ORIGINAL

Adenylate kinase 2 deficiency limits survival and regulates various genes during larval stages of *Drosophila melanogaster*

Taigo Horiguchi[1*], Miyuki Fuka[2*], Koichi Fujisawa[1,3], Ayako Tanimura[1], Keiko Miyoshi[1], Ryutaro Murakami[2], and Takafumi Noma[1]

[1]*Department of Molecular Biology, Institute of Health Biosciences, the University of Tokushima Graduate School,* [2]*Department of Applied Molecular Bioscience, Yamaguchi University,* [3]*Center for Reparative Medicine, Yamaguchi University School of Medicine*

Abstract : Adenylate kinase isozyme 2 (AK2) is located in mitochondrial intermembrane space and regulates energy metabolism by reversibly converting ATP and AMP to 2 ADPs. We previously demonstrated that disruption of the *Drosophila melanogaster* AK2 gene (*Dak2*) resulted in growth arrest during the larval stage and subsequent death. Two other groups found that human AK2 mutations cause reticular dysgenesis, a form of severe combined immunodeficiency (SCID) that is associated with severe hematopoietic defects and sensorineural deafness. However, the mechanisms underlying differential outcomes of AK2 deficiency in *Drosophila* and human systems remain unknown. In this study, effects of tissue-specific inactivation of the *Dak2* gene on *Drosophila* development were analyzed using RNAi-mediated gene knockdown. In addition, to investigate the roles of AK2 in the regulation of gene expression during development, microarray analysis was performed using RNA from first and second instar larvae of *Dak2*-deficient mutant and wild-type *D. melanogaster*. Knockdown of *Dak2* in all germ layers caused cessation of growth and subsequent death of flies. Microarray analysis revealed that *Dak2* deficiency downregulates various genes, particularly those involved in the proteasomal function and in mitochondrial translation machinery. These data indicate that adenine nucleotide interconversion by Dak2 is crucial for developmental processes of *Drosophila melanogaster*. J. Med. Invest. 61 : 137-150, February, 2014

Keywords **:** *Dak2, gene expression, in silico analysis, microarray, mitochondria*

INTRODUCTION

The enzyme adenylate kinase (AK) reversibly converts ATP+AMP to 2 ADPs and plays a role in cellular energy homeostasis (1). To date, 8 vertebrates AK isoenzymes (AK1-AK8) have been reported (2). Among these, AK2 is uniquely located in the mitochondrial intermembrane space, and is highly expressed in human liver, kidney, and heart tissues (3). AK2 provides ADP to the adenine nucleotide translocator in the inner membrane of mitochondria, which promotes the exchange of ADP with ATP.

* These authors contributed equally to this work.

List of Abbreviations
AARSs, aminoacyl tRNA synthetases ; AK, adenylate kinase ; AMPs, antimicrobial peptides ; FAC, functional annotation cluster ; GO, gene ontology ; qRT-PCR, quantitative reverse transcription polymerase chain reaction ; RD, reticular dysgenesis ; RNAi, RNA interference ; ROS, reactive oxygen species.

Received for publication November 29, 2013 ; accepted January 6, 2014.

Address correspondence and reprint requests to Takafumi Noma, MD, PhD, Professor, Department of Molecular Biology, Institute of Health Biosciences, the University of Tokushima Graduate School, 3-18-15 Kuramoto, Tokushima, 770-8504, Japan and Fax : +81-88-633-7326.

Multicellular organisms contain other high-energy phosphoryl transfer systems that are mediated by either creatine kinase or arginine kinase (ArgK) as a phosphagen kinase in mitochondrial intermembrane space of various animal cells, except for echinoderm (4).

We previously examined the metabolic roles of AK2 *in vivo*, by analyzing phenotypes resulted from mutation of the *Drosophila* AK2 gene, *Dak2*. *Drosophila* is a suitable model system for functional studies of AK2 because their mitochondrial intermembrane space lacks ArgK (5). These data indicated that *Dak2* is essential for growth and development of *Drosophila melanogaster*. Although no visible defects in the development of homozygous *Dak2*-deficient embryos were observed, hatched larvae failed to grow and died during the 1st and 2nd instar larval stages, presumably due to the depletion of maternally derived *Dak2*. Two other studies of human *AK2* gene mutant phenotypes have been reported. Specifically, Pannicke *et al.* reported that reticular dysgenesis (RD), a type of severe combined immunodeficiency, is caused by *AK2* gene mutations, with increased apoptosis and reactive oxygen species (ROS) production and decreased mitochondrial membrane potential (6). Lagresle-Peyrou *et al.* demonstrated profound hematopoietic defects and sensorineural deafness due to AK2 deficiency (7). Phenotypes of AK2 deficiency, such as viability, differ between human and *Drosophila* ; whereas the ensuing tissue-specific metabolic disorders in humans do not affect viability, these are lethal during larval stages in *Drosophila*. It was also recently reported that AK2 functions as a regulator of unfolded protein response (UPR) in the endoplasmic reticulum (ER) during cellular differentiation (8). However, these AK2-mediated metabolic changes remain largely unclear.

To clarify the metabolic roles of AK2, we initially knocked down *Dak2* gene expression during development and examined viability. Subsequently, we analyzed the effects of *Dak2* deficiency on gene expression using DNA microarrays.

METHODS

D. melanogaster stocks and breeding

The *D. melanogaster* strain containing a P-element (P-lacW) insertion at the *Dak2* locus ($Dak2^{EP2149}$/*CyO*) was obtained from the Szeged Drosophila Stock Center (Hungary). The *CyO* balancer was replaced with a balancer carrying an actin-GFP marker ($Dak2^{EP2149}$/ *CyO*, *Act-GFP*). $Dak2^{EP2149}$/ $Dak2^{EP2149}$ ($Dak2^{-/-}$) homozygous embryos and larvae were collected under a fluorescent microscope as described previously (5), and homozygous ($Dak2^{-/-}$) Ll and L2 larvae were collected for RNA isolation.

RNAi for Dak1, Dak2, and Dak3 during development

UAS-Dak1 RNAi strain (*P{TRiP.GL00177}attP2*), *UAS-Dak2* strain (*P{TRiP.GL00l96}attP2/TM3*), and *UAS-Dak3* strain (*P{TRiP.GL00490}attP2*) were provided by Bloomington Stock Center, U.S.A. Breeding of these RNAi strains with tissue-specific *GAL4* strains led to overexpression of hairpin-type double-stranded RNAs of *Dak1*, *Dak2*, and *Dak3*, respectively. The following *GAL4* strains with various regional specificities were used to induce RNAi in particular tissues and organs : *tubulin-GAL4* for ubiquitous expression, *da-GAL4* for ectodermal tissues, *24B-GAL4* for all mesodermal tissues, *48Y-GAL4* for endodermal tissues, *byn-GAL4* for ectodermal hindgut (9), *Mef2-GAL4* for somatic and visceral muscles, and *elav-GAL4* for central nervous system. These GAL4 strains were used in combination with *UAS-Dicer* construct for enhancement of RNAi effect. All these strains were provided by Bloomington Drosophila Stock Center and DGRC Kyoto Stock Center (Japan) unless otherwise indicated.

DNA microarray

Pools of 1st and 2nd instar larvae of wild-type and $Dak2^{-/-}$ mutants (2 mg each) were homogenized, and total RNA was extracted using RNeasy Mini Kits (Qiagen, Netherlands) according to the supplier's instructions. Total RNA (5 μg) from each sample was used for microarray analyses (GeneChip Drosophila Genome 2.0, Affymetrix, U.S.A).

Bioinformatic analyses of microarray data

The Gene Ontology (GO) classification system was used to analyze microarray data. The Database for Annotation, Visualization, and Integrated Discovery (DAVID) v6.7 (http : //david.abcc.ncifcrf.gov/) (10, 11) was used to evaluate biological significance and importance using the GO functional annotation cluster (FAC) tool. DAVID FAC analysis was conducted on two independent normalized gene lists containing normalized >2-fold upregulated and <0.5-fold downregulated genes. High stringency score parameter was selected to indicate confident

enrichment scores of functional significance and importance of the given pathways. The GO system in DAVID was used to identify enriched biological themes in both gene lists. Pathway tools of the Kyoto Encyclopedia of Genes and Genomes (KEGG ; http : //www.genome.jp/kegg/) were used to map genes of the "proteasome complex" cluster. The DroID database (http : //www.droidb.org/Index.jsp) was applied to obtain transcription factor-target gene interaction data of *D. melanogaster* (12), and interactions were illustrated and analyzed using Cytoscape 2.8.3 (13, 14).

RESULTS

1. Knockdown of Dak2 during D. melanogaster development

To investigate tissues and organs that are susceptible to *Dak2* knockdown in *Drosophila*, we performed ubiquitous and tissue-specific RNAi experiments by mating the *UAS-Dak2* RNAi strain with various tissue-specific *GAL4* strains. No adult flies or pupae developed when ubiquitous *Dak2* RNAi was induced with *tubulin-GAL4* driver strain. *Dak2* RNAi induced in particular tissues, such as ectodermal tissues (with *da-GAL4*), mesodermal tissues (with *24B-GAL4*), endodermal tissues (with *48Y-GAL4*), visceral and somatic muscles (with *Mef2-GAL4*), ectodermal hindgut (with *byn-GAL4*), as well as central nervous system (with *elav-GAL4*) all resulted in lethality during larval stages (Table 1). Thus, lethality caused by *Dak2* RNAi does not due to damage of some particular tissues or organs. Rather, *Dak2* is assumed to be essential for general cellular functions required for cell survival. In contrast, when RNAi for *Dak1* and *Dak3* were induced similarly, with reduction of mRNA less than 25% for each gene, these were no marked effects on viability (Table1).

Microarray analysis of Dak2 knockout mutants

Tissue-specific knockdown of *Dak2* led to eventual larval mortality, suggesting that AK2 plays a fundamental active role in developmental processes, including cell division, differentiation, and morphogenesis. To investigate gene expression changes in *Dak2* deficient larvae prior to death, we performed microarray analysis in AK2-deficient *D. melanogaster* (*Dak2*$^{-/-}$ mutants). These experiments identified 1059 upregulated (> 2-fold) and 859 downregulated (< 0.5-fold) genes compared with the wild-type control. Microarray data were confirmed using qPCR analyses of selected genes (data not shown).

Expression data was analyzed using the DAVID FAC tool, which identified 140 enriched functional clusters from 1059 upregulated genes (> 2.0-fold) under high stringency condition. Among these, the "polysaccharide binding" cluster showed the highest enrichment score (8.26), followed by "glycosyl hydrolases, family 13" (4.45) and "transmission of nerve impulse" (3.98) clusters (Figure 1A). Enrichment scores indicate the biological significance of analyzed gene groups. DAVID FAC analysis of 859 significantly downregulated genes (< 0.5-fold) revealed 128 enriched functional clusters under high stringency conditions. The "proteasome complex" cluster was the most significant biological process, followed by "membrane-enclosed lumen," "mitochondrial matrix," and "aminoacyl-tRNA ligase activity" clusters (Figure 1B).

2.1. Peritrophic membranes and other antimicrobial molecules

As shown in figure 2A, 35 genes were included in the "polysaccharide binding" cluster with common GO terms as follows : chitin binding protein, ChitBD2, chitin binding, chitin metabolic process, aminoglycan metabolic process, polysaccharide metabolic process, polysaccharide binding, and pattern binding.

Among these genes, 10 were structural constituents of peritrophic membranes, which comprise chitin and proteins that cover the midgut to facilitate digestive process and protect from invading microorganisms and parasites (15, 16). Chitin binding proteins (12 gene products of the cluster) and mucins (6 gene products of the cluster) are also peritrophic

Table 1 Effects of RNAi for adenylate kinase isozymes on viability during development of *D. melanogaster*.

	Whole body	Ectoderm	Mesoderm	Endoderm	Visceral muscle	Hindgut	Nerve
Dak1 RNAi	+	+	+	+	+	+	+
Dak2 RNAi	-	-	-	-	-	-	-
Dak3 RNAi	+	+	+	+	+	+	+

+ or - indicate viable or lethal, respectively.

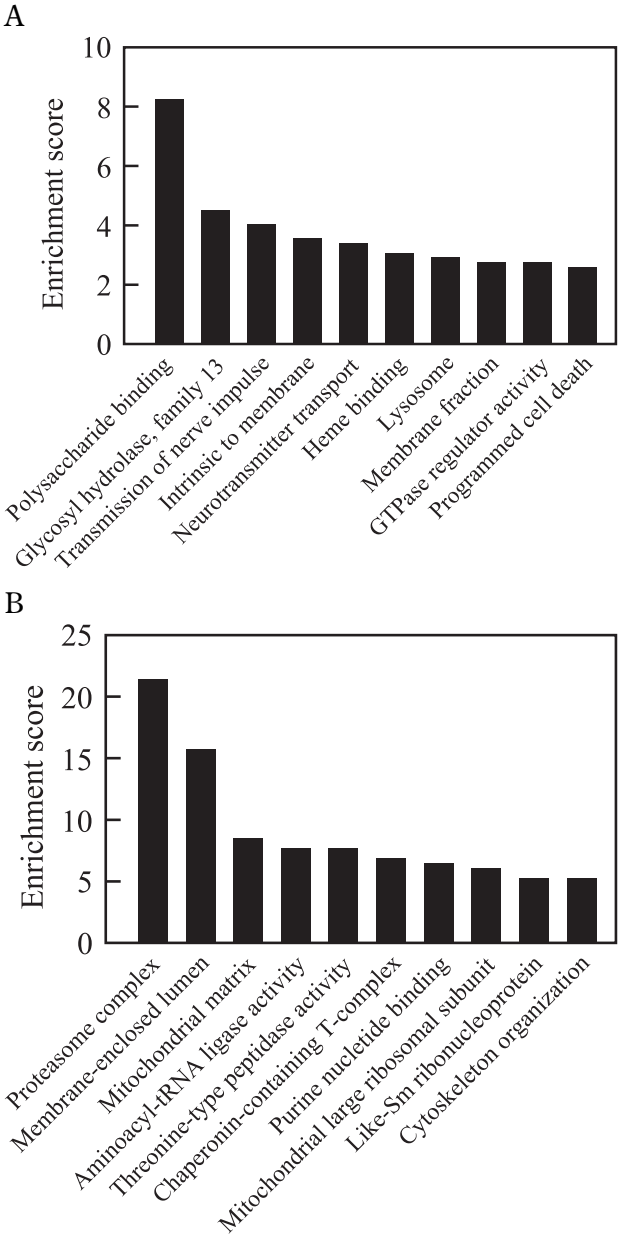


Figure 1 DAVID Functional Annotation Cluster (FAC) analyses of gene expression patterns in *Dak2* knockout flies.
(A) Major functional annotation clusters (FACs) for upregulated genes (> 2.0-fold). (B) Major FACs for downregulated genes (< 0.5-fold). Significance is determined by corresponding enrichment scores.

membrane components and were included in this cluster. In addition, the peptidoglycan-recognition proteins *PGRP-LC* and *PGRP-LF* (17), chitinase *Cht9*, the transcription factor *foxo*, the wnt binding protein *Swim*, and the scavenger receptor *Sr-Cl* were included in the cluster. Almost all of these gene products were related to defense responses.

Starvation of *Drosophila* larva affects the expression of many genes, including *Lip3*, *Pepck*, and *Thor* (18, 19). In *Dak2* knockout flies, we found changes in the expression of genes with similar tendencies (*Lip3*, 13.93-fold ; *Pepck*, 3.03-fold ; *Thor*, 3.48-fold). Starvation also induces antimicrobial peptides (AMPs) by activating the transcription factor foxo (20), which regulates some AMPs, and structural components of peritrophic membranes via the transcription factor relish (21). Taken together, these data may suggest that in *Dak2* knockout flies, relish induces AMPs and a number of peritrophic membrane components by activating foxo.

2.2. Glucose metabolism

The "glycosyl hydrolase, family 13" cluster had the second highest enrichment score, and included genes encoding 2 amylases and 6 maltases. Because maltase and amylase coordinately degrade starch into glucose, these genes may be upregulated in response to the mitochondrial dysfunction caused by *Dak2* knockout (Figure 2B). Accordingly, upregulation of the glycolysis enzymes aldolase (2.30-fold) and hexokinase (2.00-fold) were also observed. The expression of 6-phosphofructo-2-kinase (*Pfrx*), which synthesizes fructose 2,6-bisphosphate (a potent stimulator of glycolysis) was also upregulated (2.14-fold) in *Dak2*$^{-/-}$ mutants. This may reflect the metabolic switch from oxidative phosphorylation to less efficient anaerobic glycolysis in *Dak2* knockout fruit flies.

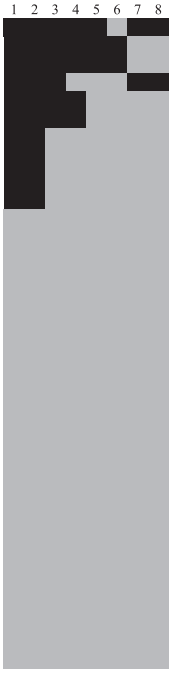
2.3. Synapse transmission

The "synapse transmission" cluster gave the third highest enrichment score (Figure 2C). This cluster included 31 genes, including those encoding Ca^{2+} channel (*cac*) and Ca^{2+} dependent phospholipid binding proteins (*Syt1*, *Syt4*, and *Syt7*), and common GO terms included synaptic transmission, cell-cell signaling, and transmission of nerve impulses. Although it is unclear why genes related to synapse transmission were upregulated under conditions of mitochondrial dysfunction in our microarray data, it has been reported that oxidative stress induced autophagy followed by synapse development (22). Hence, upregulation of several autophagy genes in *Dak2* knockout flies implies that ROS induced synapse development.

2.4. Proteasome

Among clusters of downregulated genes, those related to the proteasome produced the highest enrichment score (21.50 ; Figure 3A). Subsequent KEGG map analyses of these genes (Figure 4) indicated uniform downregulation of many structural components of the proteasome, suggesting lower

A



Symbol	ID	Fold change	Molecular function	Biological process
foxo	CG3143	2.00	Transcription factor	Response to stress
Swim	CG3074	2.46	Wnt-protein binding	Wnt receptor signaling
Sr-CI	CG4099	2.00	Scavenger receptor	Defense response
Cht9	CG10531	2.00	Chitinase	Chitin catabolic process
PGRP-LC	CG4432	2.00	Peptidoglycan receptor	Immune response; response to stress
PGRP-LF	CG4437	3.03	Peptidoglycan recdptor	Negative regulation of JNK cascade
CG7710	CG7710	2.46	Chitin binding	Chitin metabolic process
Peritrophin-15b	CG31893	2.14	Chitin binding	Chitin metabolic process
CG14300	CG14300	2.00	Chitin binding	Chitin metabolic process
CG7715	CG7715	2.46	Chitin binding	Chitin metabolic process
CG6947	CG6947	2.83	structural constituent of peritrophic membrane	Chitin metabolic process
CG17824	CG17824	2.46	structural constituent of peritrophic membrane	Chitin metabolic process
Cht4	CG3986	3.03	Chitin binding	Chitin metabolic process
CG10140	CG10140	2.83	Chitin binding	Chitin metabolic process
CG7017	CG7017	2.14	structural constituent of peritrophic membrane	Chitin metabolic process
Muc11A	CG32656	3.03	extracellular matrix structural constituent	Chitin metabolic process
Muc18A	CG7876	2.00	extracellular matrix structural constituent	Chitin metabolic process
Muc18B	CG7874	6.50	extracellular matrix structural constituent	Chitin metabolic process
Muc26B	CG13990	2.14	extracellular matrix structural constituent	Neuron projection morphogenesis
Muc68E	CG33265	2.83	extracellular matrix structural constituent	Chitin metabolic process
Muc96D	CG31439	2.64	extracellular matrix structural constituent	Chitin metabolic process
CG6996	CG6996	2.83	structural constituent of peritrophic membrane	Chitin metabolic process
CG6403	CG6403	2.14	Chitin binding	Chitin metabolic process
CG5883	CG5883	2.30	structural constituent of peritrophic membrane	Chitin metabolic process
CG17147	CG17147	4.29	structural constituent of peritrophic membrane	Chitin metabolic process
CG7252	CG7252	2.46	structural constituent of peritrophic membrane	Chitin metabolic process
CG14957	CG14957	2.30	Chitin binding	Chitin metabolic process
CG4835	CG4835	2.30	Chitin binding	Chitin metabolic process
Obst-F	CG7306	4.00	structural constituent of peritrophic membrane	Chitin metabolic process
Obst-G	CG9781	2.83	Chitin binding	Chitin metabolic process
Obst-H	CG1717	4.29	Chitin binding	Chitin metabolic process
Obst-J	CG7348	2.14	Chitin binding	Chitin metabolic process
CG7248	CG7248	3.25	structural constituent of peritrophic membrane	Chitin metabolic process
CG13075	CG13075	5.66	unknown	Not known
CG17145	CG17145	3.25	structural constituent of peritrophic membrane	Chitin metabolic process

B

Symbol	ID	Fold change	Molecular function	Biological process
Amyrel	CG8221	4.92	Alpha-amylase activity	Carbohydrate metabolic process
Amy-p	CG18730	2.64	Alpha-amylase activity	Carbohydrate metabolic process
Mal-A1	CG8696	4.92	Alpha-glucosidase activity	Glucose metabolic process
Mal-A3	CG8695	3.25	Alpha-glucosidase activity	Glucose metabolic process
Mal-A4	CG8693	4.00	Alpha-glucosidase activity	Carbohydrate metabolic process
Mal-A5	CG30359	2.64	Catalytic activity	Carbohydrate metabolic process
Mal-A6	CG30360	2.46	Catalytic activity	Carbohydrate metabolic process
Mal-B2	CG14935	3.25	Alpha-glucosidase activity	Carbohydrate metabolic process

C

Symbol	ID	Fold change	Molecular function	Biological process
Nrg	CG1634	2.30	Calcium ion binding	Neuron differentiation
hh	CG4637	3.73	Cell surface binding; protein binding	Sensory organ development
cac	CG1522	2.46	Voltage-gated calcium channel activity	Exocytosis
spict	CG12292	2.14	Mg ion transmembrane transporter activity	Regulation of synaptic growth
CG1909	CG1909	2.30	Acetylcholine receptor binding	Synaptic transmission
Sh	CG12348	3.25	Voltage-gated cation channel activity	Regulation of synaptic activity
Sap47	CG8884	2.46	Unknown	Regulation of short-term neuronal synaptic plasticity
Cdase	CG1471	3.03	Ceramidase activity	Synaptic vesicle exocytosis
Rim	CG7321	3.73	Small GTPase regulator activity	Neuromuscular synaptic transmission
Syx8	CG4109	2.14	SNAP receptor activity	Neurogenesis
Gad1	CG14994	2.00	Glutamate decarboxylase activity	Synapse assembly
Syt4	CG10047	2.46	Calcium-dependent phospholipid binding	Regulation of neurotransmitter secretion
Unc-13	CG2999	2.30	Diacylglycerol binding	Synaptic vesicle exocytosis
Syt1	CG3139	2.14	Protein binding; calcium ion binding	Synaptic vesicle endocytosis
RtGEF	CG10043	2.30	Guanyl-nucleotide exchange factor activity	Regulation of synapse organization
Syt7	CG2381	2.46	Calcium-dependent phospholipid binding	Synaptic vesicle exocytosis
Cirl	CG8639	3.25	Latrotoxin receptor activity	G-protein coupled receptor signaling pathway.
tomosyn	CG17762	2.14	Syntaxin-1 binding	Regulation of neuromuscular synaptic transmission
Ace	CG17907	2.00	Acetylcholinesterase activity	Synaptic transmission
Rph	CG11556	2.46	Protein transporter activity	Synaptic vesicle endocytosis
l(2)gl	CG2671	2.00	Myosin II binding; myosin binding	Sensory organ development
Syx17	CG7452	2.64	NAP receptor activity	Neurotransmitter secretion
CG7708	CG7708	2.30	Choline transmembrane transporter activity	Acetylcholine biosynthetic process
Caps	CG18026	2.00	Phospholipid binding	Synaptic transmission
Frq1	CG5744	2.30	Calcium ion binding	Synaptic transmission
cpx	CG32490	2.00	Neurotransmitter transporter activity	Regulation of neurotransmitter secretion
gfA	CG32538	2.46	Cation-selective channel activity	Synaptic transmission
Unc-13-4A	CG32381	2.30	Unknown	Neurotransmitter secretion
Tbh	CG1543	2.64	Oxidoreductase activity	Learning or memory
hiw	CG32592	2.30	Protein binding	Negative regulation of synaptic growth
Syn	CG3985	2.30	Catalytic activity; ATP binding	Olfactory learning; memory

Figure 2 DAVID heat map analysis of biologically significant FACs containing upregulated genes in *Dak2* knockout flies.
Gray and black shading indicates positive and unconfirmed correlations of annotated gene and functional GO terms, respectively. (A) Polysaccharide binding ; GO terms for shading block No.1, IPR002557, chitin binding protein, peritrophin-A ; No.2, SM00494, ChtBD2 ; No.3, GO, 0008061, chitin binding ; No.4, GO : 0006030, chitin metabolic processes ; No.5, 0006022, aminoglycan metabolic process ; No.6, GO : 0005976, polysaccharide metabolic process ; No.7, GO : 0030247, polysaccharide binding ; No.8, GO : 0001871, pattern binding. (B) Glycosyl hydrolases, family 13, catalytic region. GO terms for shading block No.1, IPR006589, glycosyl hydrolase, family 13, subfamily, catalytic region ; No.2, SM00642, Aamy ; No.3, IPR006047, glycosyl hydrolase, family13, catabolic region. (C) Transmission of nerve impulses ; GO terms for shading block No.1, GO : 0007268, synaptic transmission ; No.2, GO : 0007267, cell-cell signaling ; No.3, GO : 0019226, transmission of nerve impulse.

A

Symbol	ID	Fold change	Molecular function	Biological process
TER94	CG2331	0.44	Protein binding	Proteasomal ubiquitin-dependent protein catabolic process
Ufd1-like	CG6233	0.47	Unknown	Proteasomal ubiquitin-dependent protein catabolic process
CG13349	CG13349	0.47	Ubiquitin binding	Lateral inhibition
Uch-L3	CG3431	0.44	Endopeptidase activity	Protein deubiquitination
Rpn5	CG1100	0.50	Endopeptidase activity	Proteolysis
Pomp	CG9324	0.20	Unknown	Proteasomal ubiquitin-dependent protein catabolic process
Rpt4	CG3455	0.44	Endopeptidase activity	Proteolysis
Pros26	CG4097	0.47	Endopeptidase activity	Ubiquitin-dependent protein catabolic process
Prosalpha5	CG10938	0.44	Endopeptidase activity	Cellular process
Prosbeta1	CG8392	0.47	Endopeptidase activity	Response to DNA damage stimulus
Rpn6	CG10149	0.50	Endopeptidase activity	Proteolysis
Prosbeta7	CG12000	0.47	Endopeptidase activity	Response to DNA damage stimulus
Pros28.1	CG3422	0.41	Endopeptidase activity	Response to DNA damage stimulus
Pros45	CG1489	0.44	Endopeptidase activity	Response to DNA damage stimulus
Prosbeta5	CG12323	0.44	Endopeptidase activity	Mitotic spindle organization
Rpn7	CG5378	0.41	Endopeptidase activity	Cellular process; proteolysis
Rpn1	CG7762	0.44	Endopeptidase activity	Mitotic spindle organization
Rpn2	CG11888	0.41	Endopeptidase activity	Response to DNA damage stimulus
Rpn12	CG4157	0.44	Endopeptidase activity	Proteolysis
Rpn3	CG10484	0.38	Endopeptidase activity	Proteolysis
Pros54	CG7619	0.47	Endopeptidase activity	Response to DNA damage stimulu
Prosalpha7	CG1519	0.47	Endopeptidase activity	Response to DNA damage stimulus
Tbp-1	CG10370	0.38	Endopeptidase activity	Cellular process; proteolysis
CG13779	CG13779	0.38	Peptidase activity	Neurogenesis
Rpn11	CG18174	0.41	Endopeptidase activity	Proteasomal ubiquitin-dependent protein catabolic proces
Pros29	CG9327	0.41	Endopeptidase activity	Mitotic spindle elongation
Pros35	CG4904	0.44	Endopeptidase activity	Ubiquitin-dependent protein catabolic process
Rpn9	CG10230	0.44	Endopeptidase activity	Regulation of exit from mitosis; proteolysis
Prosbeta2	CG3329	0.44	Endopeptidase activity	Response to DNA damage stimulus
Prosbeta3	CG11981	0.38	Endopeptidase activity	Response to DNA damage stimulus
Rpt3	CG16916	0.44	Endopeptidase activity	Response to DNA damage stimulus
Mov34	CG3416	0.50	Endopeptidase activity	Mitotic spindle organization; proteolysis
CG17331	CG17331	0.41	Endopeptidase activity	Response to DNA damage stimulus
Prosalpha1	CG18495	0.18	Endopeptidase activity	Response to DNA damage stimulus
Pros26.4	CG5289	0.44	Endopeptidase activity	Mitotic spindle organization

C

Symbol	ID	Fold change	Molecular function	Biological process
RpLP0-like	CG1381	0.41	Structural constituent of ribosome	Translation
CG13096	CG13096	0.41	Structural constituent of ribosome	Translation
CG3107	CG3107	0.50	Metalloendopeptidase activity	Proteolysis
CG7433	CG7433	0.35	4-aminobutyrate transaminase activity	Cellular amino acid metabolic process
mtDNA-helicase	CG5924	0.44	5'-3' DNA helicase activity; ATP binding	Mitochondrial genome maintenance
Roe1	CG6155	0.50	Unfolded protein binding	Neurogenesis
mtSSB	CG4337	0.29	Single-stranded DNA binding	Mitochondrial genome maintenance
Hsp60	CG12101	0.47	Unfolded protein binding	Mitochondrion organization
CG10399	CG10399	0.50	Hydroxymethylglutaryl-CoA lyase activity	Leucine metabolic process
P32	CG6459	0.47	Unknown	Regulation of calcium-mediated signaling
RpS11	CG8857	0.50	Structural constituent of ribosome	Mitotic spindle organizatio
CG4866	CG4866	0.38	Structural constituent of ribosome	Translation
mRpL3	CG8288	0.50	Structural constituent of ribosome	Translation
mRpL10	CG11488	0.50	Structural constituent of ribosome	Translation
mRpL11	CG3351	0.44	Structural constituent of ribosome	Translation
mRpL12	CG5012	0.47	chromatin binding	Cell growth; mitochondrion organization
mRpL15	CG5219	0.44	Structural constituent of ribosome	Translation
mRpL16	CG3109	0.47	Structural constituent of ribosome	Translation
mRpL17	CG13880	0.47	Structural constituent of ribosome	Translation
mRpL18	CG12373	0.33	Structural constituent of ribosome	Mitochondrial translation
mRpL20	CG11258	0.44	Structural constituent of ribosome	Translation
mRpL22	CG4742	0.50	Structural constituent of ribosome	Translation
mRpL36	CG18767	0.47	Structural constituent of ribosome	Translation
mRpL40	CG5242	0.47	Structural constituent of ribosome	neurogenesis
mRpL32	CG12220	0.47	Structural constituent of ribosome	Translation
mRpL44	CG2109	0.50	Structural constituent of ribosome	neurogenesis
mRpL47	CG9378	0.50	Structural constituent of ribosome	Translation
mRpL50	CG8612	0.33	Structural constituent of ribosome	Mitochondrial translation
mRpL51	CG13098	0.44	Structural constituent of ribosome	Translation
mRpL54	CG9353	0.38	Structural constituent of ribosome	Mitochondrial translation
mRpS6	CG15016	0.47	Structural constituent of ribosome	Translation
mRpS10	CG4247	0.44	Structural constituent of ribosome	Translation
mRpS16	CG8338	0.50	Structural constituent of ribosome	Translation
mRpS18A	CG31450	0.47	Structural constituent of ribosome	Translation
mRpS18B	CG10757	0.44	Structural constituent of ribosome	Translation
mRpS18C	CG9688	0.47	Structural constituent of ribosome	Translation
mRpS30	CG8470	0.44	Structural constituent of ribosome	Translation
mRpS33	CG10406	0.50	Structural constituent of ribosome	Translation
mRpS35	CG2101	0.44	Structural constituent of ribosome	Translation
tko	CG7925	0.16	Structural constituent of ribosome	Response to hypoxia; response to mechanical stimulus
bonsai	CG4207	0.50	Structural constituent of ribosome	Regulation of growth

B

Symbol	ID	Fold change	Molecular function	Biological process
Tim9a	CG1660	0.44	Protein transmembrane transporter activity	Protein targeting to mitochondrion; protein transport
AIF	CG7263	0.38	Oxidoreductase activity	Mitochondrion organization
Tim9b	CG33066	0.47	Protein transmembrane transporter activity	Cellular response to hypoxia
Adk2	CG3140	0.07	Adenylate kinase activity	Neurogenesis
Tim8	CG1728	0.50	Protein transmembrane transporter activity	Protein targeting to mitochondrion
cup	CG11181	0.50	Translation regulator activity	Macromolecule biosynthetic process
Ski6	CG15481	0.38	3'-5'-exoribonuclease activity	Regulation of gene expression
P32	CG6459	0.47	Unknown	Regulation of calcium-mediated signaling
Rpd3	CG7471	0.31	Histone deacetylase activity	Regulation of RNA metabolic process
mia	CG10390	0.47	Unknown	Regulation of gene expression; spermatogenesis
rept	CG9750	0.35	DNA helicase activity	Negative regulation of gene expression
CG3107	CG3107	0.50	Metalloendopeptidase activity	Proteolysis
Nopp140	CG7421	0.47	Unknown	Neurogenesis
CG11583	CG11583	0.31	Unknown	Neurogenesis
pont	CG4003	0.31	DNA helicase activity	Positive regulation of gene expression
Skeletor	CG32922	0.50	Structural molecule activity.	Spindle assembly
tko	CG7925	0.16	Structural constituent of ribosome	Response to hypoxia; response to mechanical stimulus
mRpL3	CG8288	0.50	Structural constituent of ribosome	Translation
mRpL10	CG11488	0.50	Structural constituent of ribosome	Translation
mRpL11	CG3351	0.44	Structural constituent of ribosome	Translation
mRpL12	CG5012	0.47	Chromatin binding	Cell growth; mitochondrion organization
mRpL15	CG5219	0.44	Structural constituent of ribosome	Translation
mRpL16	CG3109	0.47	Structural constituent of ribosome	Translation
mRpL17	CG13880	0.47	Structural constituent of ribosome	Translation
mRpL18	CG12373	0.33	Structural constituent of ribosome	Mitochondrial translation
mRpL20	CG11258	0.44	Structural constituent of ribosome	Translation
mRpL22	CG4742	0.50	Structural constituent of ribosome	Translation
mRpL32	CG12220	0.47	Structural constituent of ribosome	Translation
mRpL36	CG18767	0.47	Structural constituent of ribosome	Translation
mRpL40	CG5242	0.47	Structural constituent of ribosome	Neurogenesis
mRpL44	CG2109	0.50	Structural constituent of ribosome	Neurogenesis
mRpL47	CG9378	0.50	Structural constituent of ribosome	Translation
mRpL50	CG8612	0.33	Structural constituent of ribosome	Mitochondrial translation
mRpL51	CG13098	0.44	Structural constituent of ribosome	Translation
mRpL54	CG9353	0.38	Structural constituent of ribosome	Mitochondrial translation
mRpS6	CG15016	0.47	Structural constituent of ribosome	Translation
mRpS10	CG4247	0.44	Structural constituent of ribosome	Translation
mRpS16	CG8338	0.50	Structural constituent of ribosome	Translation
mRpS18A	CG31450	0.47	Structural constituent of ribosome	Translation
mRpS18B	CG10757	0.44	Structural constituent of ribosome	Translation
mRpS18C	CG9688	0.47	Structural constituent of ribosome	Translation
mRpS30	CG8470	0.44	Structural constituent of ribosome	Translation
mRpS33	CG10406	0.50	Structural constituent of ribosome	Translation
mRpS35	CG2101	0.44	Structural constituent of ribosome	Translation
Rpb7	CG31155	0.44	Protein binding	Transcription from RNA polymerase II promoter
Rpb8	CG11246	0.47	DNA-directed RNA polymerase activity	Mitotic G2 DNA damage checkpoint
CG3756	CG3756	0.41	DNA-directed RNA polymerase activity	Transcription from RNA polymerase III promoter
Nxt1	CG12752	0.47	Unknown	Poly(A)+ mRNA export from nucleus
Rat1	CG10354	0.25	5'-3' exonuclease activity	Neurogenesis
NHP2	CG5258	0.41	SnoRNA binding	rRNA processing
Mcm10	CG9241	0.35	Chromatin binding	Chromatin silencing; DNA replication
l(3)07882	CG5824	0.50	SnoRNA binding	Ribosomal small subunit biogenesis
l(2)37Cg	CG10685	0.50	DNA-directed RNA polymerase activity	Transcription from RNA polymerase I promoter
CG7993	CG7993	0.38	Unknown	Neurogenesis
HP4	CG8044	0.50	Unknown	Chromatin organization
Nsun2	CG6133	0.35	tRNA (cytosine-5-)-methyltransferase activity	Short-term memory
CG11837	CG11837	0.44	rRNA (adenine-N6,N6-)-dimethyltransferase activity	Neurogenesis
Ns1	CG3983	0.50	GTP binding	Regulation of insulin receptor signaling pathwa
l(1)1Bi	CG6189	0.29	DNA-directed DNA polymerase activity	Transcription, DNA-dependent
mtDNA-helicase	CG5924	0.44	5'-3' DNA helicase activity; ATP binding	Mitochondrial genome maintenance
Mcm7	CG4978	0.44	3'-5' DNA helicase activity	Mitotic G2 DNA damage checkpoint
Roe1	CG6155	0.50	Unfolded protein binding	Neurogenesis
hoip	CG3949	0.33	mRNA binding	Nervous system development
esc	CG14941	0.50	Histone methyltransferase activity	Chromatin silencing
His2Av	CG5499	0.35	DNA binding	Response to DNA damage stimulus
Nop60B	CG3333	0.41	Pseudouridylate synthase activity	rRNA processing
CG6724	CG6724	0.38	Unknown	Neurogenesis
Rrp42	CG8395	0.50	3'-5'-exoribonuclease activity	Neurogenesis
Fib	CG9888	0.50	mRNA binding	Centrosome organization
wcd	CG7989	0.44	Unknown	rRNA processing
wbl	CG7225	0.22	Protein homodimerization activity	Regulation of multicellular organism growth
Dbp73D	CG9680	0.47	ATP-dependent RNA helicase activity	Neurogenesis
Mcm5	CG4082	0.41	3'-5' DNA helicase activity	Chromosome condensation
Bin1	CG6046	0.47	Transcription corepressor activity	Negative regulation of transcription
Caf1	CG4236	0.23	Protein binding	Negative regulation of cellular biosynthetic process
CG7433	CG7433	0.35	4-aminobutyrate transaminase activity	Cellular amino acid metabolic process
SC35	CG5442	0.50	Protein binding	Regulation of alternative mRNA splicing, via spliceosome
CG8545	CG8545	0.44	S-adenosylmethionine-dependent methyltransferase activity	rRNA processing
CG4866	CG4866	0.38	Structural constituent of ribosome	Translation
bonsai	CG4207	0.50	Structural constituent of ribosome	Regulation of growth
Mcm3	CG4206	0.44	3'-5' DNA helicase activity	Mitotic G2 DNA damage checkpoint
CG1542	CG1542	0.38	Unknown	rRNA processing
bys	CG1430	0.38	Unknown	Neurogenesis
smt3	CG4494	0.47	protein binding	Cellular response to endogenous stimulus
Cdc6	CG5971	0.44	DNA clamp loader activity	Pre-replicative complex assembly
Hrb87F	CG12749	0.41	Sequence-specific DNA binding	Regulation of alternative mRNA splicing, via spliceosome
CG3071	CG3071	0.50	Unknown	Retrograde vesicle-mediated transport, Golgi to ER
CG5033	CG5033	0.41	Ribonucleoprotein complex binding	Regulation of hemocyte proliferation
Hsp60	CG12101	0.47	unfolded protein binding	Cellular response to heat; mitochondrion organization
CG10399	CG10399	0.50	Hydroxymethylglutaryl-CoA lyase activity	Leucine metabolic process
Nop56	CG13849	0.50	Unknown	Neurogenesis
pit	CG6375	0.33	ATP-dependent RNA helicase activity	Cell proliferation; cell growth
CG8939	CG8939	0.50	rRNA methyltransferase activity	rRNA processing
CG12909	CG12909	0.38	Unknown	Not known
sqd	CG16901	0.47	mRNA binding	Regulation of RNA metabolic process
CG7185	CG7185	0.44	mRNA binding	Regulation of alternative mRNA splicing
CstF-50	CG2261	0.38	Unknown	mRNA cleavage
dom	CG9696	0.50	Protein binding	Negative regulation of gene expression
B52	CG10851	0.50	Protein binding	Regulation of insulin receptor signaling pathway
mtSSB	CG4337	0.29	Single-stranded DNA binding	Mitochondrial genome maintenance
CG6712	CG6712	0.44	RNA binding	Not known
CG7637	CG7637	0.41	Unknown	Not known
Surf6	CG4510	0.31	Heme transporter activity	Neurogenesis
Hel25E	CG7269	0.41	ATP-dependent RNA helicase activity	Regulation of alternative mRNA splicing, via spliceosome

D

Symbol	ID	Fold change	Molecular function	Biological process
Tim9b	CG33066	0.47	Protein transmembrane transporter activity	Response to hypoxia
CG1750	CG1750	0.47	Methionyl-tRNA formyltransferase activity	Conversion of methionyl-tRNA to N-formyl-methionyl-tRNA
CG8235	CG8235	0.41	tRNA binding	Not known
CG17259	CG17259	0.47	ATP binding	Seryl-tRNA aminoacylation
CG15100	CG15100	0.47	Methionine-tRNA ligase activity	Neurogenesis
Slimp	CG31133	0.47	tRNA binding	Mitochondrion morphogenesis
Aats-ala	CG13391	0.44	Alanine-tRNA ligase activity	Alanyl-tRNA aminoacylation
CG5706	CG5706	0.41	Phenylalanine-tRNA ligase activity	Phenylalanyl-tRNA aminoacylation
Aats-lys	CG12141	0.47	Lysine-tRNA ligase activity	Lysyl-tRNA aminoacylation
CG4573	CG4573	0.41	Glutamate-tRNA ligase activity	Glutamyl-tRNA aminoacylation
Aats-ile	CG11471	0.50	Isoleucine-tRNA ligase activity	Isoleucyl-tRNA aminoacylation
Aats-arg	CG9020	0.38	Arginine-tRNA ligase activity	Arginyl-tRNA aminoacylation
CG2263	CG2263	0.38	Phenylalanine-tRNA ligase activity	Phenylalanyl-tRNA aminoacylation
CG33123	CG33123	0.38	Leucine-tRNA ligase activity	Neurogenesis
Aats-asn	CG10687	0.50	Asparagine-tRNA ligase activity	Asparaginyl-tRNA aminoacylation
Aats-val	CG4062	0.41	Glutamate-tRNA ligase activity	Neurogenesis
Aats-glupro	CG5394	0.47	Glutamate-tRNA ligase activity; proline-tRNA ligase activity	Prolyl-tRNA aminoacylation; glutamyl-tRNA aminoacylation
Aats-cys	CG8431	0.44	Cysteine-tRNA ligase activity	Cysteinyl-tRNA aminoacylation
Aats-gly	CG6778	0.50	Glycine-tRNA ligase activity	Glycyl-tRNA aminoacylation
Aats-gln	CG10506	0.50	Glutamine-tRNA ligase activity	Glutaminyl-tRNA aminoacylation

Figure 3 DAVID heat map analysis of biologically significant FACs containing downregulated genes in *Dak2* knockout flies.
Gray and black shading indicates positive and unconfirmed correlations of annotated genes and functional GO terms, respectively. (A) Proteasome ; GO terms for shading block No.1, proteasome ; No.2, dme03050, proteasome ; No.3, GO : 0000502, proteasome complex. (B) Intracellular organelle lumen. GO terms for shading block No.1, GO : 0070013, intracellular organelle lumen ; No.2, GO : 0043233, organelle lumen ; No.3, GO : 0031974, membrane-enclosed lumen. (C) Mitochondrial matrix ; GO terms for shading block No.1, ribosomal protein ; No.2, GO : 003279, ribosomal subunit ; No.3, GO : 0005761, mitochondrial ribosome ; No.4, GO : 0000313, organellar ribosome ; No.5, GO : 0003735, structural constituent of ribosome ; No.6, GO : 0005840, ribosome ; No.7, GO : 0005759, mitochondrial matrix ; and No.8, GO : 0031980, mitochondrial lumen. (D) Aminoacyl-tRNA ligase ; GO terms for shading block No.1, aminoacyl-tRNA synthetase ; No.2, GO : 0006418, tRNA aminoacylation for protein translation ; No.3, GO : 0043039, tRNA aminoacylation ; No.4, GO : 0043038, amino acid activation ; No.5, dme00970, aminoacyl-tRNA biosynthesis ; No.6, GO : 0016876, ligase activity, forming aminoacyl-tRNA and related compounds ; No.7, GO : 0016875, ligase activity, forming carbon-oxygen bonds ; and No.8, GO : 0004812, aminoacyl-tRNA ligase activity.

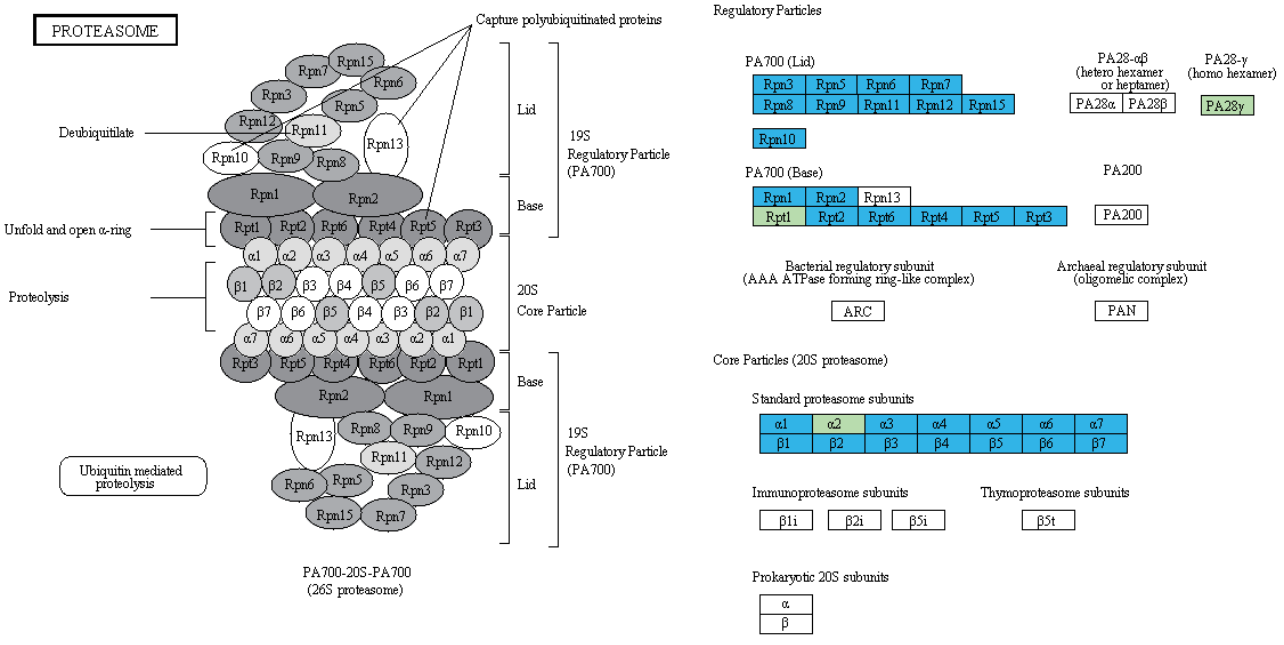


Figure 4 KEGG pathway map analysis of the proteasome.
KEGG pathway map analysis was performed using the gene list that contains > 2-fold upregulated and < 0.5-fold downregulated genes compared with wild type flies, and obtained result for the proteasome is shown. Blue boxes indicate genes with expression levels < 0.5-fold of those in wild type flies, and green boxes indicate genes with expression levels < 1-fold and > 0.5-fold of those in wild type flies.

proteasomal activity in *Dak2* mutant flies. The proteasome comprises the main proteolytic enzymes of the ubiquitin-protease system. These components of the multi-subunit 26S proteasome complex included those of 20S catalytic core and 19S regulatory particles (23).

2.5. Mitochondrial ribosome

The cluster with second highest enrichment score (15.78) of downregulated genes was related to "Membrane enclosed lumen," with the common GO terms intracellular organelle lumen, organelle lumen, and membrane enclosed lumen (Figure 3B). Of the 105 genes included in this cluster, 31 encoded structural constituents of mitochondrial ribosomes. Genes related to RNA editing, cell cycle, transcription, and neurogenesis were also included in this cluster.

The cluster with third highest enrichment score (8.46) related to the "Mitochondrial matrix" (Figure 3C). This cluster also comprised genes encoding mitochondrial ribosomal proteins. Other mitochondrial ribosome subunits that were not listed in the cluster were also downregulated, suggesting reduced mitochondrial translation activity in *Dak2* knockout flies.

2.6. Aminoacyl tRNA biosynthesis

The fourth cluster of downregulated genes was associated with "aminoacyl-tRNA ligase activity." Of the 20 genes in this cluster, 13 encoded aminoacyl-tRNA synthetases (AARSs, Figure 3D), further indicating reduced overall translational activity. All AARSs on the list were cytosolic, although expression of mitochondrial AARSs were also generally lower (0.93-0.41 fold), albeit insignificantly, than in wild type flies.

DISCUSSION

The enzyme encoded by *Dak2* is unique to mitochondrial intermembrane space and reversibly interconverts ATP and AMP to 2 ADP. Mammals express the additional adenine nucleotide metabolizing enzyme mitochondrial creatine kinase (MtCK) in the same cellular compartment, which may accommodate AK deficiencies. Because no phosphagen kinases are present in mitochondrial intermembrane space of *D. melanogaster* except *Dak2*, *Dak2* mutant fruit flies are a useful model for investigations of AK2 (5).

Drosophila development and energy demand

In our previous study, *Dak2* knockout caused larval mortality in *Drosophila* flies (5). In the present study, tissue-specific roles of AK2 during larval development were further investigated by analyzing the effects of *Dak2* knockdown in various tissue-specific *GAL4* strains. We found that all tissues/organs examined were susceptible to the *Dak2* RNAi and resulted in larval mortality, suggesting that *Dak2* is essential for development in most of these tissues.

As a source of ATP, glycolysis predominates over the TCA cycle in stem cells and tumor cells, even in the presence of normal mitochondria (24, 25). However, glycolysis in *Dak2* mutant *D. melanogaster* failed to sustain normal growth and histogenesis, despite increased expression of glycolytic aldolase (2.3-fold) and hexokinase (2.0-fold). These data clearly demonstrate that *Dak2* is crucial for mitochondrial function during development in *Drosophila* flies.

Mitochondrial gene expression and hearing loss

DAVID analyses of downregulated genes in *Dak2* mutants revealed relatively high enrichment scores for FAC mitochondrial translation machinery, indicating impairment of mitochondrial translation in *Dak2* knockout flies.

In a report published in 1992, mutation of mitochondrial $tRNA^{Leu(UUR)}$ was shown to cause deafness (26), and thereafter many reports about mitochondrial tRNA and hearing impairment have been reported (27-29). Furthermore, mutations in mitochondrial rRNA transfer and mitochondrial aminoacyl tRNA synthetase genes were identified as causative of deafness (30, 31). Mutation of the mitochondrial ribosomal protein S12 (*technical knockout*, *tko*) was also reported as a cause of deafness in fruit flies (32). Taken together, these observations strongly indicate functional associations between defects of mitochondrial translation machinery and hearing loss.

Zheng *et al.* suggested that impaired mitochondrial translational activity reduces the expression of electron transport chain complexes, leading to deficiencies of ATP production and increased production of reactive oxygen species (ROS) in cochlear and vestibular cells. Enhanced ROS production damages mitochondrial and cellular proteins, lipids, and nucleic acids, and also auditory structure (27). Microarray analyses of *Dak2* knockout flies indicated

downregulation of multiple genes related to mitochondrial translation machinery, suggesting that *AK2* mutations may also cause failure of mitochondrial translational machinery and hearing loss in RD patients (7). The *Drosophila* counterpart of mammalian auditory systems is known as Johnston's organ, which comprises specialized clusters of mechanosensory neurons (33). However, it is unclear whether *Dak2* knockout impairs auditory function in flies (34).

Proteasome activity and immunodeficiency

We found that the downregulated gene cluster with the highest enrichment score was related to subunits of the proteasome (Figure 1B). It is accepted that the proteasome plays important roles in immune regulatory systems involving antigen processing and inflammatory responses (35, 36).

Fruit fly immunity is predominantly mediated by toll and IMD pathways (37). In the toll pathway, infectious signals of gram-positive bacteria, yeast, and fungi are primarily transmitted to transcription factors such as dif and dorsal. Subsequently, these factors are translocated into the nucleus, where they promote transcription of target genes such as drosomycin. Activities of these transcription factors are regulated by cactus, a *Drosophila* homolog of mammalian I-κB. Cactus binds to dif and dorsal to inactivate their activities, and in response to infection, it is degraded by the proteasome, and the transcription factors are activated and released into the nucleus. Hence, insufficient proteasomal activity leads to reduced expression of target genes (38). The importance of the proteasome to immune function has also been demonstrated in humans (39). Thus, *AK2* mutations in RD patients may also lower proteasomal activity following inactivation of NF-κB, which is the human counterpart of dif and dorsal, leading to impaired regulation of target immune related genes.

Inhibition of mitochondrial complex I reduces ATP production and suppresses the activity of the proteasome (40). Knockdown of *AK2* in immune cells resulted in deficiencies in ATP production (8). Hence, the present observations of ATP deficiency in *Dak2* mutant flies may also be relevant to humans.

Barjaktarevic *et al.* argued that inactivation of the transcription factor *Gfi-1* (growth factor independence 1) caused immunodeficiency, incomplete development of mouse inner ears, and reticular dysgenesis (41). Proteasomal regulation of Gfi-1 activity (42) further implies the involvement of the proteasome in the expression of genes that are required for developments of the immune system.

Reduced proteasome activity was also expected to lower drosomycin expression in *Drosophila.* However, the present microarray data show that *Dak2* mutation led to a 5-fold increase in drosomycin expression. Drosomycin is usually induced by infections of gram-positive bacteria and ROS such as H_2O_2 (43). Although the relationship between ROS production and AK2 depletion remains unclear, elevated ROS in *AK2* mutants (6) may reflect induction of drosomycin by ROS.

To determine whether dorsal and senseless (*Drosophila* homolog of *Gfi-1*) regulate the proteasome and the mitochondrial translational system, we examined transcription factor binding to promoters of the genes included in the clusters "proteasome complex" (Figure 5A), "membrane-enclosed lumen" (Figure 5B), "mitochondrial matrix" (Figure 5C), and "aminoacyl-tRNA ligase activity" (Figure 5D) using the DroID database, which estimates transcription factor-target gene interactions (12). These *in silico* analyses revealed that almost all promoters contain corresponding putative binding sites.

In summary, the present bioinformatics analyses clearly demonstrate that *AK2* mutations inhibit proteasomal expression, leading to suppression of NF-κB and target immune genes. Accordingly, we propose a working hypotheses for the effects of AK2 deficiency on cellular responses in *D. melanogaster* and human systems as shown in Figures 6A and 6B, respectively. In *Drosophila* flies, *Dak2* deficiency results in decreased ATP levels, followed by inhibited proteasome activity. Consequently, the activities of transcription factors dif and dorsal are decreased, while the inhibitory activities of senseless are increased, resulting in decreased expression of target genes for proteasome subunits and mitochondrial translational systems. Furthermore, *Dak2* depletion induces production of ROS, accumulation of AMP, and expression of drosomycin independently of the toll pathway. In humans, *AK2* mutation reduces proteasome activity by lowering ATP levels and inhibits NF-κB activity while activating Gfi-1. Hence, transcriptional insufficiencies in cells with inactive AK2 reduce the expression of proteasome subunits and proteins of mitochondrial and hematopoietic translational systems, resulting in sensorineural deafness and impaired immunity, respectively. Further analyses are required to confirm these hypotheses.

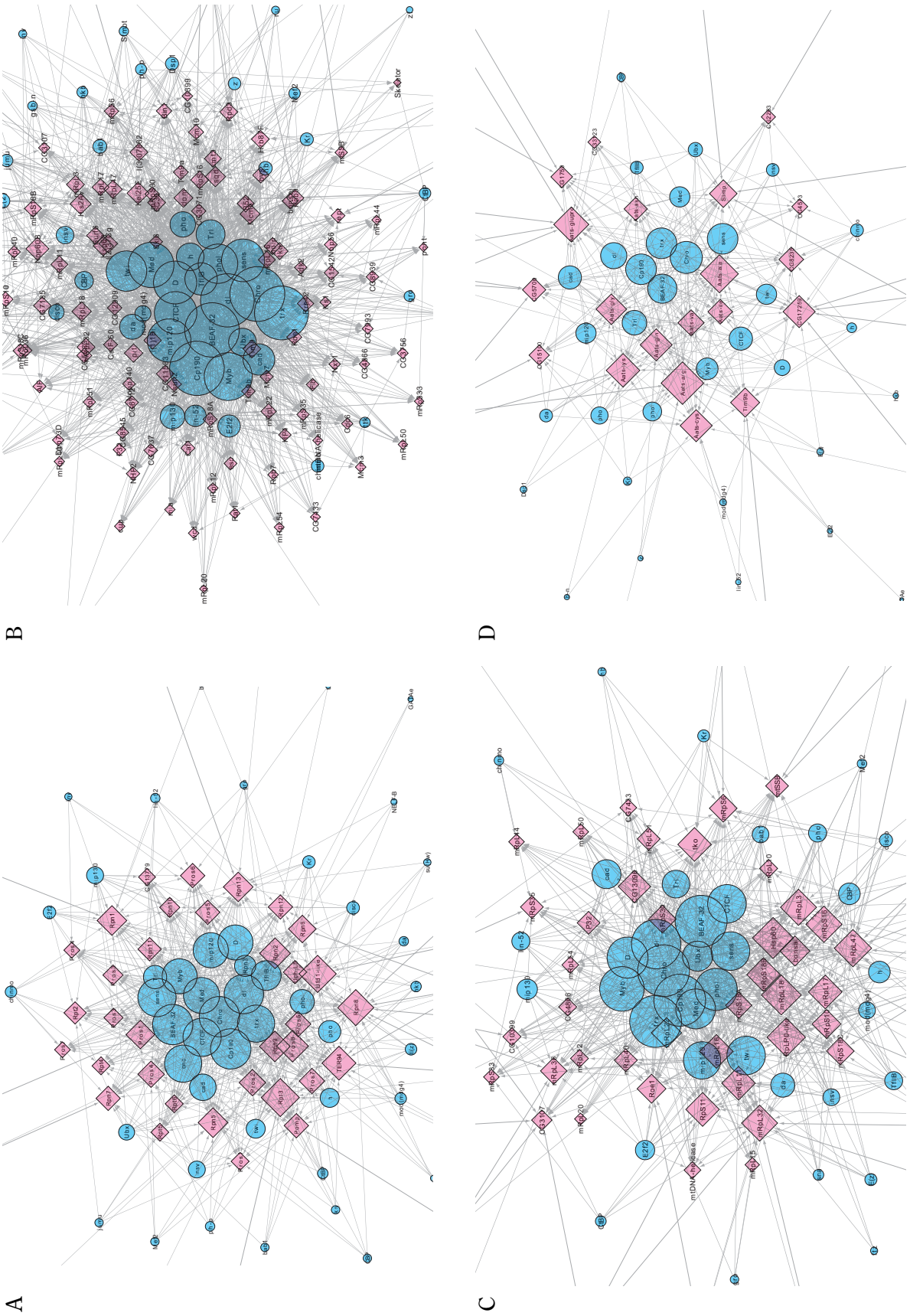


Figure 5 Predicted interactions between transcription factors and their target genes.
Transcription factors (circles) and target genes (squares) listed in Figure 3 are connected with arrows. Sizes of circles and squares indicate the numbers of interactions between them.
(A) Proteasome complex ; (B) Membrane-enclosed lumen ; (C) Mitochondrial matrix ; (D) Aminoacyl-tRNA ligase activity.

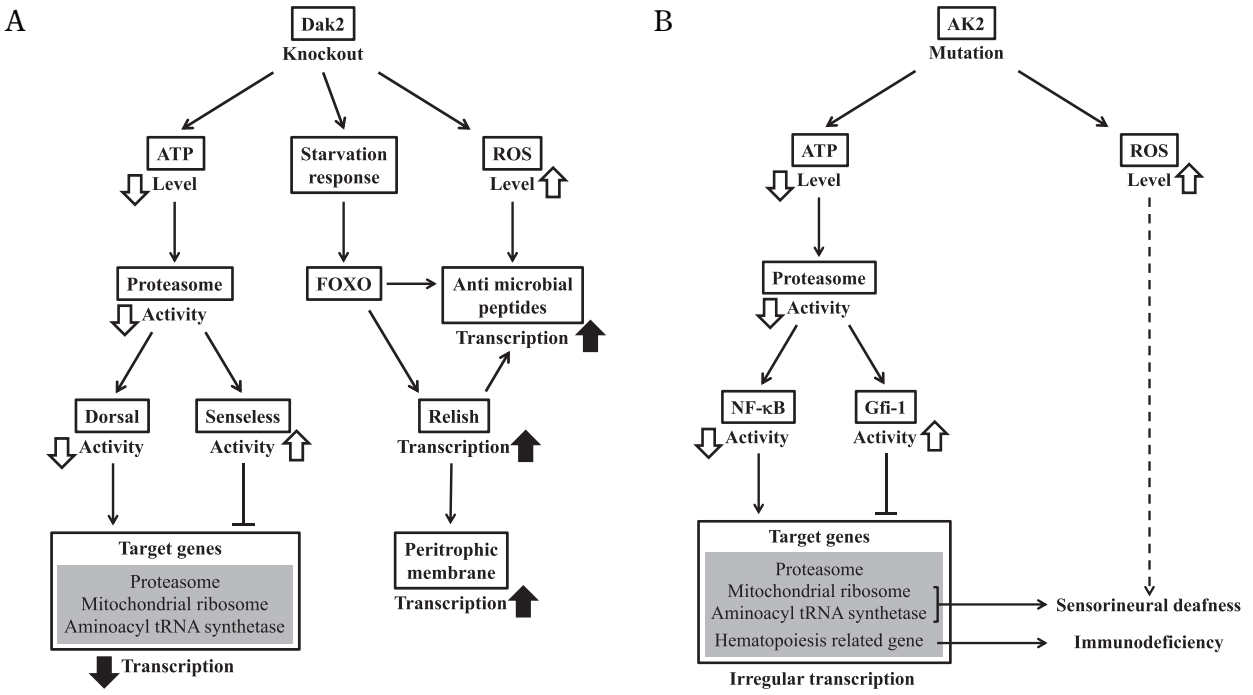


Figure 6 Working model of AK2 deficiency-induced phenotypic modifications in *D. melanogaster* and human.
(A) Model for the effects of *Dak2* knockout in *D. melanogaster* ; (B) Model for the effects of *AK2* defects on humans. The hypothetical model for pathogenic mechanism of reticular dysgenesis in humans is based on published reports (6, 7). Increased and decreased expression levels and activities are depicted with filled upward arrows and filled downward arrows, respectively. Predicted changes in levels and activities are depicted with open arrows. Speculated causalities are interconnected with black arrows.

CONCLUSIONS

Dak2 is an essential gene for the development of *D. melanogaster*. Deletion of *Dak2* resulted in perturbations of morphogenesis and larval development by affecting gene expression patterns and downregulating genes encoding proteasomal subunits and mitochondrial translation machinery, thus reducing target gene expression. The present analyses indicate the utility of *Dak2* knockout flies in investigations of mechanisms of RD phenotypes and may facilitate future developments of therapeutic strategies for RD patients.

AUTHOR'S CONTRIBUTIONS

TN and RM prepared the experimental design. TH, MF and KF performed the experiments. TH, MF, KF, AT and KM analyzed the experimental data. TH, MF, KF, RM and TN wrote the manuscript. All authors read and approved the final manuscript.

CONFLICT OF INTEREST

The authors declare that they have no competing interests.

ACKNOWLEDGEMENTS

This work was supported, in part, by a grant-in-aid for scientific research (no. 16591858) from the Ministry of Education, Culture, Sports, Science, and Technology of Japan, and the President discretion research budget of the University of Tokushima.

REFERENCES

1. Noma T : Dynamics of nucleotide metabolism as a supporter of life phenomena. J Med Invest 52 : 127-136, 2005
2. Panayiotou C, Solaroli N, Xu Y, Johansson M, Karlsson A : The characterization of human adenylate kinases 7 and 8 demonstrates differences in kinetic parameters and structural organization among the family of adenylate kinase isoenzymes. Biochem J 433 : 527-534, 2011

3. Noma T, Song S, Yoon YS, Tanaka S, Nakazawa A : cDNA cloning and tissue-specific expression of the gene encoding human adenylate kinase isozyme 2. Biochim Biophys Acta 1395 : 34-39, 1998
4. Ratto A, Shapiro BM, Christen R : Phosphagen kinase evolution-Expression in echinoderms. Eur J Biochem 186 : 195-203, 1989
5. Fujisawa K, Murakami R, Horiguchi T, Noma T : Adenylate kinase isozyme 2 is essential for growth and development of *Drosophila melanogaster*. Comp Biochem Physiol B Biochem Mol Biol 153 : 29-38, 2009
6. Pannicke U, Hönig M, Hess I, Friesen C, Holzmann K, Rump EM, Barth TF, Rojewski MT, Schulz A, Boehm T, Friedrich W, Schwarz K : Reticular dysgenesis (aleukocytosis) is caused by mutations in the gene encoding mitochondrial adenylate kinase 2. Nat Genet 41 : 101-105, 2009
7. Lagresle-Peyrou C, Six EM, Picard C, Rieux-Laucat F, Michel V, Ditadi A, Demerens-de Chappedelaine C, Morillon E, Valensi F, Simon-Stoos KL, Mullikin JC, Noroski LM, Besse C, Wulffraat NM, Ferster A, Abecasis MM, Calvo F, Petit C, Candotti F, Abel L, Fischer A, Cavazzana-Calvo M : Human adenylate kinase 2 deficiency causes a profound hematopoietic defect associated with sensorineural deafness. Nat Genet 41 : 106-111, 2009
8. Burkart A, Shi X, Chouinard M, Corvera S : Adenylate kinase 2 links mitochondrial energy metabolism to the induction of the unfolded protein response. J Biol Chem 286 : 4081-4089, 2012
9. Lengyel JA, Iwaki DD : It takes guts : the *Drosophila* hindgut as a model system for organogenesis. Dev Biol 243 : 1-19, 2002
10. Huang da W, Sherman BT, Lempicki RA : Bioinformatics enrichment tools : paths toward the comprehensive functional analysis of large gene lists. Nucleic Acids Res 37 : 1-13, 2008
11. Huang da W, Sherman BT, Lempicki RA : Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. Nat Protoc 4 : 44-57, 2009
12. Murali T, Pacifico S, Yu J, Guest S, Roberts GG 3rd, Finley RL Jr : DroID 2011 : a comprehensive, integrated resource for protein, transcription factor, RNA and gene interactions for *Drosophila*. Nucleic Acids Res 39 : D736-D743, 2011
13. Shannon P, Markiel A, Ozier O, Baliga NS, Wang JT, Ramage D, Amin N, Schwikowski B, Ideker T : Cytoscape : a software environment for integrated models of biomolecular interaction networks. Genome Res 13 : 2498-2504, 2003
14. Cline MS, Smoot M, Cerami E, Kuchinsky A, Landys N, Workman C, Christmas R, Avila-Campilo I, Creech M, Gross B, Hanspers K, Isserlin R, Kelley R, Killcoyne S, Lotia S, Maere S, Morris J, Ono K, Pavlovic V, Pico AR, Vailaya A, Wang PL, Adler A, Conklin BR, Hood L, Kuiper M, Sander C, Schmulevich I, Schwikowski B, Warner GJ, Ideker T, Bader GD : Integration of biological networks and gene expression data using Cytoscape. Nat Protoc 2 : 2366-2382, 2007
15. Wang P, Granados RR : Molecular structure of the peritrophic membrane (PM) : identification of potential PM target sites for insect control. Arch Insect Biochem Physiol 47 : 110-118, 2001
16. Terra WR : The origin and functions of the insect peritrophic membrane and peritrophic gel. Arch Insect Biochem Physiol 47 : 47-61, 2001
17. Girardin SE, Philpott DJ : Mini-review : the role of peptidoglycan recognition in innate immunity. Eur J Immunol 34 : 1777-1782, 2004
18. Zinke I, Kirchner C, Chao L, Tetzlaff MT, Pankratz MJ : Suppression of food intake and growth by amino acids in *Drosophila* : the role of *pumpless*, a fat body expressed gene with homology to vertebrate glycine cleavage system. Development 126 : 5275-5284, 1999
19. Zinke I, Schütz CS, Katzenberger JD, Bauer M, Pankratz MJ : Nutrient control of gene expression in *Drosophila* : microarray analysis of starvation and sugar-dependent response. EMBO J 21 : 6162-6173, 2002
20. Becker T, Loch G, Beyer M, Zinke I, Aschenbrenner AC, Carrera P, Inhester T, Schultze JL, Hoch M : FOXO-dependent regulation of innate immune homeostasis. Nature 463 : 369-373, 2010
21. Karpac J, Younger A, Jasper H : Dynamic coordination of innate immune signaling and insulin signaling regulates systemic responses to localized DNA damage. Dev Cell 20 : 841-854, 2011
22. Milton VJ, Sweeney ST : Oxidative stress in synapse development and function. Dev Neurobiol 72 : 100-110, 2012
23. Xie Y : Structure, assembly and homeostatic

regulation of the 26S proteasome. J Mol Cell Biol 2 : 308-317, 2010
24. Warburg O : On the origin of cancer cells. Science 123 : 309-314, 1956
25. Zhou W, Choi M, Margineantu D, Margaretha L, Hesson J, Cavanaugh C, Blau CA, Horwitz MS, Hockenbery D, Ware C, Ruohola-Baker H : HIF1α induced switch from bivalent to exclusively glycolytic metabolism during ESC-to-EpiSC/hESC transition. EMBO J 31 : 2103-2116, 2012
26. van den Ouweland JM, Lemkes HH, Ruitenbeek W, Sandkuijl LA, de Vijlder MF, Struyvenberg PA, van de Kamp JJ, Maassen JA : Mutation in mitochondrial $tRNA^{Leu(UUR)}$ gene in a large pedigree with maternally transmitted type II diabetes mellitus and deafness. Nat Genet 1 : 368-371, 1992
27. Zheng J, Ji Y, Guan MX : Mitochondrial tRNA mutations associated with deafness. Mitochondrion 12 : 406-413, 2012
28. Fischel-Ghodsian N : Mitochondrial deafness mutations reviewed. Hum Mutat 13 : 261-270, 1999
29. Guan MX : Molecular pathogenetic mechanism of maternally inherited deafness. Ann N Y Acad Sci 1011 : 259-271, 2004
30. Pierce SB, Gersak K, Michaelson-Cohen R, Walsh T, Lee MK, Malach D, Klevit RE, King MC, Levy-Lahad E : Mutations in *LARS2*, encoding mitochondrial leucyl-tRNA synthetase, lead to premature ovarian failure and hearing loss Perrault syndrome. Am J Hum Genet 92 : 614-620, 2013
31. Pierce SB, Chisholm KM, Lynch ED, Lee MK, Walsh T, Opitz JM, Li W, Klevit RE, King MC : Mutations in mitochondrial histidyl tRNA synthetase *HARS2* cause ovarian dysgenesis and sensorineural hearing loss of Perrault syndrome. Proc Natl Acad Sci USA 108 : 6543-6548, 2011
32. Toivonen JM, O'Dell KM, Petit N, Irvine SC, Knight GK, Lehtonen M, Longmuir M, Luoto K, Touraille S, Wang Z, Alziari S, Shah ZH, Jacobs HT : *technical knockout*, a Drosophila model of mitochondrial deafness. Genetics 159 : 241-254, 2001
33. Kamikouchi A, Shimada T, Ito K : Comprehensive classification of the auditory sensory projections in the brain of the fruit fly *Drosophila melanogaster*. J Comp Neurol 499 : 317-356, 2006
34. Kamikouchi A, Inagaki HK, Effertz T, Hendrich O, Fiala A, Göpfert MC, Ito K : The neural basis of Drosophila gravity-sensing and hearing. Nature 458 : 165-171, 2009
35. Basler M, Kirk CJ, Groettrup M : The immunoproteasome in antigen processing and other immunological functions. Curr Opin Immunol 25 : 74-80, 2013
36. Karin M, Ben-Neriah Y : Phosphorylation meets ubiquitination : the control of NF-κB activity. Annu Rev Immunol 18 : 621-663, 2000
37. Charroux B, Royet J : Drosophila immune response : From systemic antimicrobial peptide production in fat body cells to local defense in the intestinal tract. Fly 4 : 40-47, 2010
38. Lemaitre B, Hoffmann J : The host defense of *Drosophila melanogaster*. Ann Rev Immunol 25 : 697-743, 2007
39. Wang X, Luo H, Chen H, Duguid W, Wu J : Role of proteasomes in T cell activation and proliferation. J Immunol 160 : 788-801, 1998
40. Höglinger GU, Carrard G, Michel PP, Medja F, Lombès A, Ruberg M, Friguet B, Hirsch EC : Dysfunction of mitochondrial complex I and the proteasome : interactions between two biochemical deficits in a cellular model of Parkinson's disease. J Neurochem 86 : 1297-1307, 2003
41. Barjaktarevic I, Maletkovic-Barjaktarevic J, Kamani NR, Vukmanovic S : Altered functional balance of Gfi-1 and Gfi-1b as an alternative cause of reticular dysgenesis? Med Hypotheses 74 : 445-448, 2010
42. Marteijn JA, van der Meer LT, Van Emst L, de Witte T, Jansen JH, van der Reijden BA : Diminished proteasomal degradation results in accumulation of Gfi1 protein in monocytes. Blood 109 : 100-108, 2007
43. Wu SC, Liao CW, Pan RL, Juang JL : Infection-induced intestial oxidative stress triggers organ-to-organ immunological communication in *Drosophila*. Cell Host Microbe 11 : 410-417, 2012