladyshev, *Science*, 2003, **300**, 1439–1443.
12 A. V. Lobanov, D. E. Fomenko, Y. Zhang, A. Sengupta, D. L. Hatfield and V. N. Gladyshev, *Genome Biol.*, 2007, **8**, R198.
13 A. V. Lobanov, D. L. Hatfield and V. N. Gladyshev, *Biochim. Biophys. Acta*, 2009, **1790**, 1424–1428.
14 M. Mariotti, P. G. Ridge, Y. Zhang, A. V. Lobanov, T. H. Pringle, R. Guigo, D. L. Hatfield and V. N. Gladyshev, *PLoS ONE*, 2012, **7**, e33066.
15 X.-M. Xu, B. A. Carlson, R. Irons, H. Mix, N. Zhong, V. N. Gladyshev and D. L. Hatfield, *Biochem. J.*, 2007, **404**, 115–120.
16 X.-M. Xu, B. A. Carlson, Y. Zhang, H. Mix, G. V. Kryukov, R. S. Glass, M. J. Berry, V. N. Gladyshev and D. L. Hatfield, *Biol Trace Elem Res*, 2007, **119**, 234–241.
17 W. Leinfelder, K. Forchhammer, B. Veprek, E. Zehelein and A. Böck, *Proc. Natl. Acad. Sci. U.S.A.*, 1990, **87**, 543–547.
18 M. J. Guimarães, D. Peterson, A. Vicari, B. G. Cocks, N. G. Copeland, D. J. Gilbert, N. A. Jenkins, D. A. Ferrick, R. A. Kastelein, J. F. Bazan and A. Zlotnik, *Proc. Natl. Acad. Sci. U.S.A.*, 1996, **93**, 15086–15091.
19 A. V. Lobanov, D. L. Hatfield and V. N. Gladyshev, *Protein Sci.*, 2008, **17**, 176–182.
20 M. S. Shim, J. Y. Kim, H. K. Jung, K. H. Lee, X.-M. Xu, B. A. Carlson, K. W. Kim, I. Y. Kim, D. L. Hatfield and B. J. Lee, *J. Biol. Chem.*, 2009, **284**, 32881–32894.
21 K. H. Lee, M. S. Shim, J. Y. Kim, H. K. Jung, E. Lee, B. A. Carlson, X.-M. Xu, J. M. Park, D. L. Hatfield, T. Park and B. J. Lee, *BMC Genomics*, 2011, **12**, 426.
22 S. Wang, T. B. Sim, Y.-S. Kim and Y.-T. Chang, *Curr Opin Chem Biol*, 2004, **8**, 371–377.
23 C. Virtanen and J. Woodgett, *Methods Mol. Med.*, 2008, **141**, 87–113.
24 A. Masotti, L. Da Sacco, G. F. Bottazzo and A. Alisi, *Crit Rev Food Sci Nutr*, 2010, **50**, 693–698.
25 A. Y. Sivachenko, A. Yuryev, N. Daraselia and I. Mazo, *J Bioinform Comput Biol*, 2007, **5**, 429–456.
26 M. Provenzano and S. Mocellin, *Adv. Exp. Med. Biol.*, 2007, **593**, 66–73.
27 K. Owzar, W. T. Barry and S.-H. Jung, *Clin Transl Sci*, 2011, **4**, 466–477.
28 L. A. Hooven, J. Butler, L. W. Ream and P. D. Whanger, *Biol Trace Elem Res*, 2006, **109**, 173–179.
29 R. A. Sunde and A. M. Raines, *Adv Nutr*, 2011, **2**, 138–150.
30 S. B. Ben, Q. Y. Wang, L. Xia, J. Z. Xia, J. Cui, J. Wang, F. Yang, H. Bai, M. S. Shim, B. J. Lee, L. G. Sun and C. L. Chen, *Biochemistry Mosc.*, 2011, **76**, 1030–1036.
31 P. A. Tsuji, B. A. Carlson, S. Naranjo-Suarez, M.-H. Yoo, X.-M. Xu, D. E. Fomenko, V. N. Gladyshev, D. L. Hatfield and C. D. Davis, *PLoS ONE*, 2012, **7**, e50574.
32 S. Falcon and R. Gentleman, *Bioinformatics*, 2007, **23**, 257–258.
33 A. Chatr-Aryamontri, B.-J. Breitkreutz, S. Heinicke, L. Boucher, A. Winter, C. Stark, J. Nixon, L. Ramage, N. Kolas, L. O'Donnell, T. Reguly, A. Breitkreutz, A. Sellam, D. Chen, C. Chang, J. Rust, M. Livstone, R. Oughtred, K. Dolinski and M. Tyers, *Nucleic Acids Res.*, 2013, **41**, D816–823.

34 J. H. Morris, L. Apeltsin, A. M. Newman, J. Baumbach, T. Wittkop, G. Su, G. D. Bader and T. E. Ferrin, *BMC Bioinformatics*, 2011, **12**, 436.
35 J. S. Jin, S. Baek, H. Lee, M. Y. Oh, Y. E. Koo, M. S. Shim, S. Y. Kwon, I. Jeon, S. Y. Park, K. Baek, M. A. Yoo, D. L. Hatfield and B. J. Lee, *Nucleic Acids Res.*, 2004, **32**, 2482–2493.
36 G. C. K. W. Koh, P. Porras, B. Aranda, H. Hermjakob and S. E. Orchard, *J. Proteome Res.*, 2012, **11**, 2014–2031.
37 M. Beller, D. Riedel, L. Jänsch, G. Dieterich, J. Wehland, H. Jäckle and R. P. Kühnlein, *Mol Cell Proteomics*, 2006, **5**, 1082–1094.
38 L. Liu, K. Zhang, H. Sandoval, S. Yamamoto, M. Jaiswal, E. Sanz, Z. Li, J. Hui, B. H. Graham, A. Quintana and H. J. Bellen, *Cell*, 2015, **160**, 177–190.
39 E. Kurucz, R. Márkus, J. Zsámboki, K. Folkl-Medzihradszky, Z. Darula, P. Vilmos, A. Udvardy, I. Krausz, T. Lukacsovich, E. Gateff, C.-J. Zettervall, D. Hultmark and I. Andó, *Curr. Biol.*, 2007, **17**, 649–654.
40 T. Y. Estévez-Lao and J. F. Hillyer, *Insect Biochem. Mol. Biol.*, 2014, **44**, 12–22.
41 V. Honti, G. Cinege, G. Csordás, E. Kurucz, J. Zsámboki, C. J. Evans, U. Banerjee and I. Andó, *Fly (Austin)*, 2013, **7**, 263–266.
42 M. Yamaguchi, *Int. J. Mol. Med.*, 2005, **15**, 371–389.
43 M. Yamaguchi and T. Murata, *Metab. Clin. Exp.*, 2013, **62**, 1045–1051.
44 C. E. Arboleda-Bustos and C. Segarra, *Mol. Biol. Evol.*, 2011, **28**, 2185–2195.
45 M. Isogai, H. Kurota and M. Yamaguchi, *Mol. Cell. Biochem.*, 1997, **168**, 67–72.
46 C. Nakashima and M. Yamaguchi, *J. Cell. Biochem.*, 2006, **99**, 1582–1592.
47 J. Zhou, K. Huang and X. G. Lei, *Free Radic. Biol. Med.*, 2013, **65**, 1548–1556.
48 M. E. Spencer and J. R. Guest, *J. Bacteriol.*, 1982, **151**, 542–552.
49 W. A. Bridger, W. T. Wolodko, W. Henning, C. Upton, R. Majumdar and S. P. Williams, *Biochem. Soc. Symp.*, 1987, **54**, 103–111.
50 E. Ostergaard, *J. Inherit. Metab. Dis.*, 2008, **31**, 226–229.
51 C. Thirunavukkarasu, J. P. Singh, K. Selvendiran and D. Sakthisekaran, *Cell Biochem. Funct.*, 2001, **19**, 265–271.
52 X.-M. Xu, B. A. Carlson, H. Mix, Y. Zhang, K. Saira, R. S. Glass, M. J. Berry, V. N. Gladyshev and D. L. Hatfield, *PLoS Biol.*, 2007, **5**, e4.
53 S. Castellano, N. Morozova, M. Morey, M. J. Berry, F. Serras, M. Corominas and R. Guigó, *EMBO Rep.*, 2001, **2**, 697–702.
54 F. Serras, M. Morey, B. Alsina, J. Baguñà and M. Corominas, *Biofactors*, 2001, **14**, 143–149.
55 M. Morey, F. Serras and M. Corominas, *FEBS Lett.*, 2003, **534**, 111–114.
56 F. C. Costa, M. a. V. Oliva, T. C. L. de Jesus, S. Schenkman and O. H. Thiemann, *Mol. Biochem. Parasitol.*, 2011, **180**, 47–50.

Journal Name

ARTICLE

Table 1. Top 20 up- and down-regulated genes identified in the larval stage

Gene ID	Symbol	Description	Fold change
Up-regulated			
43056	CG11893	Ecdysteroid kinase	259.35
40908	alpha-Est2	Alpha-Esterase-2	123.24
40539	CG9766	Ankyrin	71.81
31332	Rala	Ras-related protein	40.37
2768690	CG33346	Endonuclease	37.26
43786	CG2177	Metal ion transmembrane transporter	35.43
41763	CG14851	CG14851 gene product from transcript CG14851-RA	28.33
38433	Eip63E	Ecdysone-induced protein 63E	17.78
317842	CG32074	CG32074 gene product from transcript CG32074-RA	14.60
43261	CG14259	Juvenile hormone binding protein	12.72
40957	CG7900	Fatty acid amide hydrolase	10.50
5740836	CG34273	CG34273 gene product from transcript CG34273-RA	8.50
64867	Nol	No optic lobe	7.94
41762	CG14850	CG14850 gene product from transcript CG14850-RA	7.92
37920	CG11413	CG11413 gene product from transcript CG11413-RA	6.90
3355149	CG40298	CG40298 gene product from transcript CG40298-RA	6.81
41786	smp-30	Senescence marker protein-30	6.66
50271	CG13069	Sec24-related protein	6.30
38584	CG32237	CG32237 gene product from transcript CG32237-RA	6.23
37318	CG30148	CG30148 gene product from transcript CG30148-RA	6.14
Down-regulated			
34259	Fbp2	Fat body protein 2	0.0046
34472	CG7300	CG7300 gene product from transcript CG7300-RB	0.0087
45326	Lsp2	Larval serum protein 2	0.0124
33156	l(2)gl	Lethal (2) giant larvae	0.0173
39736	CG17032	CG17032 gene product from transcript CG17032-RA	0.0208
36349	Cpr49Ad	Cuticular protein 49Ad	0.0352
37538	CG3292	Alkaline phosphatase	0.0356
36400	CG8768	NAD(P)(+)-binding protein	0.0371
34182	Bace	Beta-site APP-cleaving enzyme	0.0389
35946	Cyp4p2	Cytochrome P450	0.0444
53507	Ugt86Dd	UDP-glucosyltransferase	0.0476
37622	CG30265	Na(+)-dependent inorganic phosphate cotransporter	0.0521
40470	CG11437	Phosphatidate phosphatase	0.0527
37110	GstE5	Glutathione S transferase E5	0.0559
34916	CG15253	Astacin (Peptidase family M12A)	0.0643
31915	CG32698	Carbonate dehydratase	0.0654
33810	CG11034	Dipeptidyl-peptidase	0.0708
35635	Cyp9b2	Cytochrome P450-9b2	0.0719
38129	LysP	Lysozyme P	0.0752
40583	CG31531	CG43427 gene product from transcript CG43427-RP	0.0787

Table 2. Top 20 up- and down-regulated genes in the adult stage

Gene ID	Symbol	Description	Fold change
Up-regulated			
38122	LysX	Lysozyme X	212.78
31332	Rala	Ras-related protein	73.12
33406	Ser12	Serine protease 12	34.28
38433	Eip63E	Ecdysone-induced protein 63E	27.13
40957	CG7900	Fatty acid amide hydrolase	19.99
43786	CG2177	Predicted divalent heavy-metal cations transporter	19.91
2768690	CG33346	Endonuclease	18.58
40908	alpha-Est2	Alpha-Esterase-2	17.59
40157	Ir76a	Ionotropic receptor 76a	16.29
43056	CG11893	Ecdysteroid kinase	16.18
318970	CG31832	Fibrinogen-related	14.81
43261	CG14259	Juvenile hormone binding protein	14.50
41454	CG3397	Aldo/keto reductase	8.84
3885655	CG34040	CG34040 gene product from transcript CG34040-RA	7.93
34092	CG12560	Acyltransferase	7.38
31818	Rbm13	RNA-binding motif protein 13	7.18
36434	CG13324	CG13324 gene product from transcript CG13324-RA	6.95
117460	TotX	Turandot X	6.72
41889	Rh6	Rhodopsin 6	6.66
3355149	CG40298	CG40298 gene product from transcript CG40298-RA	6.51
Down-regulated			
37099	IM23	Immune induced molecule 23	0.0084
41333	Ugt35b	UDP-glycosyltransferase 35b	0.0091
318943	CG31789	CG31789 gene product from transcript CG31789-RA	0.0178
34218	CG32984	CG32984 gene product from transcript CG32984-RA	0.0182
33156	l(2)gl	Lethal (2) giant larvae	0.0202
59225	CG17234	Serine-type endopeptidase	0.0402
34898	CG15263	CG15263 gene product from transcript CG15263-RA	0.0411
36400	CG8768	NAD(P)(+)-binding protein	0.0519
33155	CG11023	Cysteine-type peptidase	0.0546
43479	CG7567	CG7567 gene product from transcript CG7567-RA	0.0566
34872	mol	Moladietz	0.0571
34916	CG15253	Astacin (Peptidase family M12A)	0.0574
246666	Obp51a	Odorant-binding protein 51a	0.0587
36484	AttC	Attacin-C	0.0593
34836	CG18125	Spermathecal endopeptidase 2	0.0654
40583	CG31531	CG43427 gene product from transcript CG43427-RP	0.0802
326195	CG32086	CG32086 gene product from transcript CG32086-RB	0.0830
39736	CG17032	CG17032 gene product from transcript CG17032-RA	0.0841
38952	Jon66Ci	Jonah 66Ci (serine-type endopeptidase)	0.0957
34472	CG7300	CG7300 gene product from transcript CG7300-RB	0.1020

Table 3. GO enrichment analysis of DEGs in larval and adult stages

GO type	GO ID	Count[1]	Total[2]	P-value	FDR	Term
Larva						
BP	GO:0055114	33	511	3.45E-05	0.0564	oxidation-reduction process
BP	GO:0040003	15	156	8.12E-05	0.0664	chitin-based cuticle development
MF	**GO:0003824**[3]	131	2597	1.80E-10	8.13E-08	catalytic activity
MF	**GO:0020037**	22	131	3.64E-10	8.20E-08	heme binding
MF	**GO:0009055**	21	132	2.57E-09	3.87E-07	electron carrier activity
MF	**GO:0016705**	22	157	1.22E-08	1.37E-06	oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen
MF	**GO:0005506**	20	147	9.43E-08	8.51E-06	iron ion binding
MF	GO:0008010	14	91	1.86E-06	1.40E-04	structural constituent of chitin-based larval cuticle
MF	GO:0008012	3	3	3.78E-05	0.00244	structural constituent of adult chitin-based cuticle
MF	GO:0042302	14	127	9.06E-05	0.00511	structural constituent of cuticle
MF	GO:0004364	7	38	2.36E-04	0.0118	glutathione transferase activity
MF	GO:0045735	3	5	3.60E-04	0.0162	nutrient reservoir activity
Adult						
BP	GO:0006508	52	748	2.99E-05	0.0534	proteolysis
MF	GO:0004252	29	249	3.28E-07	1.59E-04	serine-type endopeptidase activity
MF	GO:0008233	46	565	5.18E-06	0.00125	peptidase activity
MF	**GO:0005506**	19	147	8.18E-06	0.00132	iron ion binding
MF	**GO:0020037**	17	131	2.32E-05	0.00276	heme binding
MF	**GO:0003824**	195	3812	2.86E-05	0.00276	catalytic activity
MF	**GO:0016705**	18	157	7.19E-05	0.00579	oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen
MF	GO:0008970	4	8	1.70E-04	0.0117	phosphatidylcholine 1-acylhydrolase activity
MF	**GO:0009055**	15	132	3.14E-04	0.0190	electron carrier activity
MF	GO:0004558	4	12	1.05E-03	0.0565	alpha-1,4-glucosidase activity

[1] Number of DEGs involved in this GO term in each stage.

[2] Number of genes involved in this GO term.

[3] Bold font represents the common GO terms enriched in both stages.

Table 4. KEGG pathway analysis of DEGs in larval and adult stages

Pathway ID	Count	Total	P-value	FDR	Pathway name
Larva					
dme00980[1]	11	59	2.98E-07	1.51E-05	Metabolism of xenobiotics by cytochrome P450
dme00982	11	59	2.98E-07	1.51E-05	Drug metabolism - cytochrome P450
dme00480	11	60	3.63E-07	1.51E-05	Glutathione metabolism
dme00040	8	44	9.88E-06	3.09E-04	Pentose and glucuronate interconversions
dme00051	4	25	0.00121	0.0253	Fructose and mannose metabolism
dme00053	4	29	0.00244	0.0435	Ascorbate and aldarate metabolism
dme00830	4	30	0.00285	0.0445	Retinol metabolism
dme00983	5	47	0.00437	0.0546	Drug metabolism - other enzymes
dme00790	3	24	0.00767	0.0871	Folate biosynthesis
dme00500	5	55	0.00952	0.0991	Starch and sucrose metabolism
Adult					
dme00500	8	55	7.90E-06	9.88E-04	Starch and sucrose metabolism
dme00040	6	44	1.07E-04	0.00464	Pentose and glucuronate interconversions
dme00052	5	31	1.11E-04	0.00464	Galactose metabolism
dme00053	4	29	7.65E-04	0.0239	Ascorbate and aldarate metabolism
dme00790	3	24	0.00306	0.0543	Folate biosynthesis
dme00980	5	59	0.00385	0.0543	Metabolism of xenobiotics by cytochrome P450
dme00982	5	59	0.00385	0.0543	Drug metabolism - cytochrome P450
dme00531	2	14	0.00507	0.0633	Glycosaminoglycan degradation
dme00983	4	47	0.00688	0.0730	Drug metabolism - other enzymes
dme00830	3	30	0.00701	0.0730	Retinol metabolism
dme00330	4	49	0.00822	0.0790	Arginine and proline metabolism

[1] Bold font represents common KEGG pathways enriched in both stages;

Table 5. The overlap of DEGs in pathways that are enriched in both larval and adult stages

Pathway ID	Pathway Name	Total	DEGs in larval stage	DEGs in adult stage	Overlapped DEGs
dme00982	Drug metabolism - cytochrome P450	59	11	5	2
dme00980	Metabolism of xenobiotics by cytochrome P450	59	11	5	2
dme00500	Starch and sucrose metabolism	55	5	8	1
dme00040	Pentose and glucuronate interconversions	44	8	6	3
dme00053	Ascorbate and aldarate metabolism	29	4	4	1
dme00830	Retinol metabolism	30	4	3	1
dme00790	Folate biosynthesis	24	3	3	1
dme00983	Drug metabolism - other enzymes	47	5	4	2

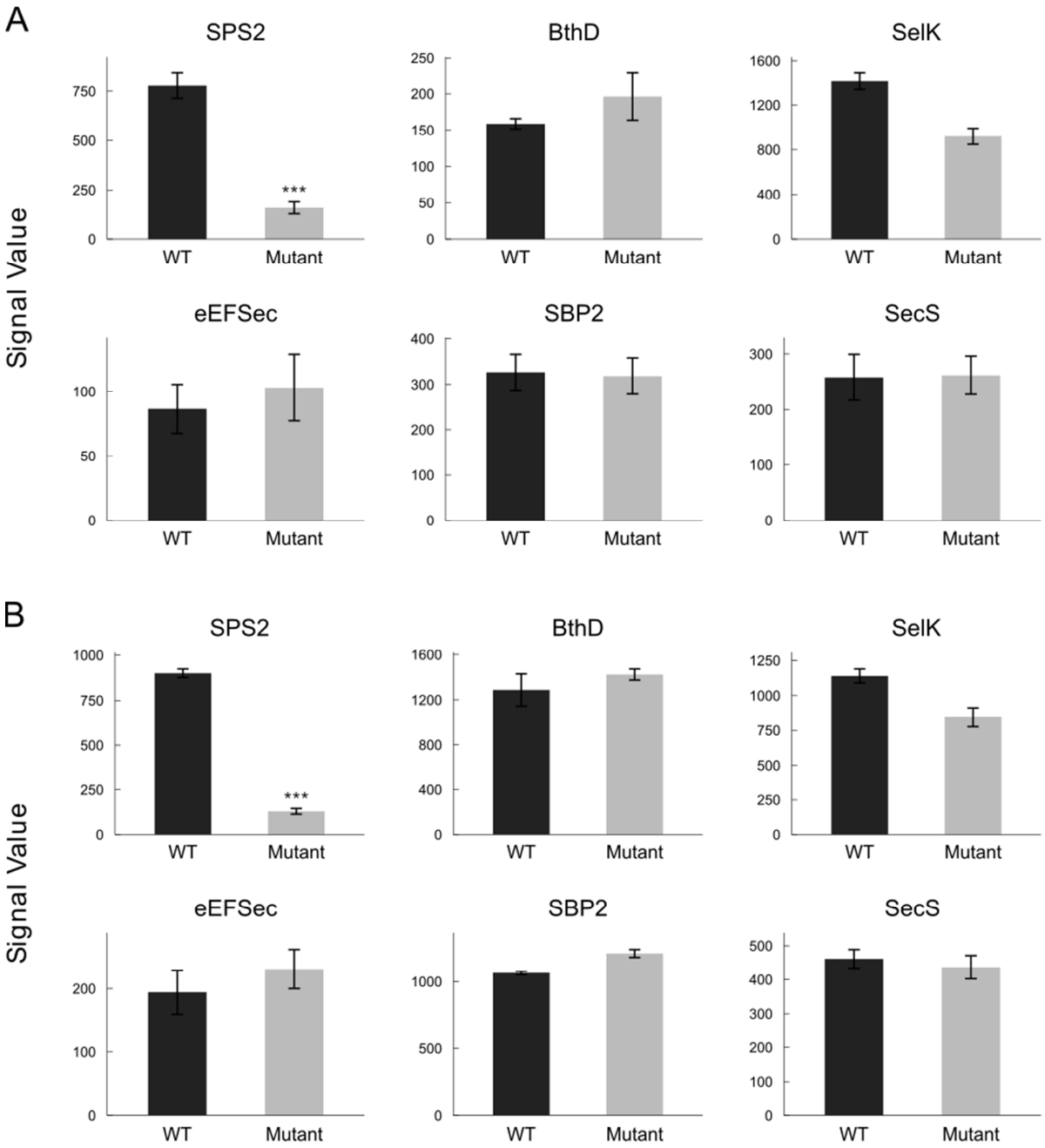

Fig. 1 Expression levels of genes involved in Sec biosynthesis and selenoproteins in SPS2 knockdown fruit flies. The y-axis represents the normalized expression values of both Sec biosynthesis genes and selenoprotein genes (including SPS2) measured in microarray. DEGs are indicated (*** indicates significance at p-value < 0.001). A. larval stage; B. adult stage.

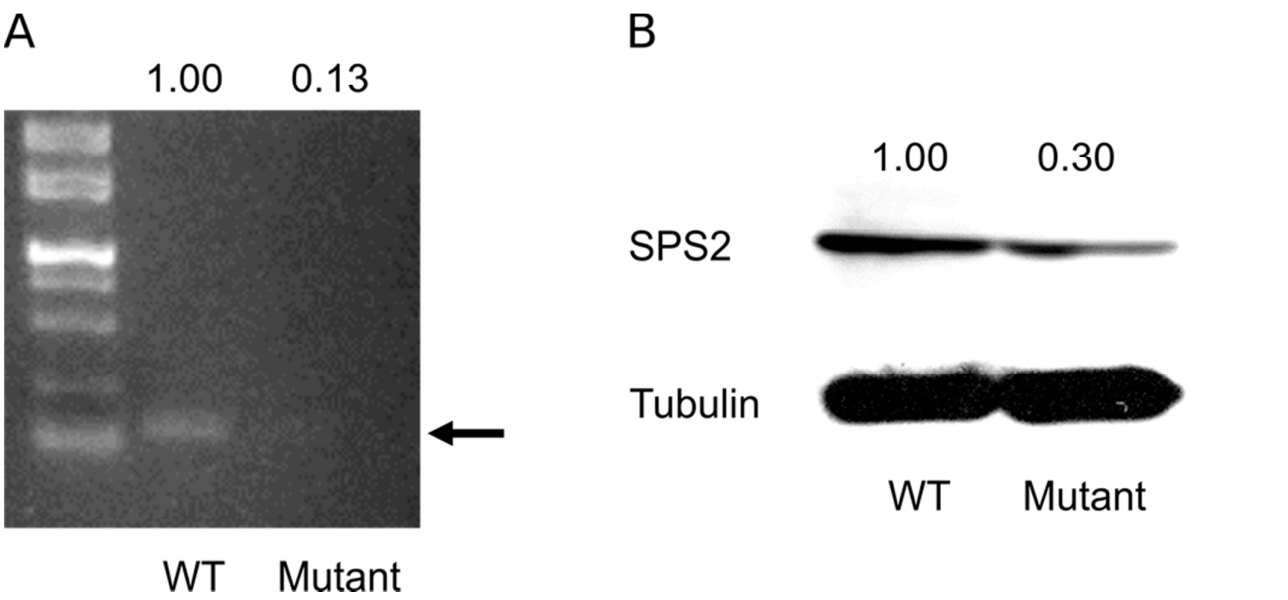

Fig. 2 Verification of SPS2 knockdown efficiency in fruit flies. A. Agarose gel electrophoresis of SPS2 mRNA RT-PCR products. The relative mRNA levels (normalized to actin and tubulin) are indicated above samples; B. Western blot analysis of SPS2 protein. The relative protein levels (normalized to tubulin) are indicated above samples.

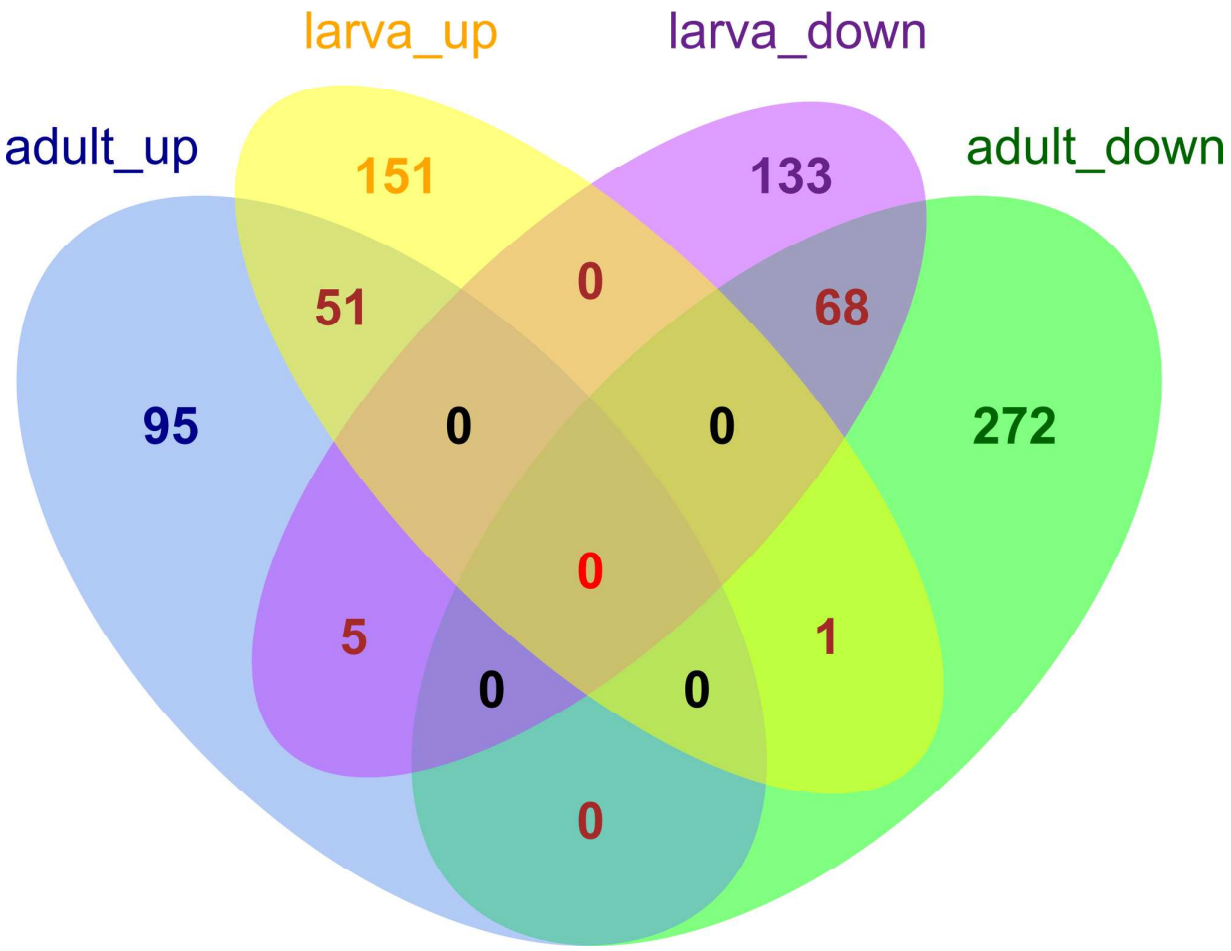

Fig. 3 Comparison of DEGs identified in larval and adult stages. The Venn diagram was used to show the overlaps of up- and down-regulated DEGs in different stages.

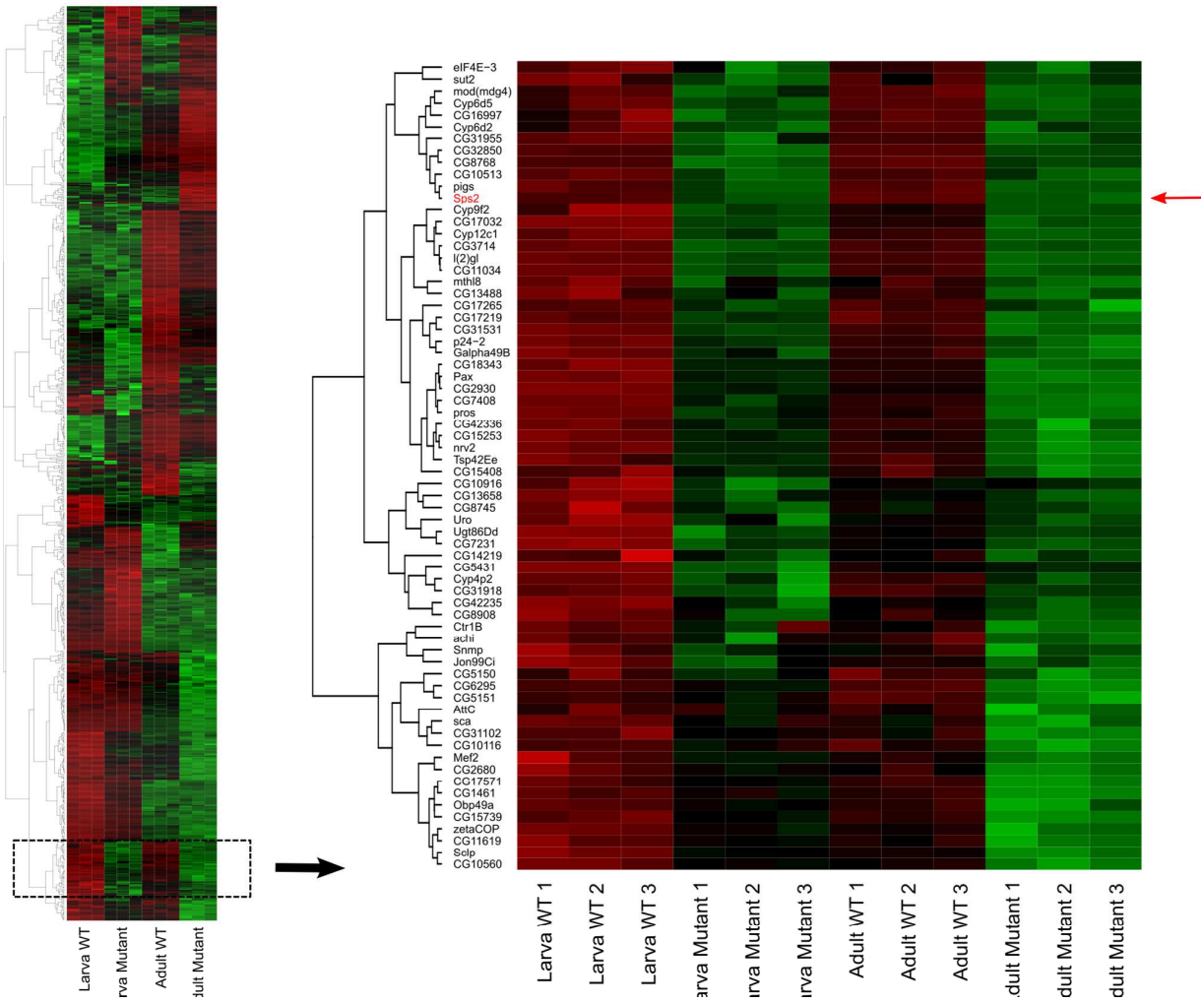

Fig. 4 A correlation heat map of DEGs. Gene expression profiles were analyzed by hierarchical clustering approach as described in the text. Red and green colors represent increased and decreased expression (transformed into z-scores), respectively, when compared with the average expression level for each gene. Each DEG is represented by a single row of colored boxes; and each sample is represented by a single column. A fraction of the map that shows genes whose expression patterns are similar to SPS2 is also shown on the right. The SPS2 gene is highlighted in red.

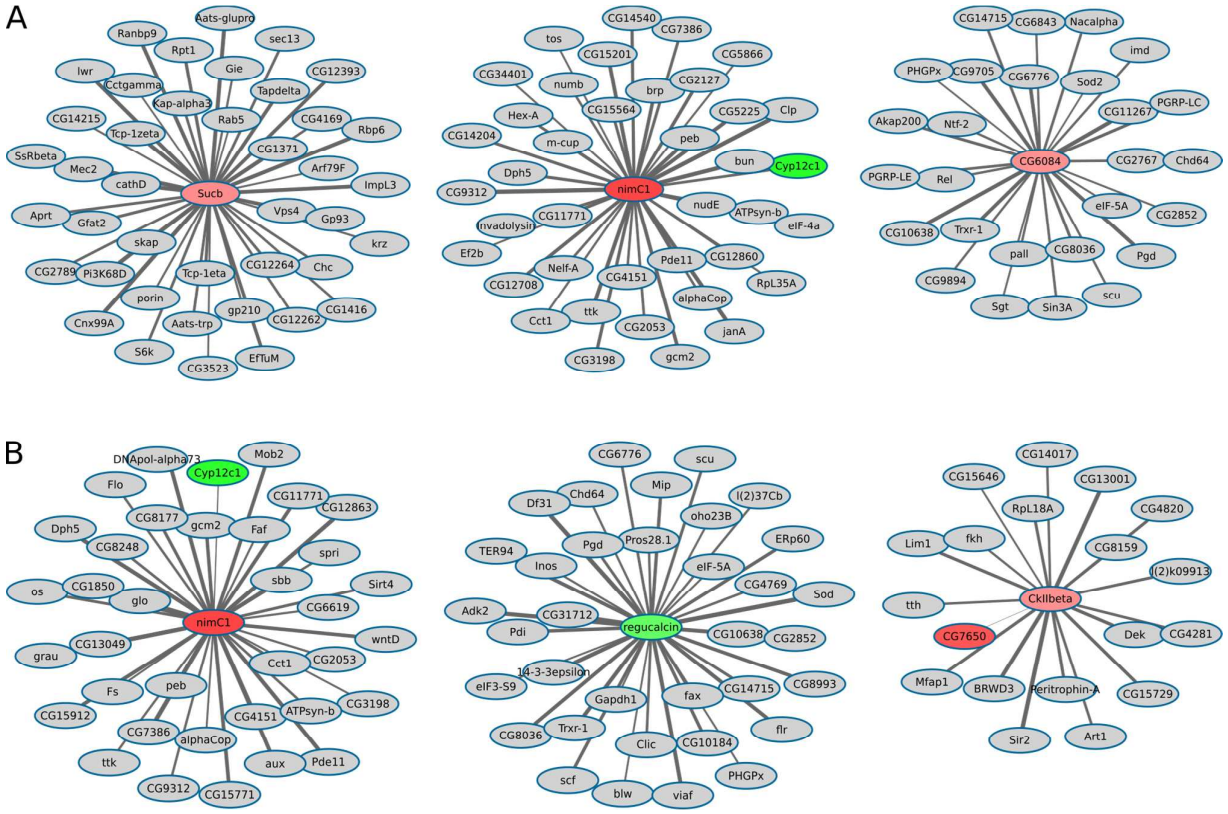

Fig. 5 The top three modules identified in different stages. A. larval stage; B. adult stage. Up- and down-regulated genes are highlighted with red and green colors, respectively. The color scale is from saturated green (significantly down-regulated) to white (no significant change) and then to saturated red (significantly up-regulated).

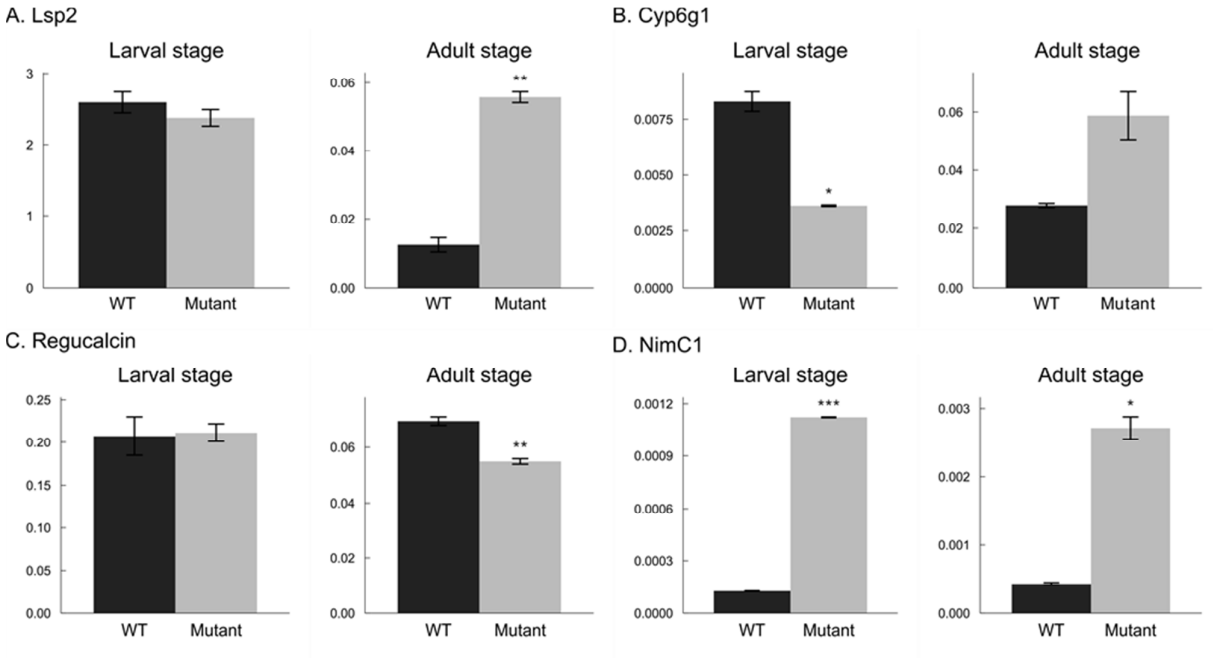

Fig. 6 Validation of the selected genes by quantitative PCR. The y-axis represents the relative mRNA level of each gene compared to actin. Statistical significance was tested by t test (*: p-value < 0.05; **: p-value < 0.01; ***: p-value < 0.001). A. Lsp2; B. Cyp6g1; C. Regucalcin; D. NimC1.