ARTICLE
Journal of Cellular Biochemistry 114:418–427 (2013)

Journal of Cellular Biochemistry

# Microarray Analysis of *Drosophila dicer-2* Mutants Reveals Potential Regulation of Mitochondrial Metabolism by Endogenous siRNAs

Do-Hwan Lim,[1] Langho Lee,[2] Chun-Taek Oh,[3] Nam-Hoon Kim,[1] Seungwoo Hwang,[2] Sung-Jun Han,[3] and Young Sik Lee[1*]

[1]*College of Life Sciences and Biotechnology, Korea University, Seoul 136-713, South Korea*
[2]*Korean Bioinformation Center, KRIBB, Daejeon 305-806, South Korea*
[3]*Drug Biology Group, Institut Pasteur Korea, Seongnam-Si, Gyeonggi-Do 463-400, South Korea*

## ABSTRACT

RNA interference is a eukaryotic regulatory mechanism by which small non-coding RNAs typically mediate specific silencing of their cognate genes. In *Drosophila*, the RNase III enzyme Dicer-2 (Dcr-2) is essential for biogenesis of endogenous small interfering RNAs (endo-siRNAs), which have been implicated in regulation of endogenous protein-coding genes. Although much is known about microRNA-based regulatory networks, the biological functions of endo-siRNAs in animals remain poorly understood. We performed gene expression profiling on *Drosophila dcr-2* null mutant pupae to investigate transcriptional effects caused by a severe defect in endo-siRNA production, and found 306 up-regulated and 357 down-regulated genes with at least a twofold change in expression compared with the wild type. Most of these up-regulated and down-regulated genes were associated with energy metabolism and development, respectively. Importantly, mRNA sequences of 39% of the up-regulated genes were perfectly complementary to the sequences of previously reported endo-siRNAs, suggesting they may be direct targets of endo-siRNAs. We confirmed up-regulation of five selected genes matching endo-siRNAs and concomitant down-regulation of the corresponding endo-siRNAs in *dcr-2* mutant pupae. Most of the potential endo-siRNA target genes were associated with energy metabolism, including the citric acid cycle and oxidative phosphorylation in mitochondria, implying that these are major metabolic processes directly affected by endo-siRNAs in *Drosophila*. Consistent with this finding, *dcr-2* null mutant pupae had lower ATP content compared with controls, indicating that mitochondrial energy production is impaired in these mutants. Our data support a potential role for the endo-siRNA pathway in energy homeostasis through regulation of mitochondrial metabolism. J. Cell. Biochem. 114: 418–427, 2013.


**KEY WORDS:** DICER-2; *DROSOPHILA*; ENDOGENOUS SIRNA; MICROARRAY; RNA INTERFERENCE

RNA interference (RNAi) is a biological process by which small non-coding RNAs regulate the expression of their cognate target genes. These small RNAs are mainly classified into two groups: microRNAs (miRNAs) and small interfering RNAs (siRNAs) [Farazi et al., 2008]. siRNAs were first thought to be exogenously derived from viral RNAs or experimentally introduced double-stranded RNAs (dsRNAs), whereas miRNAs were endogenously generated from short-hairpin RNA structures [Golden et al.,

The authors declare no conflicts of interest.

Additional supporting information may be found in the online version of this article.

Grant sponsor: National Research Foundation of Korea (NRF), Mid-Career Reseacher Program; Grant number: 2011-0016863; Grant sponsor: Seoul Scholarship Foundation; Grant sponsor: Korea Research Institute of Bioscience and Biotechnology Research Initiative Program; Grant sponsor: Korea University; Grant sponsor: NRF, Pioneer Research Center Program; Grant number: 2012-0000433.

Langho Lee's present address is Graduate School of Genome Science and Technology, University of Tennessee-Oak Ridge National Laboratory, Knoxville, TN 37996.

*Correspondence to: Prof. Young Sik Lee, PhD, College of Life Sciences and Biotechnology, Korea University, Seoul 136-713, South Korea. E-mail: ys-lee@korea.ac.kr

Manuscript Received: 4 March 2012; Manuscript Accepted: 27 August 2012

Accepted manuscript online in Wiley Online Library (wileyonlinelibrary.com): 7 September 2012

DOI 10.1002/jcb.24379 • © 2012 Wiley Periodicals, Inc.

2008]. Thus, most studies on the biological function of small RNAs have focused on miRNAs. However, an extensive collection of endogenous siRNAs (endo-siRNAs) was recently characterized as another class of endogenous regulators of gene expression in animals [Okamura and Lai, 2008]. These endo-siRNAs are derived from endogenous dsRNA precursors such as RNA with extensive hairpin structures or sense-antisense transcript hybrids, and are complementary to mRNAs expressed in *Caenorhabditis elegans*, *Drosophila*, and mammals [Okamura and Lai, 2008; Ghildiyal and Zamore, 2009]. In *Drosophila*, an RNase III enzyme Dicer-2 (Dcr-2) processes the endogenous precursor into approximately 21-nucleotide (nt) endo-siRNAs, which associate primarily with Ago2-containing effector complexes to silence endogenous protein-coding genes in somatic tissues [Okamura and Lai, 2008]. Accordingly, loss of the key components Dcr-2 or Ago2 results in a substantial reduction in endo-siRNA biogenesis and accumulation, respectively, with concomitant increase in the levels of protein-coding transcripts that are complementary to the endo-siRNAs [Czech et al., 2008; Ghildiyal et al., 2008; Kawamura et al., 2008; Okamura et al., 2008]. The cellular functions of endo-siRNAs are only beginning to be explored. We recently reported that the endo-siRNA pathway plays important roles in stress resistance and lifespan by regulating metabolic homeostasis in *Drosophila* [Lim et al., 2011]. The endo-siRNA pathway has also been reported as essential for robust development of embryos and heterochromatin formation in *Drosophila* [Fagegaltier et al., 2009; Lucchetta et al., 2009]. Studies on *C. elegans* have revealed that endo-siRNAs play roles in spermatogenesis [Pavelec et al., 2009] and in directing chromatin modifications via histone H3 lysine 9 methylation [Burkhart et al., 2011]. The endo-siRNA pathway was also suggested to promote mitotic chromosome segregation in *Drosophila* and human cells [Pek and Kai, 2011].

In this study, we report a transcriptomic comparison of gene expression between wild-type and *dcr-2* null mutant pupae to understand the cellular functions associated with endo-siRNAs in *Drosophila*. Genes involved in energy metabolism were up-regulated in *dcr-2* mutants, while genes associated with development were down-regulated. Importantly, 39% of the up-regulated genes perfectly matched with previously reported endo-siRNAs and were mainly involved in energy metabolic pathways, such as the citric acid cycle and oxidative phosphorylation (OXPHOS) in mitochondria. Our data provide insight into a role of the endo-siRNA pathway in regulating mitochondrial function in *Drosophila*.

## MATERIALS AND METHODS

### DROSOPHILA STRAINS

Flies were reared on standard cornmeal/agar media under non-crowded conditions at 25°C. Canton-S and $w^{1118}$ flies were used for developmental profiling of *dcr-2* transcripts and Dcr-2 protein, respectively. Canton-S flies were also used as $white^{+}$ ($w^{+}$) flies. To generate *dcr-2-GAL4* transgenic flies, the 5′ regulatory region of the genomic *dcr-2* rescue transgene was subcloned into pPTGAL and transformed into *Drosophila* using standard procedures [Sharma et al., 2002; Lee et al., 2004]. To analyze the expression pattern of *dcr-2* using the UAS/GAL4 system [Brand and Perrimon, 1993], the *dcr-2-GAL4* transgenic flies were crossed to flies carrying *UAS-mCD8GFP* at 25°C [Lee and Luo, 1999]. For microarray analysis, transheterozygous $dcr\text{-}2^{L811fsX}/dcr\text{-}2^{R416X}$ mutant pupae were generated by crossing between two independent *dcr-2* null mutant homozygotes (*y w eyFLP*; *FRT42D*, $dcr\text{-}2^{R416X}$ and *y w eyFLP*; *FRT42D*, $dcr\text{-}2^{L811fsX}$) [Lee et al., 2004; Lim et al., 2011]. The parental strain (*y w eyFLP*; *FRT42D*) of the *dcr-2* mutants was used as an isogenic wild-type control [Lee et al., 2004; Lim et al., 2011].

### QUANTITATIVE REVERSE TRANSCRIPTION-POLYMERASE CHAIN REACTION (RT-PCR) ANALYSIS OF TRANSCRIPT LEVELS

Total RNA was isolated from the indicated developmental stages (~100 animals each) or genotypes (20 animals each) using Trizol reagent (Invitrogen) according to the manufacturer's instructions. One microgram of total RNA from each sample was treated with DNase I (Invitrogen), and used to generate first-strand cDNA using random hexamers (Promega) and M-MLV reverse transcriptase (Invitrogen) according to the manufacturer's protocol. Quantitative PCR analysis was performed as described previously [Lim do et al., 2008]. The sequences of gene-specific primers are listed in Supplementary Table I.

### WESTERN BLOTTING

Protein extract was prepared in lysis buffer (150 mM NaCl, 20 mM Tris–HCl [pH 8.0], 0.5% NP-40, 1 mM EDTA, and EDTA-free protease inhibitor cocktail [Roche]) from each of the indicated developmental stages of $w^{1118}$ or from isogenic wild-type and transheterozygous *dcr-2* null mutant pupae. Western blot analysis was performed as described previously [Lim do et al., 2008]. The primary antibodies used were anti-Dcr-2 (1:1,000; a gift from Q. Liu), anti-R2D2 (1:1,000; a gift from Q. Liu), and anti-β-tubulin (1:5,000; Developmental Studies Hybridoma Bank).

### MICROARRAY ANALYSIS

Gene expression analysis was performed using Agilent's *Drosophila* 4 × 44K oligo microarrays (G2519F; Agilent Technologies). We analyzed three biological replicates of age-matched wild-type and transheterozygous *dcr-2* null mutant pupae. Total RNA was isolated from each biological replicate (20 animals) using Trizol reagent (Invitrogen) according to the manufacturer's protocol. The integrity of RNA was verified using a 2100 Bioanalyzer (Agilent Technologies). Total RNA was purified using an RNeasy Mini kit (Qiagen) to remove remnants from Trizol extraction, and used for cDNA chip hybridization according to a modified TIGR protocol [Hegde et al., 2000]. Images of microarray slides were acquired using an Agilent Scanner G2505B (Agilent Technologies) and processed using Agilent Feature Extraction Software (version 9.5; Agilent Technologies). The raw expression profile was normalized using the linear and LOWESS methods. Spots flagged with low reliability were excluded from further analysis. The normalized Cy5/Cy3 (mutant/wild-type) ratios were $\log_2$ transformed before statistical testing. A probe was selected to be differentially expressed if its *P*-value from the Welch *t*-test was $<0.05$ and its mean fold change value across three replicates more than twofold. All microarray data were deposited in the GEO database (www.ncbi.nih.gov/geo) under the NCBI accession number GSE35945.

### FUNCTIONAL ENRICHMENT ANALYSIS

Agilent's original probe annotations for differentially expressed genes were re-defined by mapping the identifiers in the ''Accessions'' column into transcript and gene identifiers according to the latest release of FlyBase and Entrez databases. Functional enrichment analysis was performed on the differentially expressed genes using the DAVID database version 6.7 [Huang da et al., 2009]. The enriched Gene Ontology biological processes were determined according to the criteria with involvement of at least five genes and $P < 0.05$ for each category. Functional enrichment analysis was also performed using the KEGG database to identify enrichment of genes with specific biological pathways.

### PREDICTION OF PUTATIVE TARGET GENES OF ENDO-siRNAs AND FUNCTIONAL CATEGORIES

Endo-siRNA sequences were collected from previously published studies [Czech et al., 2008; Ghildiyal et al., 2008; Kawamura et al., 2008]. To identify candidate target genes of these endo-siRNAs, the sequences were aligned on *Drosophila melanogaster* transcripts obtained from FlyBase using BLAST and BLAT programs [Altschul et al., 1997; Kent, 2002; Tweedie et al., 2009]. Default options were used for running these programs except that tile size parameter was set to eight for BLAT. A transcript was considered to match with an endo-siRNA sequence if either of the BLAST or BLAT hits was reported. For each endo-siRNA sequence, multiple candidate targets were allowed, and vice versa. Only transcripts that perfectly matched with endo-siRNA sequences were used for further analysis. The aligned transcripts were filtered to contain only the minus strand hits against the sense endo-siRNA sequences. The results were compared with the up-regulated genes in *dcr-2* mutants. Functional enrichment analysis was performed on the list of the endo-siRNA-matching up-regulated genes using DAVID.

### RT-PCR ANALYSIS OF SMALL RNAs

The relative abundance of endo-siRNAs with specific sequences was measured using a PCR-based method as described previously with some modifications [Ro and Yan, 2010; Song et al., 2011]. Briefly, small RNAs were isolated from wild-type and transheterozygous *dcr-2* null mutant pupae (6–7 animals each) using the miRNeasy Mini kit (Qiagen) and RNeasy MinElute Cleanup kit (Qiagen) according to the manufacturer's protocols. Seven hundred nanograms of small RNAs were polyadenylated using poly(A) polymerase (NEB). The poly(A)-tailed small RNAs were purified by phenol/chloroform/isoamyl alcohol extraction and ethanol precipitation. cDNA was synthesized using Superscript III (Invitrogen) and the RTQ primer consisting of adaptor sequences at the 5′ end and poly dT at the 3′ end, with 1 μg poly(A)-tailed small RNAs. Following treatment of the resulting cDNA with RNase H (Takara), semi-quantitative PCR was performed in a 20-μl reaction volume containing 1 U DNA polymerase (Enzynomics), 1 μl small RNA cDNAs, 0.25 μM each small RNA-specific primer and RTQ-UNIr primer, and 200 μM each deoxyribonucleotide triphosphate. The sequences of the primers RTQ and RTQ-UNIr as well as sequences of other small RNA-specific primers are listed in Supplementary Table II. The cycling parameters for the PCR reactions were as follows: 95°C for 2 min; 28 cycles [except for endo-siRNAs targeting *mthl8*, *Rm62*, and *w* (30 cycles)] of 95°C for 30 s, 60°C for 30 s, and 72°C for 30 s; and a final extension at 72°C for 5 min. The resulting PCR products were electrophoresed on 1.5% agarose gels and visualized with ethidium bromide.

### ATP MEASUREMENT

ATP content was determined with nine independent extracts from two pupae for each genotype, using the ATP bioluminescence assay kit HS II (Roche) as previously described [Clark et al., 2006]. Values were normalized to the protein content of each sample, measured by the Bradford method.

## RESULTS

### SPATIOTEMPORAL EXPRESSION OF DCR-2

In *Drosophila*, Dcr-2 plays a critical role in endo-siRNA-directed repression of gene expression by processing endogenous dsRNA precursors into endo-siRNAs. We first investigated the developmental expression pattern of *dcr-2* by quantitative RT-PCR analysis. The *dcr-2* transcript was detected at all developmental stages, with elevated levels in second instar larvae, pupae, and adults (Fig. 1A). Next, we confirmed the temporal expression pattern of *dcr-2* at the protein level using Western blotting. Dcr-2 protein was constitutively expressed with the lowest level at the larval stage (Fig. 1B). We generated *dcr-2-GAL4* transgenic flies expressing GAL4 under the control of the *dcr-2* promoter to determine spatial expression of *dcr-2* using the UAS/GAL4 system [Brand and Perrimon, 1993]. These transgenic flies were crossed to flies bearing *UAS-mCD8GFP*, which leads to GAL-4-dependent expression of a fusion protein composed of the extracellular and transmembrane domains of the mouse CD8 antigen (mCD8) fused to green fluorescent protein (GFP) [Lee and Luo, 1999]. Progeny pupae carrying both transgenes showed strong and ubiquitous expression of mCD8GFP (Fig. 1C), which correlates with the expression pattern of the endogenous *dcr-2* at the same developmental stage. Together, the results indicate that *dcr-2* is constitutively expressed throughout the development of *Drosophila*, with higher transcript and protein levels at late developmental stages, such as in pupae and adults.

### MICROARRAY ANALYSIS OF DCR-2 MUTANTS

Our earlier study demonstrated that *dcr-2* null mutant flies defective in endo-siRNA biogenesis had decreased stress resistance and lifespan, with abnormal lipid and carbohydrate metabolism [Lim et al., 2011]. The study also identified a limited set of proteins with altered expression in the heads and bodies of the *dcr-2* mutants. To obtain a more comprehensive picture of the cellular functions associated with endo-siRNAs, we sought to measure the genome-wide transcriptional profile of *dcr-2* null mutants and isogenic controls. To avoid the effects of undesired mutations (except for the *dcr-2* mutation), we used *dcr-2* mutants transheterozygous for $dcr\text{-}2^{L811fsX}$ and $dcr\text{-}2^{R416X}$ null alleles that abolish Dcr-2 production and lead to concomitant absence of the Dcr-2 partner R2D2 due to its destabilization [Liu et al., 2006; Lim et al., 2011] (Supplementary Fig. 1). R2D2 is required for loading of endo-siRNAs into Ago2 rather than biogenesis of endo-siRNAs [Czech et al., 2008; Marques et al., 2010]. Accordingly, transheterozygous *dcr-2* null

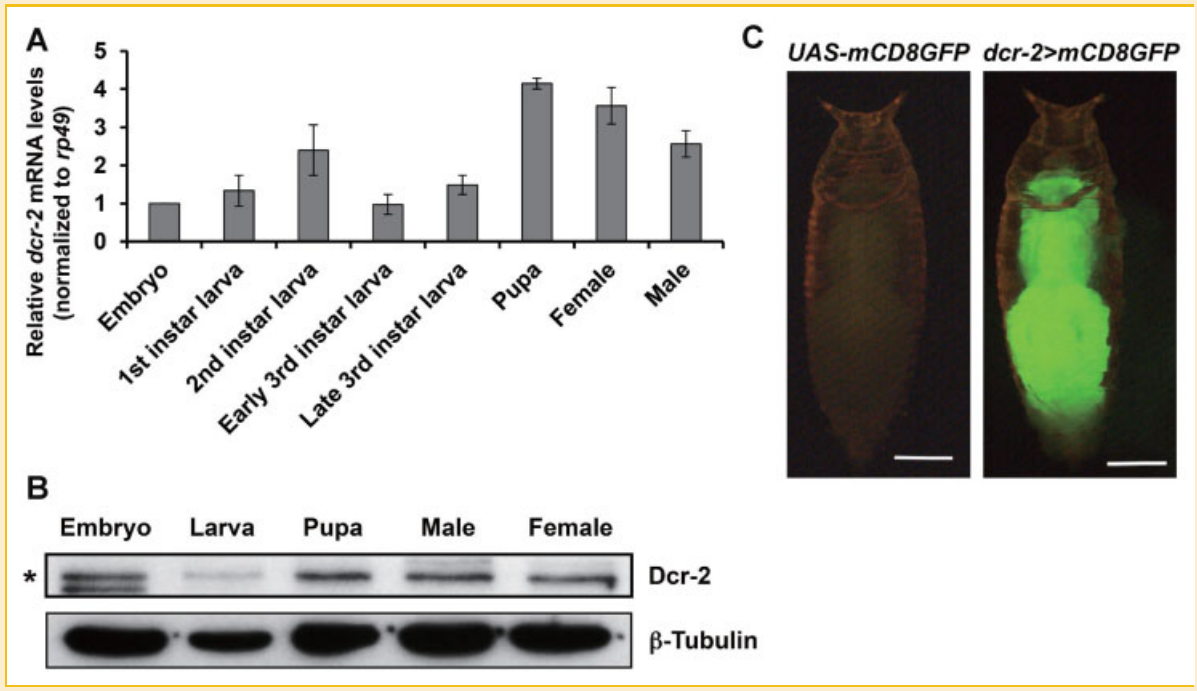


Fig. 1. Spatiotemporal expression of *dcr-2*. A: Analysis of relative *dcr-2* transcript levels at different developmental stages of wild-type animals using quantitative RT-PCR. The levels of *dcr-2* transcript were normalized to those of *rp49* transcript, which served as an internal control. The normalized *dcr-2* transcript level in embryos was arbitrarily set to 1. Data are shown as the mean ± standard error of the mean (SEM) from three independent experiments. B: Temporal expression pattern of Dcr-2 protein as determined by Western blotting. β-Tubulin was used as a loading control. An asterisk indicates Western signals corresponding to Dcr-2. C: Ubiquitous expression of *dcr-2* at the pupal stage based on the expression of *mCD8GFP* driven by *dcr-2-GAL4*. No green fluorescence was detected in the control pupa carrying the *UAS-mCD8GFP* transgene alone. Scale bar, 500 μm.

mutants would have severe defects in the endo-siRNA pathway, including endo-siRNA biogenesis [Czech et al., 2008; Ghildiyal et al., 2008; Kawamura et al., 2008; Okamura et al., 2008]. The transcriptomic analysis was also performed on pupae undergoing metamorphosis from larva to adult, which have high expression levels of *dcr-2*, and because expression of ~21-nt endo-siRNAs is increased relative to small RNAs of other sizes at the pupal stage [Fagegaltier et al., 2009]. To examine the changes in the gene expression profile of the *dcr-2* null mutant pupae, we used Agilent's *Drosophila* 4 × 44K microarrays. Among the 32,162 probes, only 20,626 probes (64%) had been described with gene symbols. Therefore, we generated a better definition of probes in the array using probe re-annotation because this process yields a more accurate interpretation of microarray data [Dai et al., 2005]. As a result, the annotated probes were increased to 23,406 (73%). Following probe re-annotation, 742 probes displayed at least 2-fold differences in expression between the wild type and *dcr-2* mutants, as well as a significant *P*-value (<0.05). Of these 742 probes, 343 and 399 showed up-regulation and down-regulation, respectively, in *dcr-2* mutants compared with controls. Because some probes represented the same gene, the differentially expressed probe list was trimmed at the gene level by selecting the probe with the largest magnitude of fold change, yielding 306 over-expressed genes and 357 under-expressed genes. The full list of the differentially regulated probes is shown in Supplementary Tables III and IV. The identified gene of note is *mus308*, which has been known to be directly regulated by endo-siRNAs derived from the *esi-2* locus [Czech et al., 2008]. In support of our transcriptomic approach, this gene was up-regulated by 1.4-fold, although it was below the stringent twofold change threshold (data not shown).

## FUNCTIONAL ENRICHMENT ANALYSIS OF DIFFERENTIALLY EXPRESSED GENES IN DCR-2 MUTANTS

The differentially expressed genes were further analyzed by DAVID with respect to the Gene Ontology biological process category [Huang da et al., 2009]. Of the 357 down-regulated genes, 138 could be categorized into 151 significantly enriched functional groups, most of which were linked to development, including cell differentiation and growth (Supplementary Table V). Among these categories, the top 10% were related to oocyte localization, neurogenesis, and organ formation (Fig. 2A). Additionally, enriched KEGG pathways in the down-regulated genes were the Notch signaling pathway and drug metabolism. For the 306 up-regulated genes, 61 genes belonged to nine significantly enriched categories, many associated with energy metabolism, such as generation of precursor metabolites and energy, the coenzyme metabolic process, the carboxylic acid biosynthetic process, hydrogen and monovalent inorganic cation transport, oxidation reduction, and the phosphate metabolic process (Fig. 2B; Supplementary Table VI). To further evaluate involvement of these up-regulated genes in energy metabolism, we performed KEGG pathway analysis. Interestingly, a remarkable enrichment for the citric acid cycle and OXPHOS

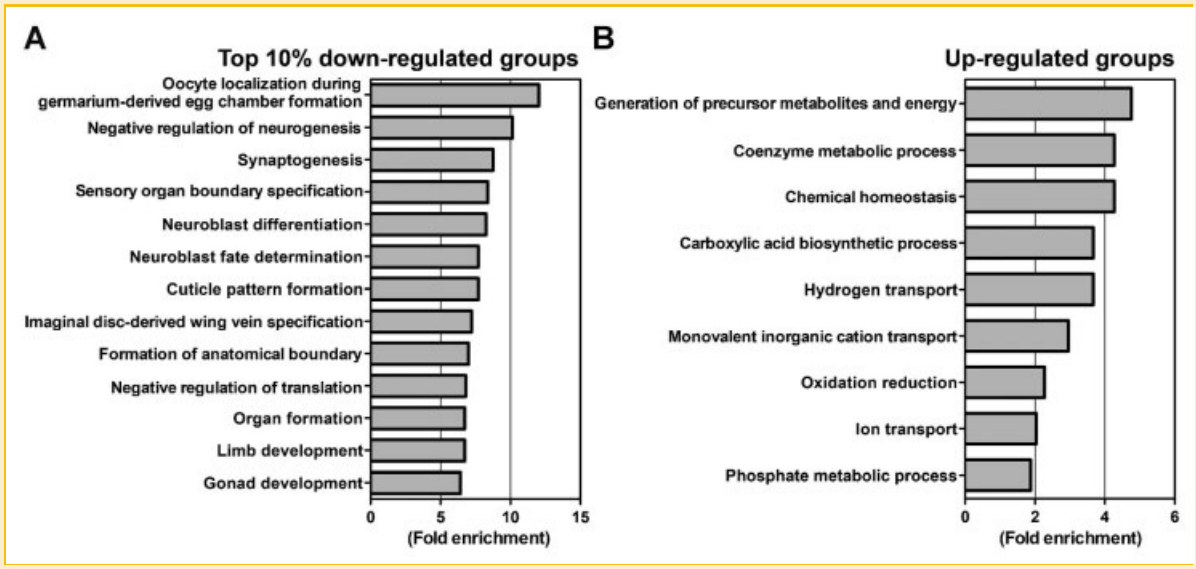


Fig. 2. Functional enrichment analysis of down- and up-regulated genes in *dcr-2* null mutant pupae. A: Of the 357 down-regulated genes, 151 biological processes were significantly over-represented, involving 138 genes. Among these biological processes, the top 10% groups are shown as a bar graph. B: Of the 306 up-regulated genes, 61 were significantly enriched for nine biological processes. Data were sorted by fold enrichment.

pathway in mitochondria was revealed (Fig. 3). Seven genes are involved in the citric acid cycle: *Scsα* (succinyl-CoA synthetase α subunit), *Nc73EF* (oxoglutarate dehydrogenase activity), *CG5028* (isocitrate dehydrogenase activity), *CG6439* (isocitrate dehydrogenase activity), *Acon* (aconitate hydratase activity), *CG1516* (pyruvate carboxylase activity), and *CG11876* (pyruvate dehydrogenase activity). Fourteen genes are associated with 5 complexes of the electron transport chain (ETC): complex I (*mt:ND1*, *mt:ND4*,

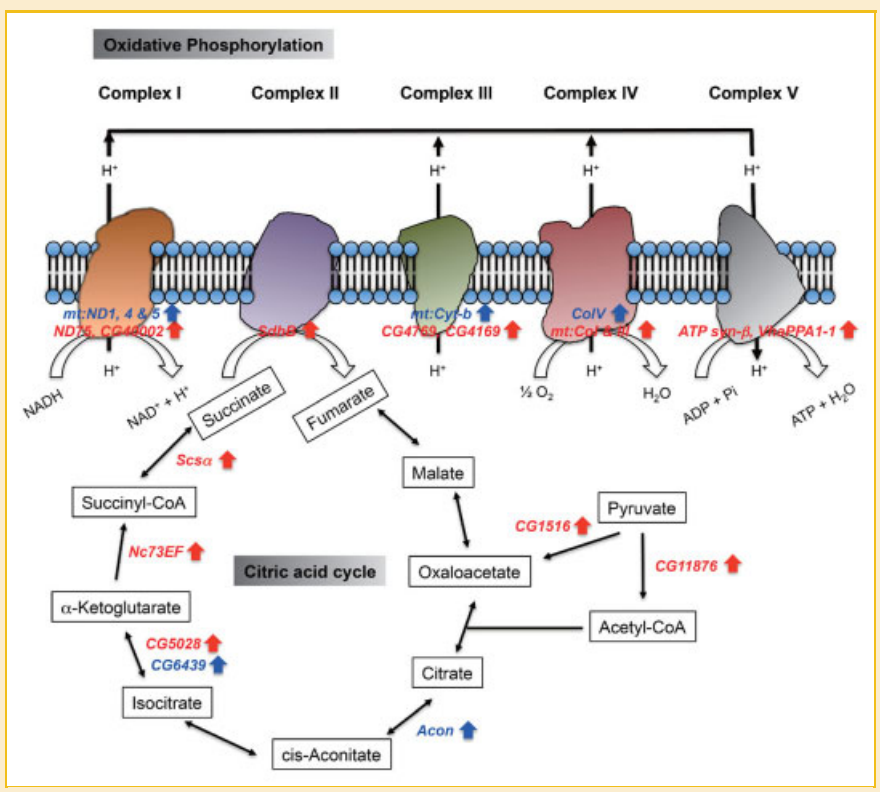


Fig. 3. The citric acid cycle and oxidative phosphorylation pathway over-represented by the genes up-regulated in *dcr-2* null mutant pupae. Endo-siRNA-matching genes are shown in red, others in blue. Upward arrows indicate up-regulation of the corresponding genes in *dcr-2* mutants compared with wild-type controls.

*mt:ND5*, *ND75*, and *CG40002*); complex II (*SdhB*); complex III (*mt:Cyt-b*, *CG4769*, and *CG4169*); complex IV (*mt:CoI*, *mt:CoIII*, and *CoIV*); and complex V (*ATP syn-β* and *VhaPPA1-1*). Together, our results suggest that the endo-siRNA pathway controls, either directly or indirectly, biological processes such as development and energy metabolism.

### ANALYSIS OF UP-REGULATED GENES MATCHING WITH PREVIOUSLY IDENTIFIED ENDO-siRNAs

Loss of the key components Dcr-2 and Ago-2 of the endo-siRNA pathway causes up-regulation of target mRNAs due to the decreased levels of complementary endo-siRNAs [Czech et al., 2008; Okamura et al., 2008]. Therefore, the up-regulated genes in *dcr-2* null mutant pupae were of greatest interest, because they could be targets of endo-siRNAs. We compared the mRNA sequences of these genes with previously reported endo-siRNA sequences [Czech et al., 2008; Ghildiyal et al., 2008; Kawamura et al., 2008]. Among 306 genes up-regulated in the *dcr-2* mutants, the mRNA transcripts of 117 genes were perfectly complementary to endo-siRNAs (Supplementary Table VII). To validate the differential expression of these endo-siRNA-matching genes obtained from microarray analysis, five genes (*Rm62*, *Su(P)*, *mthl8*, *w*, and *His2B:CG33910*) were randomly chosen and their expression levels were determined by quantitative RT-PCR. Although the extent of the changes was not identical to the microarray results, the transcript levels of all the genes, except for *His2B:CG33910*, were significantly increased in *dcr-2* null mutant pupae, compared with controls (Fig. 4A). It should be noted that although the isogenic control and *dcr-2* mutant animals carry a mutation in the *w* locus and have a white eye color that exhibits strong loss of *w* activity, *w* transcripts were detected in both genotypes by microarray and quantitative RT-PCR but at a higher level in the *dcr-2* mutant pupae than in the controls. We further verified expression of the *w* transcript by RT-PCR analysis performed on two additional different regions of a single *w* transcript annotated in the FlyBase and observed a substantial reduction of *w* transcript levels in control pupae of *dcr-2* mutants compared with those in $w^+$ Canton-S pupae (data not shown). Next, we investigated whether increased transcript levels of the selected genes in *dcr-2* null mutant pupae were accompanied by decreased levels of their complementary endo-siRNAs. Semi-quantitative RT-PCR analysis showed that loss of *dcr-2* function led to a reduction in levels of all tested endo-siRNAs, including *esi-2* siRNA, an endo-siRNA for which biogenesis was previously shown to be dependent on Dcr-2, compared with controls [Czech et al., 2008] (Fig. 4B). By contrast, *dcr-2* mutation had no effect on miRNA biogenesis as shown with the miRNA miR-8, a finding that is consistent with an essential role for Dcr-2 in biogenesis of siRNAs rather than miRNAs [Lee et al., 2004] (Fig. 4B). These results suggest that the observed up-regulation of transcripts may be a consequence of down-regulation of the corresponding endo-siRNAs. Endo-siRNAs are thought to exert their regulatory effects by homology-mediated mRNA cleavage based on previous observations in vitro and in vivo [Czech et al., 2008; Kawamura et al., 2008; Okamura et al., 2008]. However, the extent to which the elevated transcript levels observed in the *dcr-2* mutant were caused by reduced endo-siRNA-directed cleavage activity arising from a decrease in abundance of the corresponding endo-siRNAs remains to be determined.

According to the biological process, 26 of the candidate target genes belonged to five significantly enriched categories (Fig. 5; Supplementary Table VIII). Similar to the functional categories of all the up-regulated genes, most endo-siRNA-matching up-regulated genes were mainly involved in energy metabolism, including generation of precursor metabolites and energy, the coenzyme metabolic process, monovalent inorganic cation transport, the

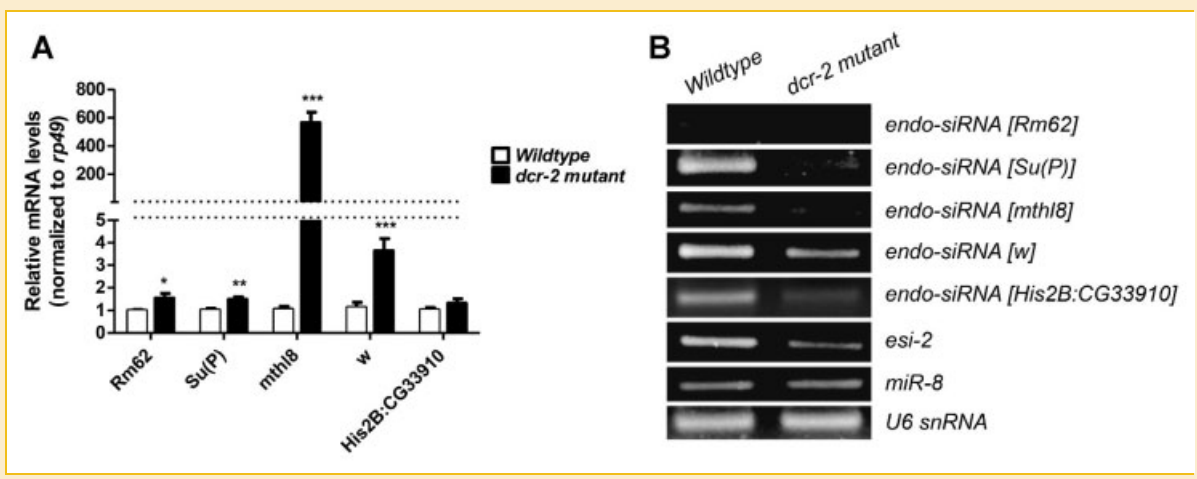


Fig. 4. Comparison of the transcript levels (A) of selected candidate target genes of endo-siRNAs as well as the levels of the corresponding endo-siRNAs (B) between wild-type and *dcr-2* null mutant pupae. A: Increase in abundance of endo-siRNA-matching transcripts in the *dcr-2* mutant. The steady-state levels of transcripts from the indicated genes were determined by quantitative RT-PCR and were normalized to *rp49* transcript levels, which served as an internal control. The normalized transcript levels of each gene were compared between the wild type and *dcr-2* mutant. Data are shown as the mean ± SEM from eight independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, compared with the wild type by Student's *t*-test. B: Decrease in abundance of endo-siRNAs in the *dcr-2* mutant as assessed by semi-quantitative RT-PCR analyses. Endo-siRNA *esi-2* and miRNA miR-8 were used as positive and negative controls, respectively. U6 snRNA served as an internal control. Representative images of three independent experiments are shown. Endo-siRNAs targeting transcripts shown in (A) are indicated according to their cognate genes, which are shown in brackets.

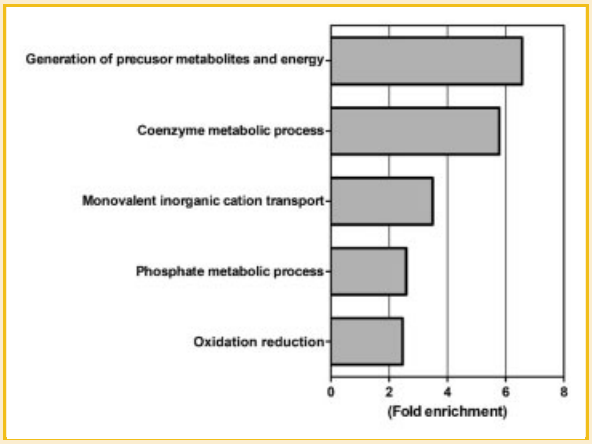


Fig. 5. Functional enrichment analysis of the candidate endo-siRNA target genes for biological processes. Among the 117 potential endo-siRNA targets, 26 were significantly enriched for five functional categories. Data are shown as fold enrichment value.

phosphate metabolic process, and oxidation reduction. Additionally, regarding the KEGG database, the citric acid cycle and OXPHOS pathway were enriched in these genes (Fig. 3). *CG11876*, *CG1516*, *CG5028*, *Nc73EF*, and *Scsα* are involved in the citric acid cycle, whereas *ND75*, *CG40002*, *SdhB*, *CG4769*, *CG4169*, *mt:CoI*, *mt:CoIII*, *ATP syn-β*, and *VhaPPA1-1* are associated with OXPHOS.

### REDUCED ATP LEVELS IN DCR-2 MUTANTS

Because most endo-siRNA-matching up-regulated genes were associated with energy metabolism, including the citric acid cycle and OXPHOS, we predicted that mitochondrial metabolic functions could be altered in *dcr-2* null mutant pupae. To test this possibility, we measured ATP levels as an indicator of mitochondrial function in the wild type and *dcr-2* mutants. The ATP levels were significantly reduced in *dcr-2* null mutant pupae compared with controls ($P < 0.01$; Fig. 6), indicating that mitochondrial energy production is impaired by loss of *dcr-2* function.

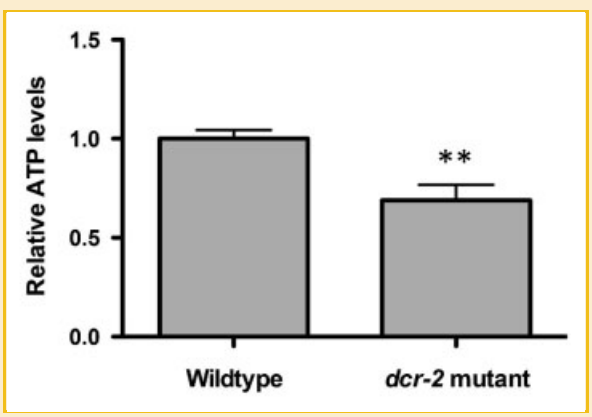


Fig. 6. ATP levels are reduced in *dcr-2* null mutant pupae. Whole-animal ATP levels were measured in wild-type and *dcr-2* mutant pupae and normalized to protein content. Data are presented as the mean ± SEM from nine independent experiments. $^{**}P < 0.01$, compared with the wild type by Student's *t*-test.

## DISCUSSION

In our previous study, *dcr-2* null mutant flies had hypersensitivity to various stresses and a shortened lifespan with altered carbohydrate and lipid metabolism [Lim et al., 2011]. To identify genes or physiological systems associated with the phenotypes of *dcr-2* mutants, we performed microarray experiments on *dcr-2* null mutant pupae in which both *dcr-2* and endo-siRNAs would otherwise have been highly expressed. Analysis of gene expression between the *dcr-2* mutants and isogenic controls identified 663 genes with at least twofold differential expression. Of these, 357 genes were down-regulated and 306 genes were up-regulated in *dcr-2* mutants compared with controls. Most of the down-regulated genes were related to development, including cell differentiation and growth. The *dcr-2* gene is constitutively expressed throughout development, suggesting pleiotropic effects of the mutated gene. Nevertheless, *dcr-2* null mutants are viable and fertile with no overt phenotypes [Lee et al., 2004], suggesting the down-regulated genes have no significant effects at the organismal level. Genes normally silenced by endo-siRNAs are presumed to be up-regulated in *dcr-2* mutants due to derepression of gene expression. Therefore, the down-regulated genes in *dcr-2* null mutant pupae may be attributable in part to a secondary effect arising from a failure of repressing key regulatory genes due to defective endo-siRNA biogenesis.

In the present study, our interest is focused on analysis of genes up-regulated in *dcr-2* null mutant pupae because they could be endo-siRNA targets. Interestingly, most of these genes encode proteins related to energy metabolism. Importantly, upon performing KEGG pathway analysis, the most significantly enriched pathways were the citric acid cycle and OXPHOS pathway. Therefore, these aspects of mitochondrial function are thought to be a significant difference between the wild type and *dcr-2* mutants. Identification of genes that could be directly regulated by the endo-siRNA pathway is essential to elucidate the cellular functions associated with endo-siRNAs. Among the 306 up-regulated genes, the mRNA transcripts of 117 genes were found to have perfect target sequences for endo-siRNAs. This number could be further increased to 216 when we allowed up to 2-nt mismatches between mRNAs and endo-siRNAs (data not shown). The up-regulation of numerous genes matching endo-siRNAs by loss of *dcr-2* function suggests broad usage of endo-siRNAs as endogenous regulators of gene expression. An increase in expression of several of these endo-siRNA-matching genes in *dcr-2* null mutant pupae was confirmed by quantitative RT-PCR. Moreover, semi-quantitative RT-PCR analysis showed concomitant decrease in the levels of the corresponding endo-siRNAs in the *dcr-2* mutants. Similar to KEGG analysis of all the up-regulated genes, KEGG analysis of the endo-siRNA-matching up-regulated genes identified the citric acid cycle and OXPHOS pathway as the most significant pathways affected by loss of *dcr-2* function.

One of the major alterations in the *dcr-2* mutants is up-regulation of genes involved in the citric acid cycle. *CG11876* encoding a subunit of pyruvate dehydrogenase complex is associated with aging in *C. elegans* [Stacpoole, 2012], and *CG5028* coding for $NAD^+$-dependent isocitrate dehydrogenase has been implicated in

resistance to oxidative stress and lifespan in *Drosophila* [Da Cunha and de Oliveira, 1996]. *Indy* (I'm not dead yet), another endo-siRNA-matching gene that is up-regulated in *dcr-2* mutants, is also linked to the citric acid cycle. *Indy* encodes a transporter of the citric acid cycle intermediates found primarily in *Drosophila* tissues important for metabolism [Rogina et al., 2000; Knauf et al., 2002]. Decreased levels of *Indy* transcripts had a strong positive effect on lifespan extension, and *Indy* long-lived flies showed decreased lipid storage and starvation resistance [Wang et al., 2009]. What is the physiological effect of higher expression levels of the citric acid cycle genes observed in *dcr-2* null mutants compared with wild-type controls? Cellular bioenergetics relies on the activity of the citric acid cycle because it provides intermediates for fatty acid synthesis and precursors of protein synthesis as well as reducing equivalents (NADH and FADH) for ATP synthesis. Such alterations in expression of the citric acid cycle genes may likely have an effect on the levels of the citric acid cycle intermediates. In agreement with this idea, our recent study demonstrated that loss of *dcr-2* function resulted in a decrease in triglyceride levels, which in turn led to hypersensitivity to starvation [Lim et al., 2011]. Additionally, up-regulation of the citric acid cycle genes may increase the activity of the citric acid cycle, leading to enhanced delivery of reducing equivalents to the mitochondrial ETC with a resulting increase in reactive oxygen species (ROS).

Another striking feature of our results is a significant up-regulation of genes encoding multiple components of the mitochondrial OXPHOS pathway, which is responsible for most of the energy needs in animals. Mitochondrial bioenergetics is also linked to oxidative stress, which often negatively correlates with lifespan across different animal species [Sedensky and Morgan, 2006]. The proteins of the mitochondrial ETC are organized into five complexes: I (NADH/ubiquinone oxidoreductase), II (succinate dehydrogenase), III (ubiquinol-cytochrome *c* reductase), IV (cytochrome *c* oxidase), and V (ATP synthase) [Saraste, 1999]. Among these respiratory complexes, complex II also links the activity of the citric acid cycle to electron transport in the membrane. Complexes I, III, and IV are involved in creating an electrochemical gradient that is used by complex V to drive ATP synthesis. In *Drosophila*, complexes I and III have been proposed to produce ROS under normal physiological conditions [Balaban et al., 2005]. In both *C. elegans* and *Drosophila*, RNAi-mediated knockdown of ETC complexes I, III, IV, and V has been found to confer longevity [Dillin et al., 2002; Lee et al., 2003; Copeland et al., 2009], although long-lived flies with reduced ETC gene expression did not consistently show increased resistance to oxidative and starvation stresses [Copeland et al., 2009]. Similar effects of ETC dysfunction on lifespan were observed in mice [Liu et al., 2005; Dell'agnello et al., 2007; Lapointe and Hekimi, 2008]. Additionally, flies and worms mutant for the *SdhB* gene encoding the subunit b of complex II were found to be hypersensitive to oxygen and short-lived [Ishii et al., 1998; Walker et al., 2006]. Aging is a complex process involving tight regulation of gene expression, and multicellular organisms are challenged with oxidative stress during their life. Our recent study revealed that defective endo-siRNA production is associated with a reduction in oxidative stress resistance and lifespan in *Drosophila* [Lim et al., 2011]. Therefore, up-regulation of genes encoding components of complexes I–V of the ETC observed in *dcr-2* null mutant pupae may have an effect on mitochondrial oxidative metabolism such as energy production and ROS generation, thereby affecting aging and susceptibility to oxidative stress. Consistent with this idea, *dcr-2* null mutant pupae had lower ATP content compared with controls, indicating that mitochondrial energy production is impaired in these mutants. Mitochondrial dysfunction is associated with aging in invertebrates and vertebrates [Lenaz et al., 2000]. The increase in ETC gene expression may also stimulate mitochondrial respiration and thus enhance spontaneous "leakage" of electrons from the ETC, ultimately leading to production of free radicals at higher levels [McCord, 2000]. Because oxidative stress contributes considerably to aging, the reduced lifespan of *dcr-2* null mutants could result from excessive oxidative stress. Although it is not yet clear how the *dcr-2*-dependent changes in expression of genes associated with the citric acid cycle and OXPHOS pathway affects the physiology of an animal, including complex traits like aging, it should be considered that the net effect of a complex interaction among enzymes, hormones, and other effectors, as well as changes in the levels of gene expression in many organs and tissues of an animal ultimately determines the final phenotype.

Endo-siRNAs might serve as endogenous regulators of various biological processes in *Drosophila*. Among these, our microarray analysis of *dcr-2* null mutants highlights significant up-regulation of genes for major metabolic processes, such as the citric acid cycle and OXPHOS pathway in mitochondria. Because metabolic pathways are complex networks, their perturbation can dramatically alter the ability of an animal to maintain its normal physiological functions. The metabolic changes can also lead to homeostatic impairment and thus compromise an animal's ability to handle environmental stresses [Vockley, 2008]. Other studies in *Drosophila* have also reported that the endo-siRNA pathway is crucial for resistance of the embryo to temperature fluctuations [Lucchetta et al., 2009] and that the RNAi machinery is involved in the heat-shock stress response [Cernilogar et al., 2011]. Therefore, the endo-siRNA pathway might have evolved to maintain robustness in an ever-changing environment by modulating gene expression, although how endo-siRNA-mediated regulation could be coordinated is unknown.

Overexpression of *dcr-2*, an opposite circumstance to that investigated in this study, enhances the activity of RNAi triggered by nuclear expressed hairpin dsRNA [Dietzl et al., 2007]. Although we did not investigate the consequences of the opposite condition, it remains to be determined whether *dcr-2* overexpression could promote endo-siRNA biogenesis and thus strengthen endo-siRNA-directed repression. In the present study, we identified transcripts whose levels were changed because of defective endo-siRNA production, providing a panel of candidate target genes of endo-siRNAs. The citric acid cycle and OXPHOS pathway were found to be major metabolic processes that could be affected directly by endo-siRNAs in *Drosophila*, suggesting a potential role for the endo-siRNA pathway in regulation of mitochondrial metabolism. Further study is required to clarify the functional mechanisms of endo-siRNA-matching genes underlying energy homeostasis and protection against aging.

## ACKNOWLEDGMENTS

We thank Q. Liu for the Dcr-2 and R2D2 antibodies. D.H.L. was supported by a Hi Seoul Science/Humanities fellowship from Seoul Scholarship Foundation and S.H. was supported by a grant from the Korea Research Institute of Bioscience and Biotechnology Research Initiative Program. This work was also supported by a National Research Foundation of Korea (NRF) grant funded by the Korea government (Ministry of Education, Science, and Technology [MEST]; 2011-0016863 to Y.S.L.), a Korea University grant (to Y.S.L.), and the Pioneer Research Center Program through the NRF funded by MEST (2012-0000433 to S.J.H.).

## REFERENCES

Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ. 1997. Gapped BLAST and PSI-BLAST: A new generation of protein database search programs. Nucleic Acids Res 25:3389–3402.

Balaban RS, Nemoto S, Finkel T. 2005. Mitochondria, oxidants, and aging. Cell 120:483–495.

Brand AH, Perrimon N. 1993. Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. Development 118:401–415.

Burkhart KB, Guang S, Buckley BA, Wong L, Bochner AF, Kennedy S. 2011. A pre-mRNA-associating factor links endogenous siRNAs to chromatin regulation. PLoS Genet 7:e1002249.

Cernilogar FM, Onorati MC, Kothe GO, Burroughs AM, Parsi KM, Breiling A, Lo Sardo F, Saxena A, Miyoshi K, Siomi H, Siomi MC, Carninci P, Gilmour DS, Corona DF, Orlando V. 2011. Chromatin-associated RNA interference components contribute to transcriptional regulation in Drosophila. Nature 480:391–395.

Clark IE, Dodson MW, Jiang C, Cao JH, Huh JR, Seol JH, Yoo SJ, Hay BA, Guo M. 2006. Drosophila pink1 is required for mitochondrial function and interacts genetically with parkin. Nature 441:1162–1166.

Copeland JM, Cho J, Lo T, Jr., Hur JH, Bahadorani S, Arabyan T, Rabie J, Soh J, Walker DW. 2009. Extension of Drosophila life span by RNAi of the mitochondrial respiratory chain. Curr Biol 19:1591–1598.

Czech B, Malone CD, Zhou R, Stark A, Schlingeheyde C, Dus M, Perrimon N, Kellis M, Wohlschlegel JA, Sachidanandam R, Hannon GJ, Brennecke J. 2008. An endogenous small interfering RNA pathway in Drosophila. Nature 453:798–802.

Da Cunha GL, de Oliveira AK. 1996. Citric acid cycle: A mainstream metabolic pathway influencing life span in Drosophila melanogaster? Exp Gerontol 31:705–715.

Dai M, Wang P, Boyd AD, Kostov G, Athey B, Jones EG, Bunney WE, Myers RM, Speed TP, Akil H, Watson SJ, Meng F. 2005. Evolving gene/transcript definitions significantly alter the interpretation of GeneChip data. Nucleic Acids Res 33:e175.

Dell'agnello C, Leo S, Agostino A, Szabadkai G, Tiveron C, Zulian A, Prelle A, Roubertoux P, Rizzuto R, Zeviani M. 2007. Increased longevity and refractoriness to Ca(2+)-dependent neurodegeneration in Surf1 knockout mice. Hum Mol Genet 16:431–444.

Dietzl G, Chen D, Schnorrer F, Su KC, Barinova Y, Fellner M, Gasser B, Kinsey K, Oppel S, Scheiblauer S, Couto A, Marra V, Keleman K, Dickson BJ. 2007. A genome-wide transgenic RNAi library for conditional gene inactivation in Drosophila. Nature 448:151–156.

Dillin A, Hsu AL, Arantes-Oliveira N, Lehrer-Graiwer J, Hsin H, Fraser AG, Kamath RS, Ahringer J, Kenyon C. 2002. Rates of behavior and aging specified by mitochondrial function during development. Science 298: 2398–2401.

Fagegaltier D, Bouge AL, Berry B, Poisot E, Sismeiro O, Coppee JY, Theodore L, Voinnet O, Antoniewski C. 2009. The endogenous siRNA pathway is involved in heterochromatin formation in Drosophila. Proc Natl Acad Sci USA 106:21258–21263.

Farazi TA, Juranek SA, Tuschl T. 2008. The growing catalog of small RNAs and their association with distinct Argonaute/Piwi family members. Development 135:1201–1214.

Ghildiyal M, Zamore PD. 2009. Small silencing RNAs: An expanding universe. Nat Rev Genet 10:94–108.

Ghildiyal M, Seitz H, Horwich MD, Li C, Du T, Lee S, Xu J, Kittler EL, Zapp ML, Weng Z, Zamore PD. 2008. Endogenous siRNAs derived from transposons and mRNAs in Drosophila somatic cells. Science 320:1077–1081.

Golden DE, Gerbasi VR, Sontheimer EJ. 2008. An inside job for siRNAs. Mol Cell 31:309–312.

Hegde P, Qi R, Abernathy K, Gay C, Dharap S, Gaspard R, Hughes JE, Snesrud E, Lee N, Quackenbush J. 2000. A concise guide to cDNA microarray analysis. Biotechniques 29:548–550; 552–554, 556 passim.

Huang da W, Sherman BT, Lempicki RA. 2009. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. Nat Protoc 4:44–57.

Ishii N, Fujii M, Hartman PS, Tsuda M, Yasuda K, Senoo-Matsuda N, Yanase S, Ayusawa D, Suzuki K. 1998. A mutation in succinate dehydrogenase cytochrome b causes oxidative stress and ageing in nematodes. Nature 394:694–697.

Kawamura Y, Saito K, Kin T, Ono Y, Asai K, Sunohara T, Okada TN, Siomi MC, Siomi H. 2008. Drosophila endogenous small RNAs bind to Argonaute 2 in somatic cells. Nature 453:793–797.

Kent WJ. 2002. BLAT—The BLAST-like alignment tool. Genome Res 12:656–664.

Knauf F, Rogina B, Jiang Z, Aronson PS, Helfand SL. 2002. Functional characterization and immunolocalization of the transporter encoded by the life-extending gene Indy. Proc Natl Acad Sci USA 99:14315–14319.

Lapointe J, Hekimi S. 2008. Early mitochondrial dysfunction in long-lived Mclk1+/− mice. J Biol Chem 283:26217–26227.

Lee T, Luo L. 1999. Mosaic analysis with a repressible cell marker for studies of gene function in neuronal morphogenesis. Neuron 22:451–461.

Lee SS, Lee RY, Fraser AG, Kamath RS, Ahringer J, Ruvkun G. 2003. A systematic RNAi screen identifies a critical role for mitochondria in C. elegans longevity. Nat Genet 33:40–48.

Lee YS, Nakahara K, Pham JW, Kim K, He Z, Sontheimer EJ, Carthew RW. 2004. Distinct roles for Drosophila Dicer-1 and Dicer-2 in the siRNA/miRNA silencing pathways. Cell 117:69–81.

Lenaz G, D'Aurelio M, Merlo Pich M, Genova ML, Ventura B, Bovina C, Formiggini G, Parenti Castelli G. 2000. Mitochondrial bioenergetics in aging. Biochim Biophys Acta 1459:397–404.

Lim do H, Kim J, Kim S, Carthew RW, Lee YS. 2008. Functional analysis of dicer-2 missense mutations in the siRNA pathway of Drosophila. Biochem Biophys Res Commun 371:525–530.

Lim DH, Oh CT, Lee L, Hong JS, Noh SH, Hwang S, Kim S, Han SJ, Lee YS. 2011. The endogenous siRNA pathway in Drosophila impacts stress resistance and lifespan by regulating metabolic homeostasis. Febs Lett 585:3079–3085.

Liu X, Jiang N, Hughes B, Bigras E, Shoubridge E, Hekimi S. 2005. Evolutionary conservation of the clk-1-dependent mechanism of longevity: Loss of mclk1 increases cellular fitness and lifespan in mice. Genes Dev 19:2424–2434.

Liu X, Jiang F, Kalidas S, Smith D, Liu Q. 2006. Dicer-2 and R2D2 coordinately bind siRNA to promote assembly of the siRISC complexes. RNA 12:1514–1520.

Lucchetta EM, Carthew RW, Ismagilov RF. 2009. The endo-siRNA pathway is essential for robust development of the Drosophila embryo. PLoS ONE 4:e7576.

Marques JT, Kim K, Wu PH, Alleyne TM, Jafari N, Carthew RW. 2010. Loqs and R2D2 act sequentially in the siRNA pathway in Drosophila. Nat Struct Mol Biol 17:24–30.

McCord JM. 2000. The evolution of free radicals and oxidative stress. Am J Med 108:652–659.

Okamura K, Lai EC. 2008. Endogenous small interfering RNAs in animals. Nat Rev Mol Cell Biol 9:673–678.

Okamura K, Chung WJ, Ruby JG, Guo H, Bartel DP, Lai EC. 2008. The Drosophila hairpin RNA pathway generates endogenous short interfering RNAs. Nature 453:803–806.

Pavelec DM, Lachowiec J, Duchaine TF, Smith HE, Kennedy S. 2009. Requirement for the ERI/DICER complex in endogenous RNA interference and sperm development in Caenorhabditis elegans. Genetics 183:1283–1295.

Pek JW, Kai T. 2011. DEAD-box RNA helicase Belle/DDX3 and the RNA interference pathway promote mitotic chromosome segregation. Proc Natl Acad Sci USA 108:12007–12012.

Ro S, Yan W. 2010. Detection and quantitative analysis of small RNAs by PCR. Methods Mol Biol 629:295–305.

Rogina B, Reenan RA, Nilsen SP, Helfand SL. 2000. Extended life-span conferred by cotransporter gene mutations in Drosophila. Science 290:2137–2140.

Saraste M. 1999. Oxidative phosphorylation at the fin de siecle. Science 283:1488–1493.

Sedensky MM, Morgan PG. 2006. Mitochondrial respiration and reactive oxygen species in mitochondrial aging mutants. Exp Gerontol 41:237–245.

Sharma Y, Cheung U, Larsen EW, Eberl DF. 2002. PPTGAL, a convenient Gal4 P-element vector for testing expression of enhancer fragments in drosophila. Genesis 34:115–118.

Song R, Hennig GW, Wu Q, Jose C, Zheng H, Yan W. 2011. Male germ cells express abundant endogenous siRNAs. Proc Natl Acad Sci USA 108:13159–13164.

Stacpoole PW. 2012. The pyruvate dehydrogenase complex as a therapeutic target for age-related diseases. Aging Cell 11:371–377.

Tweedie S, Ashburner M, Falls K, Leyland P, McQuilton P, Marygold S, Millburn G, Osumi-Sutherland D, Schroeder A, Seal R, Zhang H. 2009. FlyBase: Enhancing Drosophila Gene Ontology annotations. Nucleic Acids Res 37:D555–D559.

Vockley J. 2008. Metabolism as a complex genetic trait, a systems biology approach: Implications for inborn errors of metabolism and clinical diseases. J Inherit Metab Dis 31:619–629.

Walker DW, Hajek P, Muffat J, Knoepfle D, Cornelison S, Attardi G, Benzer S. 2006. Hypersensitivity to oxygen and shortened lifespan in a Drosophila mitochondrial complex II mutant. Proc Natl Acad Sci USA 103:16382–16387.

Wang PY, Neretti N, Whitaker R, Hosier S, Chang C, Lu D, Rogina B, Helfand SL. 2009. Long-lived Indy and calorie restriction interact to extend life span. Proc Natl Acad Sci USA 106:9262–9267.