

Potential involvement of *Drosophila* flightless-1 in carbohydrate metabolism

Jung-Eun Park^{1,#}, Jinho Jang^{2,#}, Eun Ji Lee¹, Su Jung Kim³, Hyun Ju Yoo³, Semin Lee^{2,*} & Min-Ji Kang^{1,*}

¹Department of Biomedical Sciences, University of Ulsan College of Medicine, Asan Medical Center, Seoul 05505, ²Department of Biological Engineering, School of Life Sciences, Ulsan National Institute of Science and Technology, Ulsan 44919, ³Department of Convergence Medicine, Asan Institute for Life Sciences, Asan Medical Center, University of Ulsan College of Medicine, Seoul 05505, Korea

A previous study of ours indicated that *Drosophila* flightless-1 controls lipid metabolism, and that there is an accumulation of triglycerides in *flightless-1* (*flil*)-mutant flies, where this mutation triggers metabolic stress and an obesity phenotype. Here, with the aim of characterizing the function of Flil in metabolism, we analyzed the levels of gene expression and metabolites in *flil*-mutant flies. The levels of enzymes related to glycolysis, lipogenesis, and the pentose phosphate pathway increased in *flil* mutants; this result is consistent with the levels of metabolites corresponding to a metabolic pathway. Moreover, high-throughput RNA sequencing revealed that *Drosophila* Flil regulates the expression of genes related to biological processes such as chromosome organization, carbohydrate metabolism, and immune reactions. These results showed that *Drosophila* Flil regulates the expression of metabolic genes, and that dysregulation of the transcription controlled by Flil gives rise to metabolic stress and problems in the development and physiology of *Drosophila*. [BMB Reports 2018; 51(9): 462-467]

INTRODUCTION

Flightless-1 was originally identified in *Drosophila* (1) and has been described as an actin-remodeling protein that belongs to the gelsolin protein superfamily (2-4). Flightless-1 contains a gelsolin-like actin-binding domain at the C terminus whereas a leucine-rich repeat (LRR) domain is located at the N terminus (1). The LRR domain is involved in both intramolecular

recognition and structural organization. The gelsolin like domain mediates actin-binding and protein-protein interactions. The Flightless protein has been implicated in actin filament organization during cell migration and tissue repair (5). Beyond cytoskeletal function, flightless-1 has been shown to act as a transcriptional coregulator that can either positively or negatively affect the activity of transcription factors (6-9). Flightless-1 interacts with nuclear receptor (NR), transcription coactivators, and the SWI/SNF chromatin-remodeling complex. Flightless-1 binds to BAF53, a component of SWI/SNF complexes, as well as to estrogen receptor α (ER α), thus contributing to the recruitment of SWI/SNF complexes to the promoter of ER α targets (10). In addition, flightless-1 forms a complex with NR coactivators—glucocorticoid receptor-interacting protein 1 (GRIP1) and coactivator-associated arginine methyltransferase 1 (CARM1)—which leads to the enhancement of NR function (6). In contrast, flightless-1 inhibits β -catenin-mediated transcription through interfering with the binding of FLII leucine-rich repeat-associated protein 1 (FLAP1) to p300 and β -catenin (8).

A recent study of ours has shown that *Drosophila* flightless-1 (Flil) plays a role in lipid metabolism (11). *Drosophila flil* mutants show increased levels of triglycerides and are resistant to starvation. In that study, the upregulation of Flil suppressed the mRNA expression of Desaturase-1, and conversely, the mRNA expression of Desaturase-1 increased in *flil*-mutant flies, suggesting that *Drosophila* Flil downregulates Desaturase-1 at the transcriptional level, thereby contributing to the obese phenotype of *flil* mutants. Based on these results, our purpose here was to investigate the genes whose expression levels are regulated by flightless-1 in *Drosophila*, thereby contributing to the above phenotype. Specifically, we examined the expression levels of genes related to energy metabolism, lipogenesis, and lipolysis; these genes may contribute to the obese phenotype, specifically, the increased levels of triglycerides and insulin resistance, in *flil*-mutant flies. The mRNA expression of most glycolytic-enzyme genes and of some lipogenic enzymes was found to be specifically higher in *flil*-mutant flies. Accordingly, we found that the levels of stearoyl-coenzyme A (CoA) and palmitoyl-CoA specifically increased in *flil* mutants according to metabolomic analysis. In addition, high-throughput RNA

*Corresponding authors. Semin Lee, Tel: +82-52-217-2663; Fax: +82-52-217-2299; E-mail: seminlee@unist.ac.kr; Min-Ji Kang, Tel: +82-2-3010-2797; Fax: +82-2-3010-5307; E-mail: mj kang@amc.seoul.kr

[#]These authors contributed equally to this work.

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sequencing (RNAseq) analysis of *flii*-mutant flies revealed that flightless-1 regulates the expression of genes related to chromatin organization, carbohydrate metabolic process, and proteolysis. Taken together, these results support the transcriptional role of flightless-1 in the expression of genes associated with metabolism.

RESULTS

Metabolic reprogramming in *flii* mutants

Our recent study indicates that *Drosophila flightless-1* mutant flies contain large amounts of triglycerides, which contribute to starvation resistance (11). In general, fat accumulation has been primarily attributed to food intake and energy expenditure (12, 13). Based on the fact that our previous results revealed that *flii* mutant flies do not consume more food (11), we tested whether or not *Flii* is required for the gene expression related to energy metabolism. To that end, we compared the transcript levels of glycolytic genes between seven-day-old controls and *flii*^{3/14} mutants. These *flii* mutant alleles, *flii*³ and *flii*¹⁴, have been characterized in previous studies (11, 14, 15). The *flii*³ mutant allele has a single-base substitution of Gly to Ser at amino acid position 602, which is homozygotically viable. In contrast, the *flii*¹⁴ allele is lethal during the larval and pupal stages. The mRNA expression of most glycolytic-enzyme genes, including *HexA*, *Pgi*, *Capdh*, *Pgk*, and *Eno*, specifically increased in *flii*^{3/14} mutants. Among them, the *Ald* transcript was upregulated more than sixfold in *flii*^{3/14} mutant flies, while the mRNA expression of *Ldh* significantly decreased (Fig. 1A). In contrast, the mRNA expression of tricarboxylic acid (TCA) cycle genes, *CG7430* and *Scsa*, did not significantly increase (Fig. 1B). These results showed that the mutation of *flii* in *Drosophila* accelerated glucose metabolism throughout the body. 6-Phosphogluconate dehydrogenase (*Pgd*) in the pentose phosphate pathway, which is essential for the supply of NADPH (16), was also

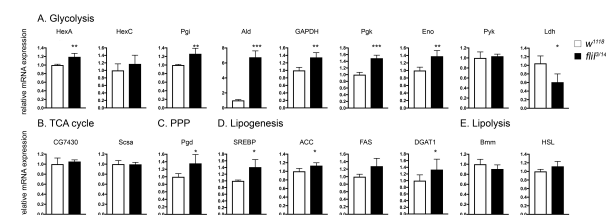


Fig. 1. The changes in expression of metabolic genes in *flii* mutants. Quantitative RT-PCR analysis of each gene in seven-day-old *w*¹¹¹⁸ flies (controls) and *flii*^{3/14} mutants. The values were normalized to *Rp49* data. (A) Relative mRNA abundance of glycolytic-enzyme genes. (B) Relative mRNA expression of TCA cycle genes. (C) Relative expression of *Pgd* mRNA. (D) Relative expression levels of lipogenic genes. (E) Relative mRNA expression of lipolytic genes. Data are presented as mean \pm SE from at least five independent experiments; **P* < 0.05, ***P* < 0.01, and ****P* < 0.001.

upregulated in *flii*^{3/14} mutant flies (Fig. 1C). The byproduct of NADPH produced by 6-Phosphogluconate dehydrogenase activity may be utilized for lipid synthesis. In addition, we found that the mRNA expression of lipogenic genes, such as sterol-regulatory element-binding protein (*SREBP*) and its downstream target genes, *fatty acid synthase* (*FAS*) and *acetyl-CoA carboxylase* (*ACC*), was slightly higher in *flii*^{3/14} mutants (Fig. 1D). In line with these data, we found that the mRNA expression of *DGAT1*, which catalyzes the conversion of diacylglycerol and fatty acyl CoA to triglycerides, was significantly increased (Fig. 1D). In contrast, the mRNA expression levels of lipolytic genes, *brummer* (*Bmm*) and *hormone-sensitive lipase* (*HSL*), did not significantly change (Fig. 1E). Altogether, these results suggest that *Flii* is necessary for the regulation of glucose metabolism and the transcription of lipogenic genes.

Metabolomic analysis of *flii* mutants reveals a change in precursors of long-chain fatty acids

In order to understand how changes in gene expression

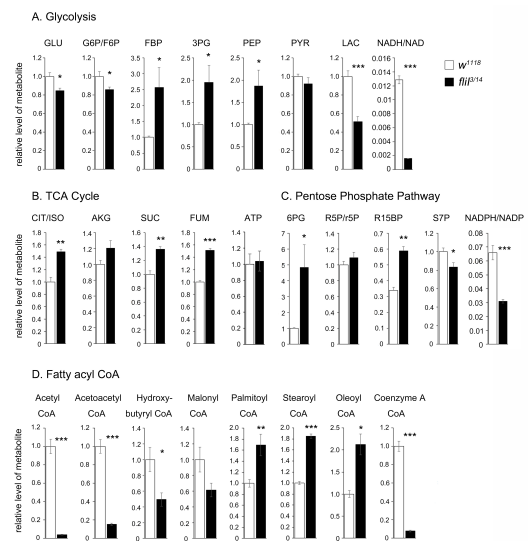


Fig. 2. *Flii* mutants show increased levels of precursors of long-chain fatty acids. The metabolites from seven-day-old *w*¹¹¹⁸ flies (controls) and *flii*^{3/14} mutants were monitored by LC-MS/MS. (A) The amounts of intermediates of glycolysis. The values of each metabolite amount were normalized to the total protein level. (B) The relative levels of intermediates of the TCA cycle. These amounts significantly increased in *flii*^{3/14} mutants compared to *w*¹¹¹⁸ flies, the controls. (C) The levels of intermediates of the pentose phosphate pathway (PPP). The ratio of NADPH/NADP involving a byproduct of the PPP, which was strongly diminished in *flii*^{3/14} mutant flies. The relative levels of fatty acyl CoAs are illustrated in (D). The amounts of precursors of long-chain fatty acyl-CoAs, such as palmitoyl-CoA, stearoyl-CoA, and oleoyl-CoA, were considerably larger in *flii*^{3/14} mutants relative to *w*¹¹¹⁸ flies (controls). Data are presented as mean \pm SE from at least three independent experiments; **P* < 0.05, ***P* < 0.01, and ****P* < 0.001.

influence fat metabolism in *flii* mutants, we compared the metabolic profiles of control and *flii*^{3/14} flies through mass spectrometry.

Specifically, we examined the intermediates of glycolysis, of the TCA cycle, of the pentose phosphate pathway, and the coenzymes involved in the metabolism of fatty acids. In agreement with the results of mRNA expression analysis by quantitative PCR, two important metabolic pathways, glycolysis and the TCA cycle, differed between *w*¹¹¹⁸ flies (controls) and *flii*^{3/14} mutants (Fig. 2A, B). Although lactate was shown to be downregulated in *flii*^{3/14} mutants, the amounts of glycolysis intermediates, such as fructose-1,6-bisphosphate, 3-phosphoglycerate, and phosphoenolpyruvate, increased relative to controls. *Flii*^{3/14} mutants also manifested slight but significant increases in the amounts of TCA cycle intermediates, namely citrate/isocitrate, succinate, and fumarate. Nevertheless, the total ATP level did not differ between the two groups. In addition, we found that a pentose phosphate pathway intermediate, 6-phosphogluconate, was significantly upregulated in *flii*^{3/14} mutant flies. The amount of ribulose-1,5-bisphosphate, which is formed from ribulose 5-phosphate in the cooperative pentose phosphate pathway, significantly increased in *flii*^{3/14} mutant flies. A previous study suggests that ribulose-1,5-bisphosphate stimulates phosphofructokinase-1 and inhibits its opposing enzyme, fructose 1,6-bisphosphatase (17); this mechanism may contribute to glycolysis and the pentose phosphate pathway. NADPH is generated by the pentose phosphate pathway and is utilized for fatty acid synthesis. *Flii*^{3/14} mutants also manifested a significant reduction in the

ratio of NADPH/NADP (Fig. 2C).

The accumulation of long-chain acyl CoAs is frequently observed in obesity or type 2 diabetes (18, 19). Given that *flii*^{3/14} mutants had an obesity-like phenotype, we tested whether this phenotype in *flii*^{3/14} mutants directly affects the amounts of long-chain acyl CoAs. Although seven-day-old *flii*^{3/14} mutants showed decreased levels of coenzyme A and short-chain acyl CoA compared to the wild type, long-chain acyl CoAs, such as palmitoyl-CoA, oleoyl-CoA, and stearoyl-CoA, were found to be upregulated in *flii*^{3/14} mutant flies (Fig. 2D).

Differences in the expression levels of metabolic genes and relevant metabolites between *w*¹¹¹⁸ flies (control) and *flii*^{3/14} mutants, as well as the changes in *flii*^{3/14} mutants are summarized in Fig. 3. In particular, increased levels of mRNA or a metabolite in *flii*^{3/14} mutants are labeled in red, downregulation is indicated with blue, and unchanged ones are shown in black.

Given that *Drosophila* flightless-1 represses the expression of Desaturase-1 (11), whose preferred substrates are palmitoyl-CoA and stearoyl-CoA, we believe that the elevated amounts of long-chain acyl CoAs contributed to the obesity-like phenotype of *flii*^{3/14} mutant flies.

Drosophila flightless-1 regulates the expression of genes related to chromatin remodeling

In order to gain a genome-wide view of the changes in gene expression specifically induced by flightless-1, we performed a differentially expressed gene (DEG) analysis of RNA-seq data from seven-day-old *w*¹¹¹⁸ control and *flii*^{3/14} adult flies. Only

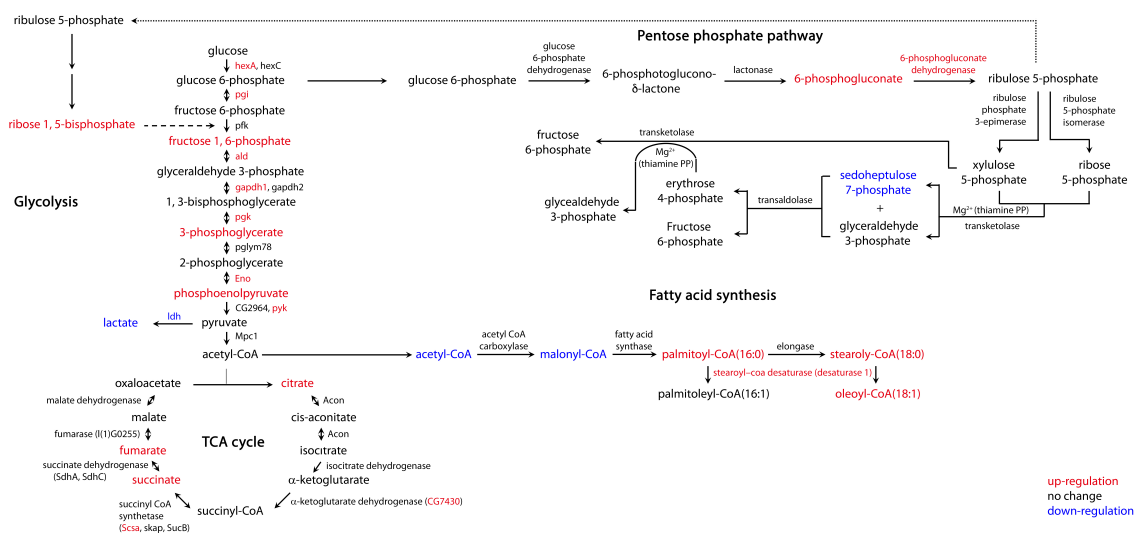


Fig. 3. A schematic diagram of glucose metabolism and lipid metabolism according to the data from Figs. 1 and 2. The red color indicates that mRNA or metabolite levels were significantly upregulated in terms of statistics in *flii*^{3/14} mutants, the blue color denotes downregulation in *flii*^{3/14} mutants, and black indicates no change.

Table 1. GO terms of genes regulated in *flightless-1* mutant flies

GO term	P value	Benjamini	Genes
Downregulated			
GO:0051276 - chromosome organization	3.98×10^{-7}	7.44×10^{-5}	CG33822, CG33810, CG33825, RHI, CG33816, CG33828, CG33861, CG33831, CG33858
GO:0031057 - negative regulation of histone modification	8.97×10^{-7}	8.39×10^{-5}	CG33822, CG33810, CG33825, CG33816, CG33828, CG33831
GO:0006334 - nucleosome assembly	1.58×10^{-6}	9.87×10^{-5}	CG33822, CG33810, CG33850, CG33825, CG33816, CG33828, CG33861, CG33844, CG33831, CG33858, CG33847
GO:0005975 - carbohydrate metabolic process	2.70×10^{-6}	1.26×10^{-4}	MAL-A7, MAL-A6, TOBI, MAL-A8, AMY-P, MAL-A1, AMY-D, AMYREL, CHT8, MAL-B1
GO:0006342 - chromatin silencing	1.39×10^{-4}	5.19×10^{-3}	CG33822, CG33810, CG33850, CG33825, CG33816, CG33828, CG33844, CG33831, CG33847
Upregulated			
GO:0006508 - proteolysis	3.74×10^{-9}	7.29×10^{-7}	CG9897, CG32523, ANCE-4, SPH93, CG42694, CG8539, CG1304, CG17239, SER6, CG15254, CG11842, GAMMATRY, CG9676, CG31267, CG7829, CG31681, CG3088, CG14529, ZETATRY, CG17475, CG8329, CG11911
GO:0050830 - defense response to Gram-positive bacterium	1.53×10^{-5}	1.49×10^{-3}	LYSP, LYSE, LYSD, SPH93, LYSB, GNPB1, IM23
GO:0019835 - cytolysis	9.95×10^{-5}	6.45×10^{-3}	LYSP, LYSE, LYSD, LYSB
GO:0016998 - cell wall macromolecule catabolic process	2.32×10^{-4}	1.13×10^{-2}	LYSP, LYSE, LYSD, LYSB

the transcripts with adjusted P values (Benjamini-Hochberg correction) < 0.05 and a fold change > 2 in gene expression between the two groups were selected for subsequent Gene Ontology (GO) enrichment analysis. According to this criterion, 181 genes were downregulated in *flil* mutants, while 160 genes were upregulated (see Table S1). The Gene Ontology (GO) terms with adjusted $P < 0.05$ are listed in Table 1. Specifically, GO terms enriched in the downregulated genes included those involved in chromosome organization (GO:0051276, adjusted P value of 7.44×10^{-5}), the negative regulation of histone modification (GO:0031057, adjusted P value of 8.39×10^{-5}), nucleosome assembly (GO:0006334, adjusted P value of 9.87×10^{-5}), and a carbohydrate metabolic process (GO:0005975, adjusted P value of 1.26×10^{-4}). This indicates that *Drosophila* Flil not only regulates specific gene expression related to glucose metabolism, but also seems to regulate the gene expression in general under certain conditions by reorganizing chromatin structure. Prominent among the genes whose expression increased significantly were those encoding proteolytic enzymes (GO:0006508, adjusted P value of 7.29×10^{-7}), proteins related to a defense response to a gram-positive bacterium (GO:0050830, adjusted P value of 1.49×10^{-3}), and proteins associated with cytolysis (GO:0019835, adjusted P value of 6.45×10^{-3}). The activators of Imd signaling (PGRP-LC) and lysozymes (LysB, -D, and -E) were highly expressed in *flil* mutant flies (Table S1). An abnormally active immune response is associated with the upregulation of stress response

factors, all of which have been previously linked to immune function, including the translational repressor 4E-BP/Thor (20). These data are in agreement with those of our recent study suggesting that the mRNA level of 4E-BP is increased in *flil*^{3/14} mutants (11). The RNA-seq. results were further validated through quantitative real-time PCR (Supplemental Fig. 2). These observations indicate that the transcriptional activity of flightless-1 controls the expression of genes related to chromatin remodeling, metabolism, and immune responses.

DISCUSSION

Here, we reveal the transcriptional influence of *Drosophila* Flil on the expression of genes including those participating in chromatin remodeling, carbohydrate metabolic processes, and immune responses. Unexpectedly, our RNAseq analysis suggests that *Drosophila* flightless-1 regulates the expression of histones, which are basic nuclear proteins that are responsible for the nucleosome structure of the chromosomal fiber (21, 22). Histones are required for the condensation of nucleosome chains into higher-order structures. They also function as regulators of individual gene transcription through chromatin remodeling. In addition, the genes whose expression decreased in *flightless-1* mutants, *rhino* and *CREG*, are known to control gene transcription through producing Piwi-interacting RNA (23) or inhibiting transcription factor binding (24). Thus, although *Drosophila* flightless-1, as a transcription factor, may regulate the expression of genes related to specific pathways

including glucose metabolism, immune responses, or proteolysis, it seems to regulate gene expression by reorganizing the chromatin structure under certain conditions. These phenomena need to be validated through further studies.

Given that *flii*^{3/14} mutants show features of a metabolic disorder (11), we analyzed the changes in metabolites in the metabolic pathways including glycolysis, the TCA cycle, pentose phosphate pathway, and fatty acid synthesis (Fig. 2). Through this assay, we found that the NADPH/NADP ratio was clearly lower in the mutants. NADPH is an essential cofactor in *de novo* lipogenesis, which requires NADPH as a reducing agent for the conversion of acetyl-CoA into fatty acids (25-27). The amounts of NADPH in *flii*^{3/14} mutants were relatively low as compared to those in *w*¹¹¹⁸ flies. We can explain this result as a form of supply and demand: NADPH is mainly generated by the pentose phosphate pathway. Our results indicate that the level of ribulose 1,5-phosphate, which is a product of the pentose phosphate pathway, significantly increased in *flii*^{3/14} mutant flies (Fig. 2). In addition, mRNA expression of the gene encoding one of the NADPH-producing enzymes, phosphogluconate dehydrogenase (Pgd), increased slightly but significantly in *flii* mutants (Fig. 1C). Therefore, we can theorize that in *flii*^{3/14} mutants, NADPH and acetyl-CoA were rapidly consumed for the elongation of long-chain fatty acids, driving the increase in fat contents throughout the body. Among the intermediates of glycolysis, the amount of lactate significantly decreased in *flii*^{3/14} mutant flies; this is consistent with the observed reduction in *Ldh* mRNA expression. As shown in Fig. 3, the levels of expression of glycolytic genes and glycolysis intermediates were significantly increased in *flii*^{3/14} mutant flies compared to in control flies. However, the mRNA expression levels of tricarboxylic acid (TCA) cycle genes and the levels of TCA cycle intermediates were only slightly changed. Thus, we believe that the increased amounts of glycolytic intermediates were not consumed for lactate production but were instead mostly converted to acetyl-CoA for the elongation of long-chain fatty acids.

Given that *flii*^{3/14} mutant flies showed increased levels of triglycerides, we expected that the amounts of lipogenic enzymes, such as SREBP, FAS, and ACC, would be greater in *flii*^{3/14} mutant flies, but there were slight and significant differences in the expression of these proteins between *w*¹¹¹⁸ and *flii*^{3/14} flies. Emerging evidence indicates that the activity of a metabolic enzyme can be regulated post-translationally under certain conditions without changing the expression level (28-30). We assumed that an enzyme's activity can somewhat contradict its expression level because enzymes may be regulated by post-translational modifications in the mutant. Therefore, in future studies, it is necessary to test the activities of the enzymes that we examined in this study.

In summary, we analyzed the expression levels of metabolic genes and the levels of metabolites in *flii*-mutant flies. These results support previous findings showing that *Drosophila* Flil serves as a key regulator of lipid metabolism. In addition,

RNAseq analysis revealed the transcriptional targets of Flil in *Drosophila*, which include the genes related to chromosome organization, carbohydrate metabolism, proteolysis, and immune responses.

MATERIALS AND METHODS

Plasmids and fly strains

All *Drosophila* stocks were raised at 25°C on a standard cornmeal medium containing 4.94% molasses, 3.8% cornmeal, 1.6% yeast, and 1.2% agar. Genes were expressed in *Drosophila* through the standard Gal4/UAS system. Fly strains *w*¹¹¹⁸ (stock number 5905), *flii*³ (stock number 4730), and *flii*¹⁴/FM6 (stock number 7481) were obtained from the Bloomington Stock Center.

Quantitative RT-PCR

Total RNA was isolated from five female flies using the TRIzol Reagent (Invitrogen, USA), and 200 ng of RNA was transcribed using the ReverTra Ace qPCR RT Kit (Toyobo Co., Japan). Quantitative PCR amplification was run for 40 cycles by means of the TOPreal™ qPCR 2X PreMIX (SYBR Green with high ROX) and a LightCycler® 480 Real-Time PCR System. *Rp49* served as a reference for normalization. Relative quantification of mRNA was performed through the comparative C_T method (31). The primers are listed in Supplemental Table 2. Detailed information is included in the Supplemental Material.

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CONFLICTS OF INTEREST

The authors have no conflicting interests.

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