Metabolic and transcriptome responses of RNAi-mediated AMPKα knockdown in Tribolium castaneum

Heng Jiang1, Nan Zhang1, Caihong Ji1, Xiangkun Meng1, Kun Qian1, Yang Zheng1 and Jianjun Wang1,2*

Abstract

Background: The AMP-activated protein kinase (AMPK) is an intracellular fuel sensor for lipid and glucose metabolism. In addition to the short-term regulation of metabolic enzymes by phosphorylation, AMPK may also exert long-term effects on the transcription of downstream genes through the regulation of transcription factors and coactivators. In this study, RNA interference (RNAi) was conducted to investigate the effects of knockdown of TcAMPKα on lipid and carbohydrate metabolism in the red flour beetle, Tribolium castaneum, and the transcriptome profiles of dsTcAMPKα-injected and dsEGFP-injected beetles under normal conditions were compared by RNA-sequencing.

Results: RNAi-mediated suppression of TcAMPKα increased whole-body triglyceride (TG) level and the ratio between glucose and trehalose, as was confirmed by in vivo treatment with the AMPK-activating compound, 5-Aminoimidazole-4-carboxamide1-β-D-ribofuranoside (AICAR). A total of 1184 differentially expressed genes (DEGs) were identified between dsTcAMPKα-injected and dsEGFP-injected beetles. These include genes involved in lipid and carbohydrate metabolism as well as insulin/insulin-like growth factor signaling (IIS). Real-time quantitative polymerase chain reaction analysis confirmed the differential expression of selected genes. Interestingly, metabolism-related transcription factors such as sterol regulatory element-binding protein 1 (SREBP1) and carbohydrate response element-binding protein (ChREBP) were also significantly upregulated in dsTcAMPKα-injected beetles.

Conclusions: AMPK plays a critical role in the regulation of beetle metabolism. The findings of DEGs involved in lipid and carbohydrate metabolism provide valuable insight into the role of AMPK signaling in the transcriptional regulation of insect metabolism.

Keywords: Tribolium castaneum, AMPK, RNA interference, Transcriptome, Lipid metabolism, Carbohydrate metabolism IIS pathway

* Correspondence: wangjj@yzu.edu.cn
1 College of Horticulture and Plant Protection, Yangzhou University, Yangzhou 225009, China
2 Joint International Research Laboratory of Agriculture and Agri-Product Safety of the Ministry of Education, Yangzhou University, Yangzhou 225009, China

© The Author(s). 2020 Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons licence, and indicate if changes were made. The images or other third party material in this article are included in the article’s Creative Commons licence, unless indicated otherwise in a credit line to the material. If material is not included in the article’s Creative Commons licence and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this licence, visit http://creativecommons.org/licenses/by/4.0/. The Creative Commons Public Domain Dedication waiver (http://creativecommons.org/publicdomain/zero/1.0/) applies to the data made available in this article, unless otherwise stated in a credit line to the data.
Background
The survival of all organisms depends on the maintenance of energy homeostasis. AMP-activated protein kinase (AMPK) is a cellular energy sensor conserved across all eukaryotic species [13]. As a serine/threonine protein kinase complex, AMPK consists of a catalytic subunit \( \alpha \) and two regulatory subunits, \( \beta \) and \( \gamma \), and is activated in response to energy stress by sensing increases in ADP/ATP and AMP/ATP ratios, which leads to the activation of ATP-generating catabolic pathways including glycolysis and fatty acid oxidation and the inhibition of ATP-consuming anabolic pathways such as gluconeogenesis, fatty acid, and protein synthesis [17]. While nucleotide-dependent phosphorylation of Thr172 in the \( \alpha \) subunit by liver kinase B1 (LKB1) is the principal event required for full activation of AMPK in mammalian cells [19, 70], several studies have revealed the nucleotide-independent regulation of AMPK via the phosphorylation of Thr172 by calcium/calmodulin-dependent kinase kinase 2 in mammals (CAMKK2) [20, 23, 69].

Lipids and carbohydrates are major sources for energy storage and supply in cells. Under aerobic conditions, most energy is derived from fatty acids oxidation and the rest of energy is obtained from glucose oxidation. In contrast, glycolysis plays an important role in ATP production under anoxic condition [51]. Activation of AMPK occurs in response to stress circumstances such as starvation, exercise, hypoxia and ischemia, heat shock, and oxidative stress [9, 11, 18, 45, 47, 49, 67]. It is well known that AMPK can regulate lipid and carbohydrate homeostasis via direct phosphorylation of multiple downstream effectors. Acetyl-CoA carboxylase (ACC), the first rate-limiting enzyme in fatty acid synthesis, glycerol-3-phosphate acyltransferases (GPAT), the rate-limiting enzyme in triglyceride (TG) synthesis, and 3-hydroxy-3-methylglutaryl-CoA reductase (HMGR), the rate-limiting enzyme of cholesterol biosynthesis pathway, can be inhibited by AMPK through phosphorylation in rats [8, 42, 68]. Subsequently, AMPK was found to promote lipid absorption and release by directly phosphorylating lipases like hormone-sensitive lipase (HSL) and adipocyte-triglyceride lipase (ATGL) in mice and Caenorhabditis elegans [1, 44, 64]. On the other hand, AMPK also stimulates glycolysis via the phosphorylation and activation of 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase 3 (PFKFB3) and 6-phosphofructo-2-kinase (PFK2) in human tissues [37, 38]. Additionally, AMPK activation was also shown to increase whole-body insulin sensitivity by phosphorylation of insulin receptor substrate-1 (IRS-1) in mouse [24] and insulin receptor (InR) in rodent muscle [10]. Given the functional attributes of AMPK in lipid and carbohydrate metabolism, AMPK is considered as an important therapeutic target for treating metabolic diseases including obesity and type 2 diabetes [43].

Although the role of AMPK in the regulation of cell metabolism is well studied in mammals, related research is still limited in insects. Notably, AMPK can regulate energy balance via modulation of transcriptional expression of metabolic enzymes in the long term, however, its downstream transcriptional pathways remains largely elusive [7]. Recently, we reported the transcriptional and post-translational activation of TcAMPKα by oxidative, heat and cold stresses in the red flour beetle, Tribolium castaneum [25]. In this study, RNAi was employed to determine the roles of TcAMPKα in lipid and carbohydrate metabolism. Comparison, annotation and classification of DEGs between dsTcAMPKα treatment and control groups were also conducted by high-throughput transcriptome sequencing to identify metabolism-related genes modulated by AMPK in T. castaneum.

Results
Effects of TcAMPKα suppression on TG, glucose and trehalose levels
RNAi was conducted to determine the effects of TcAMPKα knockdown on TG, glucose and trehalose levels. The injection of 20-day-old larvae with dsTcAMPKα reduced transcription levels by 95.50% ± 1.86% (ANOVA, df = 2, 4, F = 97.506, \( P \) value = 0.0027) on the sixth day after injection. TG measurement showed that the TG level in dsTcAMPKα group (9.03 ± 0.76 mmol/mgprot) was significantly increased by 53.49% ± 8.61% (ANOVA, df = 2, 4, \( F \) = 7.917, \( P \) value = 0.04813) when compared with the dsEGFP group (5.96 ± 0.78 mmol/mgprot) (Fig. 1a).

Similarly, increased glucose level by 62.34% ± 11.61% (ANOVA, df = 2, 4, \( F \) = 81.96, \( P \) value = 0.04580) was observed in the beetles injected with dsTcAMPKα (25.85 ± 6.12 μmol/g) when compared to the control beetles (8.32 ± 0.08 μmol/g) (Fig. 1b). However, the trehalose level in dsTcAMPKα group (3.73 ± 0.10 mg/g) was significantly reduced by 8.56% ± 3.01% (ANOVA, df = 2, 4, \( F \) = 9.357, \( P \) value = 0.0377) than that in dsEGFP group (4.08 ± 0.05 mg/g) (Fig. 1c). These data suggested that RNAi of TcAMPKα increased TG production and the ratio between glucose and trehalose (Fig. 2a).

Effects of AICAR treatment on TG, glucose and trehalose levels
To confirm the RNAi results, the 20-day-old larvae were treated with an activator of AMPK 5-Aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR), and the TG, glucose and trehalose levels were measured. The results showed that the levels of TG and glucose in AICAR group were significantly decreased by 34.60% ± 5.74% (ANOVA, df = 2, 4, \( F \) = 10.770, \( P \) value = 0.03045) and 41.89% ± 2.27% (ANOVA, df = 2, 4, \( F \) = 93.320, \( P \) value = 0.0006), respectively, compared with injection buffer (IB)
group, whereas the trehalose level increased by 17.07% ± 4.02% (ANOVA, df = 8.910, P value = 0.0405) in beetles treated with AICAR. These data suggested that activation of TcAMPK decreased TG production and the ratio between glucose and trehalose (Figs. 1 d-f; 2b).

Transcriptome sequence and reads mapping

The dsTcAMPKα and dsEGFP groups were analyzed by RNA-Seq (three independent biological replicates of each treatment). A mean of 23,570,938 clean reads were generated among six independent libraries (T01-T06) (Table 1). Evaluation of clean data quality showed that the GC counts ranged from 42 to 45% and Q30 ratios were > 93%, indicating a high level of data quality. The alignment of clean reads to the reference genome database of *T. castaneum* showed that 83.50 and 77.15% reads of the dsEGFP and dsTcAMPKα groups were aligned on average, respectively (Table 2).

Correlation analysis was conducted with Pearson’s Correlation Coefficient (R value) to evaluate the
and Table S3). The log 2-fold variation range of DEGs upregulated and 835 downregulated unigenes (Fig. 4a, b) values. 1184 DEGs were obtained including 349 script sequence per Million base pairs sequenced identified based on their Fragments Per Kilobase of transcript sequence per Million base pairs sequencing data.

Table 2 Summary of the transcriptome sequencing data from the controls and dsTcAMPKα treated samples

| Samples | ID | Clean Read Number | Clean Base Number | GC (%) | Q30 (%) |
|---------|----|-------------------|-------------------|--------|---------|
| EGFP1   | T01 | 21,746,430        | 6,523,929,000     | 42.79  | 93.39   |
| EGFP2   | T02 | 20,376,206        | 6,112,861,800     | 42.86  | 93.94   |
| EGFP3   | T03 | 26,557,829        | 7,967,348,700     | 43.85  | 92.88   |
| dsTcAMPKα1 | T04 | 24,192,194        | 7,257,658,200     | 44.91  | 93.25   |
| dsTcAMPKα2 | T05 | 23,215,801        | 6,964,740,300     | 45.01  | 92.85   |
| dsTcAMPKα3 | T06 | 25,337,170        | 7,601,151,000     | 45.54  | 92.75   |

Further analysis revealed that the three samples in each group had similar expression distributions of reads, while the control and treatment groups had significantly different expression distributions (Fig. 3b).

Further annotation of expressed unigenes revealed that a total of 14,095 unigenes out of 31,944 unique sequences were annotated and classified into at least one database of Non-redundant (Nr), Eukaryotic Orthologous Groups (KOG), Clusters of Orthologous Groups of proteins (COG), Kyoto Encyclopedia of Genes and Genomes (KEGG), Protein family (Pfam), Gene Ontology (GO) and Swiss-Prot databases (Figure S1, Figure S2 and Table S3). In molecular function category, most of the DEGs significantly downregulated with the expression log 2-fold change from 1.02 to 96.147, P value = 0.00061), a key regulator of glucose metabolism classifications such as “Lipid transport and metabolism”, “Carbohydrate transport and metabolism”, “Amino acid transport and metabolism”, “Energy production and conversion” and “Secondary metabolites biosynthesis, transport and catabolism” (Table 3). Furthermore, most DEGs involved in protein translation were downregulated such as some ribosomal proteins (Table 3). Similarly, among the 42 DEGs in the post-translational modification class, 35 DEGs were downregulated, including 3 heat shock proteins (Hsps), whereas the phosphatidylinositol 4,5-bisphosphate 3-kinase (PIK3) and InR2 involved in the signal transduction mechanisms were upregulated.

Expression of genes involved in lipid metabolism, carbohydrate metabolism and insulin signaling

Besides the analysis of the entire gene set, we specifically checked for up- or downregulation of lipid and carbohydrate metabolism genes in our set of DEGs. The results showed that the insect adipose triacylglycerol lipase homologue, brummer (Accession no. TC011935), which is responsible for the first step of TG hydrolysis, was significantly downregulated with the expression log2-fold change of ~2.20 (Fold change: 0.22; ANOVA, df1, 4, F = 5.152, P value = 1.60 × 10^-39), whereas two fatty acid synthetase genes (FAS1 and FAS2) (Accession no. TC015337 and TC015340) (Fold change: 2.03 and 3.22, ANOVA, df1, 4, F = 9.182 and 157.306, P value = 0.03878 and 0.00023) involved in fatty acid biosynthetic pathways, and the transcription factor ChREBP (Accession no. TC010471) (Fold change: 2.27, ANOVA, df1, 4, F = 96.147, P value = 0.00061), a key regulator of glucose and lipid metabolism and fat storage [62], were upregulated with the expression log2-fold change from 1.02 to 1.76 (Fold change from 2.03 to 3.39) (Table 4). Knockdown of TcAMPKα also caused upregulation of genes involved in IIS pathway, including PI3K, IRS1 and InR2 (Accession no. TC011996, TC034013 and TC010784) (Fold change: 2.22, 2.19 and 2.46; ANOVA, df1, 4, F = 86.102, 14.636 and 34.155, P value = 0.00075, 0.01869 and 3.37 × 10^-08). To confirm the reliability of the DEG

Table 1 Summary of the transcriptome sequencing data from the controls and dsTcAMPKα treated samples

| Samples | ID | Clean Read Number | Clean Base Number | GC (%) | Q30 (%) |
|---------|----|-------------------|-------------------|--------|---------|
| EGFP1   | T01 | 21,746,430        | 6,523,929,000     | 42.79  | 93.39   |
| EGFP2   | T02 | 20,376,206        | 6,112,861,800     | 42.86  | 93.94   |
| EGFP3   | T03 | 26,557,829        | 7,967,348,700     | 43.85  | 92.88   |
| dsTcAMPKα1 | T04 | 24,192,194        | 7,257,658,200     | 44.91  | 93.25   |
| dsTcAMPKα2 | T05 | 23,215,801        | 6,964,740,300     | 45.01  | 92.85   |
| dsTcAMPKα3 | T06 | 25,337,170        | 7,601,151,000     | 45.54  | 92.75   |

Changes in gene expression profiles

To identify the effects of knock-down of TcAMPKα on global unigene expression patterns of T. castaneum, DEGs between dsTcAMPKα and dsEGFP groups were identified based on their Fragments Per Kilobase of transcript sequence per Million base pairs sequenced (FPKM) values. 1184 DEGs were obtained including 349 upregulated and 835 downregulated unigenes (Fig. 4a, b and Table S3). The log2-fold variation range of DEGs was between ~6.07 and 3.75 (Fold change from ~67.18 to 13.45, P value from 1.27 × 10^-67.18 to 3.75 × 10^-3.75).

DEGs were classified by searching against GO and KOG databases. GO term enrichments revealed that 3 heat shock proteins (Hsps), whereas the phosphatidylinositol 4,5-bisphosphate 3-kinase (PIK3) and InR2 involved in the signal transduction mechanisms were upregulated.
data, the expression levels of these DEGs were determined using RT-qPCR (Fig. 6 and Table 4). Gene expression levels validated by RT-qPCR showed the high consistency with transcriptome sequencing.

To be as inclusive as possible, less strict screening criteria (Fold change $|\log_2(treatment/control)| > 0.5$) were applied, and additional lipid and carbohydrate metabolism-related genes with the expression changed in transcriptome were selected for further verification using RT-qPCR. The results showed that five genes involved in fatty acid and tri-glyceride biosynthetic pathways including three FAS (FAS3–5) (Accession no. TC011522, TC015400 and TC000238) (Fold change: 1.73, 1.55 and 1.50; ANOVA, df$_{1, 4}$, F = 1.717, 0.785 and 2.781, $P$ value = 0.26021, 0.42567 and 0.17070), one ACC (Accession no. TC015612) (Fold change: 1.77; ANOVA, df$_{1, 4}$, F = 0.785; $P$ value = 0.42567).
2.539, P value = 0.18632), one glycerol-3-phosphate
acyltransferases (GPAT) (Accession no. TC004512)
(Fold change: 1.59; ANOVA, df 1, 4 , F = 8.092, P value =
0.04665), four trehalase (TRE) genes responsible for
trehalase hydrolysis (Accession no. TC006698, LOC659620,
TC004791 and TC006697) (Fold change: 1.86, 1.48, 1.60 and
1.86; ANOVA, df 1, 4 , F = 9.491, 0.699, 25.660 and 9.491, P
value = 0.03690, 0.45001, 0.00715 and 0.03690), two genes
involved in gluconeogenesis including pyruvate carboxylase
(PC) (Accession no. TC032730) (Fold change: 1.73; ANOVA,
df 1, 4 , F = 19.919, P value = 0.01113) and phosphoenolpyr-
uvate carboxykinase (PEPCK) (Accession no. TC009072)
(Fold change: 1.23; ANOVA, df 1, 4 , F = 1.491, P value =
0.28918) were significantly upregulated (Table 4). Interest-
ingly, SREBP1(Accession no. TC007163) (Fold change: 1.93;
ANOVA, df 1, 4 , F = 9.490, P value = 0.03691), the master
regulator of lipid homeostasis, and SREBP cleavage-
activating protein (SCAP) (Accession no. TC013456) (Fold
change: 1.75; ANOVA, df 1, 4 , F = 10.483, P value = 0.03174),
a central regulator of lipogenesis that controls the activity of
SREBP [53] were also significantly upregulated (Table 4).

Discussion
In insects, the energy for growth and development is
mainly derived from the storage and utilization of lipids
and carbohydrates in specific tissues such as fat body,
midge and oenocytes [39]. TG is the master form of
lipids stored in fat body and plays an important role in
energy storage and release [63]. Meanwhile, glucose
(monosaccharide) and trehalose (disaccharide) provide
energy through glycolysis [48]. Given that AMPK acti-
vates ATP-generating pathways and inhibits energy-
consuming processes under conditions of low energy
status [60], the attenuation of AMPK signaling in insects
may disrupt the energy balance in vivo. In this study, we
found that the TG and glucose levels in dsTcAMPKα-
injected T. castaneum were significantly increased com-
pared with dsEGFP-injected insects, while a decreased
trehalose content was observed in dsTcAMPKα-injected
insects. These results were further confirmed by in vivo
AICAR treatment, which resulted in the decreased TG
and glucose levels and increased trehalose content. Simi-
larly, AICAR-induced AMPK activation resulted in sig-
nificantly decreased TG level in lean and obese rodents,
in vivo [6]. In human HepG2 cells, the kinase-inactive
AMPKα increased lipid content and prevent the metfor-
min from decreasing lipid accumulation [74]. However,
lower TG levels were observed in D. melanogaster with
reduced AMPK function during fed conditions [27]. On
the other hand, while activation of AMPK triggered a

Fig. 5 KOG function classifications of the differentially expressed unigenes. The X-axis represents names of 25 groups, and the Y-axis corresponds
to the number of unigenes in the group
reduction in glucose levels in vivo in mice [72], and increased hepatic glucose production was observed in AMPKα2 knockout mice [3], knockdown of hypothalamic AMPK activity in male Sprague-Dawley rats led to a significant suppression of glucose production [73]. These results indicate the complexity of the role of AMPK in the regulation of metabolic processes.

As an energy sensor that regulates cellular metabolism, AMPK not only has acute effects on metabolic enzymes by direct phosphorylation, but also shows long term action to change the transcriptional levels of metabolic proteins and enzymes. For example, activation of AMPK in liver and adipocytes can downregulate FAS activity and inhibit lipogenesis [36]. Activation of AMPK by 4-Hydroxyderricin and xanthoangelol downregulates GPAT in 3 T3-L1 cells [75], an enzyme necessary for triacylglycerol synthesis [66]; Similarly, in C. elegans, AAK-2 can inhibit fat synthesis under stress condition by downregulating lipid synthesis-related genes such as Δ9 fatty acid desaturases which can produce monounsaturated fatty acids to constitute TGs [55, 65]. To investigate the downstream transcriptional pathways of AMPK in T. castaneum, the transcriptomes of dsTcAMPKα-injected and dsEGFP-injected larvae under normal conditions were compared. Global gene expression profiles of dsTcAMPKα group were distinct from dsEGFP group with 1184 DEGs. Of particular interest is the upregulation of genes involved in fatty acid and TG biosynthetic pathways, such as fatty acyl-CoA reductase, desaturase, and monooxygenase.

| Categories                                      | Up-regulated DEGs | Down-regulated DEGs |
|------------------------------------------------|-------------------|---------------------|
| Signal transduction mechanisms                 | 26                | 23                  |
| Translation, ribosomal structure and biogenesis | 1                 | 42                  |
| Posttranslational modification, protein turnover, chaperones | 7                | 35                  |
| Amino acid transport and metabolism            | 4                 | 30                  |
| Lipid transport and metabolism                 | 5                 | 17                  |
| Energy production and conversion               | 1                 | 23                  |
| Secondary metabolites biosynthesis, transport and catabolism | 5               | 12                  |
| Carbohydrate transport and metabolism          | 1                 | 6                   |

Table 3 DEGs involved in different functional categories of KOG database

Jiang et al. BMC Genomics (2020) 21:655 Page 7 of 13
Table 4 DEGs encoding metabolism related proteins and transcription factors/co-activators from *T. castaneum* responding to dsTcAMPKα treatment

| Unigene name | padj       | Description (blast)                  | Length (ORF bp) | Log2Ratio Transcriptome | Log2Ratio qRT-PCR |
|--------------|------------|--------------------------------------|------------------|-------------------------|-------------------|
| Lipid metabolism |           |                                      |                  |                         |                   |
| FAS1         | 0.038775298| fatty acid synthase                   | 12,981           | 1.02                    | 0.37              |
| FAS2         | 0.056982009| fatty acid synthase                   | 6522             | 1.76                    | 0.71              |
| FAS3         | 0.260213539| fatty acid synthase                   | 7152             | 0.79                    | 1.05              |
| FAS4         | 0.425672932| fatty acid synthase                   | 6630             | 0.63                    | 1.16              |
| FAS5         | 0.170696071| fatty acid synthase                   | 6450             | 0.58                    | 1.53              |
| ACC          | 0.186316184| acetyl-CoA carboxylase                | 7005             | 0.83                    | 0.10              |
| GPAT3        | 0.046646995| glycerol-3-phosphate acyltransferases | 1440             | 0.67                    | 1.51              |
| Brummer      | 1.60E-05   | triacylglycerol lipase                | 1635             | −2.20                   | −0.37             |
| Carbohydrate metabolism | |                                      |                  |                         |                   |
| TRE1–1       | 0.036902117| Trehalase1–1                          | 1662             | 0.89                    | 1.46              |
| TRE1–3       | 0.450009964| Trehalase1–3                          | > 507            | 0.56                    | 0.58              |
| TRE1–4       | 0.007152475| Trehalase1–4                          | 1812             | 0.68                    | 0.59              |
| TRE2         | 0.036902117| Trehalase2                            | 1647             | 0.89                    | 1.61              |
| Insulin signaling pathway | |                                      |                  |                         |                   |
| IRS1         | 0.018687502| insulin receptor substrate            | 2760             | 1.13                    | 0.63              |
| InR2         | 0.004419643| insulin-like receptor                 | 4185             | 1.09                    | 0.23              |
| P13K         | 3.37E-08   | phosphatidylinositol 4,5-bisphosphate 3-kinase | 3186             | 1.02                    | 0.63              |
| Transcription factor and co-activator | |                                      |                  |                         |                   |
| SCAP         | 0.031742692| sterol regulatory element binding protein cleavage-activating protein | 3783            | 0.81                    | 1.12              |
| SREBP1       | 0.036908768| sterol regulatory element binding protein 1 | 3078            | 0.94                    | 1.74              |
| ChREBP       | 0.000060393| carbohydrate response-element-binding protein | 510              | 1.18                    | 1.94              |

Fig. 6 Comparison of gene expression patterns obtained by RNA-Seq and RT-qPCR
metabolism enzymes might contribute to the increased TG and glucose levels and decreased trehalose content in dsEGFP-injected beetles.

AMPK activation has been reported to cause a reduction in transcriptional activity of several metabolism-related transcription factors. As a key transcription factor that regulates cellular lipogenesis in liver, skeletal muscle and adipose tissue, insulin-activated SREBP1 acts in synergy with glucose-sensitive ChREBP, which mediates the response to dietary carbohydrates and is essential for regulating lipogenic gene expression [46]. AMPK-inhibited SREBP1 can block expression of some gluconeogenic and lipogenic genes, whereas SREBP1 overexpression can increase their transcription [12, 31, 54]. Activation of AMPK by metformin or an adenosine analogue suppresses the expression of SREBP1. In metformin-treated rats, hepatic expression of SREBP1 mRNAs and protein is reduced [76]. A recent study indicated that AMPK interacts with and directly phosphorylates SREBP1, suppresses SREBP1 cleavage and nuclear translocation, and represses SREBP1 target gene Fas expression in hepatocytes in response to metformin treatment, leading to reduced lipogenesis [34]. On the other hand, AMPK also phosphorylates Ser568 and reduce DNA binding capacity and promote nuclear exclusion of murine ChREBP [28, 29]. Interestingly, the promotion of PEPCK expression is associated with increased expression of SREBP-1 and ChREBP in high fat acid (HFFA)-treated hepatocytes [32], and a recent study also revealed the role of ChREBP in gluconeogenesis [58]. In this study, SREBP1, ChREBP and SCAP were significantly upregulated in dsTcAMPKα-injected insects, which in turn might modulated the expression of genes involved in lipid and carbohydrate metabolism.

IIS pathway was involved in the regulation of glucose and lipid metabolism (Saltie and Kahn 2001) [50]. In addition to the regulation of lipid synthesis, studies reveal expanding roles for SREBP1 in controlling pathways for insulin resistance [34], in which the pathological process involves a series of cascades, including defective activation of IRS and PI3K [22, 30, 59]. Overexpression of SREBP1 decreased Irs-1 mRNA levels in a dose-dependent manner, and SREBP1 knockdown led to an upregulation of IRS-1 levels [34]. Further luciferase reporter assay confirmed that Irs-1 promoter activity was repressed by SREBP1 overexpression [34]. However, in liver with Nonalcoholic fatty liver disease (NAFLD), IRS-1 expression was enhanced and correlated positively with SREBP1 expression. In contrast, IRS-2 expression decreased by 50% and was not correlated with SREBP1 [31]. In sebocytes, insulin-like growth factor-1 (IGF-1) induces SREBP-1 expressions at both mRNA and protein levels in a PI3K-dependent manner, accompanied by an increase in the transcription of SREBP-1 target genes such as FAS [57]. Additionally, insulin-stimulated endogenous ChREBP expression was also observed in HepG2 and primary hamster hepatocytes (Sirek et al. 2009) [56]. In this study, we observed the up-regulation of IIS-related DEGs like IRS1, InR2 and PI3K in dsTcAMPKa-injected beetles, which might result in the upregulation of SREBP1 and ChREBP. Interestingly, it has been reported that knockdown in expression of insulin like peptide 2 (ILP2) caused a decrease in TRE mRNA levels in T. castaneum [71], suggesting the positive control of TRE transcription by IIS signaling. Further study is needed to clarify the mechanism of upregulation of IIS signaling in dsTcAMPKa-injected insects.

Conclusion
This study confirmed that AMPK has an important role in the regulation of beetle metabolism. Specifically, our study showed that knockdown of AMPK causes alteration in expression levels of genes involved in lipid and carbohydrate metabolism as well as IIS signaling. Such investigations will help us understand the function of AMPK in transcriptional regulation of insect metabolism.

Methods

Experimental insects
The Georgia-1 (GA-1) strain of T. castaneum was reared at 30°C and 50% relative humidity in 5% yeasted flour under standard conditions as described previously [15, 33].

Double-strand RNA synthesis and injection
Gene specific primers (Table S1) with T7 promoter were designed to synthesize the dsRNAs targeting nucleotides 844–1285 (442 bp) of the ORF region of the TcAMPKa using TranscriptAid™ T7 High Yield Transcription Kit (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer’s instructions. The synthesized dsRNAs were diluted in diethyl pyrocarbonate (DEPC)-treated water with a concentration of 2 μg/μL, and about 200 ng of dsRNA in 200 nL IB was injected into 20-day-old larvae using a Nanoliter 2010 injector system (WPI, Sarasota, FL, USA) under a stereomicroscope. A total of 10 unsexed insect larvae were collected as a sample for RNA extraction at six- days post-injection. The dsEGFP-injected larvae (CK group) were set as controls in all injection experiments. Three biologically independent replicates were carried out with at least 100 insects (≥ 200 mg) in each replicate.

Triglyceride (TG) measurement
Total TG levels were determined using the liquid TG (GPO-PAP) method [4]. Briefly, each replicate with 10 injected larvae were homogenized in 270 μL of PBS (0.1 mol/L PH 7.4) and centrifuged at 2500 rpm for 10 min. The supernatant was collected, and the TG level was analyzed by using TG Assay Kit (catalogue no. A110–1,
Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer’s instructions. Three independent biological replicates and four technical replicates were performed for every treatment.

Glucose and trehalose measurement
Total glucose levels were measured using glucose content assay kit (catalogue no. BC2500, Solarbio Science & Technology, Beijing, China) according to the manufacturer’s instructions. Briefly, 10 larvae on day 6 after injection of dsTcAMPKα or dsEGFP were weighed and homogenized in 0.3 mL double distilled water. The supernatant was collected after centrifugation at 8000 g for 10 min and used to quantify the level of glucose at 505 nm with Multiskan FC microplate reader (Thermo Fisher Scientific, Waltham, MA, USA). Three independent biological replicates and 4 technical replicates were performed for every treatment.

Total trehalose level was measured using Trehalose content detection kit (catalogue no. A149–1-1, Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer’s instructions. Six days after injection of dsTcAMPKα or dsEGFP, 10 larvae were homogenized in 0.3 mL extraction solution and incubated at room temperature for 45 min. The supernatant was collected after centrifugation at 8000 g for 10 min, and used to quantify the level of trehalose at 620 nm with Multiskan FC microplate reader (Thermo Fisher Scientific, Waltham, MA, USA). Three independent biological replicates and four technical replicates were performed for every treatment.

AICAR treatment
To investigate the effects of AMPK activation on lipids and carbohydrates metabolism, 20-day-old larvae were injected with 200 nL of 1.6 mg/mL AICAR or IB as control. AMPK could be activated immediately by AICAR treatment among a couple of hours [40]. The total amounts of TG, glucose and trehalose were determined at one-hour post-injection.

RNA extraction, library construction and sequencing
Total RNA was isolated from 10 larvae of dsTcAMPKα or dsEGFP group on the sixth day after injection using TRIzol Reagent (Invitrogen, USA), and digested by RNase-free DNase I (Takara, Dalian, China) to remove genomic DNA contaminants. To ensure the quality of the samples for transcriptome sequencing, concentration and integrity of RNA samples were checked using a Nanodrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE) and an Agilent Bioanalyzer 2100 (Agilent, Palo Alto, CA), respectively. The qualified RNA samples were used for mRNA preparation and cDNA library construction. Three independent biological replicates were performed for every treatment. cDNA libraries were sequenced as described by Meng et al. (2019) [41].

Differentially expressed genes analysis
To validate the DEGs from the RNA-sequencing, RT-qPCR reactions were performed on the Bio-Rad CFX 96 Real-time PCR system using TB Green™ Premix Ex Taq™ (Takara, Dalian, China) and gene specific primers (Table S1). The stably expressed gene encoding ribosomal protein S3 (rps3, GenBank: CB335975) was used as a reference gene [5]. PCR conditions were set as an initial incubation of 95 °C for 30s, 40 cycles of 95 °C for 5 s and 60 °C for 30s, and a final melting curve analysis was performed. The mRNA levels were normalized to reference gene with the 2^ΔΔCT method Livak and Schmittgen [35]. The means and standard errors (mean ± SE) for each time point were obtained from the average of at least three biologically independent sample sets.

Reverse transcription quantitative real-time polymerase chain reaction (RT-qPCR)
To validate the DEGs from the RNA-sequencing, RT-qPCR reactions were performed on the Bio-Rad CFX 96 Real-time PCR system using TB Green™ Premix Ex Taq™ (Takara, Dalian, China) and gene specific primers (Table S1). The stably expressed gene encoding ribosomal protein S3 (rps3, GenBank: CB335975) was used as a reference gene [5]. PCR conditions were set as an initial incubation of 95 °C for 30s, 40 cycles of 95 °C for 5 s and 60 °C for 30s, and a final melting curve analysis was performed. The mRNA levels were normalized to reference gene with the 2^ΔΔCT method Livak and Schmittgen [35]. The means and standard errors (mean ± SE) for each time point were obtained from the average of at least three biologically independent sample sets.

Statistical analysis
Statistical analysis was performed using Graphpad Prism 6 (GraphPad Software Inc., San Diego, USA) by one-way analysis of variance, followed by Tukey’s Honestly Significant Difference test. All data are presented as the mean ± SE.
Supplementary information
Supplementary information accompanies this paper at https://doi.org/10.1186/s12864-020-07070-3.

Received: 28 April 2020 Accepted: 14 September 2020
Published online: 23 September 2020

References
1. Ahmadian M, Abbott MJ, Tang T, Hudak CS, Kim Y, Bruss M, Herrerstein MK, Lee HY, Samuel VT, Shulman GI. Desminutrin/ATGL is regulated by AMPK and is required for a brown adipose phenotype. Cell Metab. 2011;13:739–48.
2. Anders S, Huber W. Differential expression analysis for sequence count data. Genome Biol. 2010;11:R106.
3. Andreelli F, Forzet M, Knauf C, Cani PD, Perin C, Iglesias MA, Pilott B, Bado A, Tronche F, Methieux G. Liver adiponexine monophosphate-activated kinase a2 catalytic subunit is a key target for the control of hepatic glucose production by adiponexine and leptin but not insulin. Endocrinology. 2006;147:2432–41.
4. Annini G, Botasso BM, Ciacci D, Donato MF, Tripodi A. Liquid triglycerides (GPO-PAP). Med Diagn. 1982;9:115.
5. Arakane Y, Specht CA, Kramer KJ, Muthukrishnan S, Beeman RW. Chitin synthases are required for survival, fecundity and egg hatch in the red flour beetle, Tribolium castaneum. Insect Biochem Mol Biol. 2008;38:599–62.
6. Bergeron R, Previs SF, Cline GW, Perret P, Russell RR III, Young LH, Shulman GI. Effect of 5-aminoimidazole-4-carboxamide-1-B-D-ribofuranoside infusion on in vivo glucose and lipid metabolism in lean and obese Zucker rats. Diabetes. 2001;50:1076–82.
7. Cantó C, Auwerx J. AMP-activated protein kinase and its downstream transcriptional pathways. Cell Mol Life Sci. 2010;67:3407–23.
8. Carling D, Zammit VA, Hardie DG. A common bicyclic protein kinase cascade inactivates the regulatory enzymes of fatty acid and cholesterol biosynthesis. FEBS Lett. 1987;232:217–22.
9. Choi SL, Kim SJ, Lee KT, Kim J, Mu J, Bimbaram MJ, Kim SS, Ha J. The regulation of AMP-activated protein kinase by H2O2. Biochem Biophys Res Commun. 2001;287:92–7.
10. Chopra I, Li H, Wang H, Webster KA. Phosphorylation of the insulin receptor by AMP-activated protein kinase. AMPK) promotes ligand-independent activation of the insulin signalling pathway in rodent muscle. Diabetologia. 2012;55:783–94.
11. Fisher JS, Gao J, Han DH, Holloszy JO, Nolte LA. Activation of AMPK kinase enhances sensitivity of muscle glucose transport to insulin. Am J Physiol Endocrinol Metab. 2002;282:E18–23.
12. Foretz M, Guichard C, Ferré P, Foufelle F. Sterol regulatory element binding protein-1c is a major mediator of insulin action on the hepatic expression of glucokinase and lipogenesis-related genes. Proc Natl Acad Sci U S A. 199996:12377–42.
13. Garcia D, Shaw RJ. AMPK mechanisms of cellular energy sensing and restoration of metabolic balance. Mol Cell. 2017;66:789–800.
14. Grönke S, Mildner A, Fellert S, Tennagels N, Petry S, Müller G, Jäckle H, Kühlheim RP. Brummer lipase is an evolutionary conserved fat storage regulator in Drosophila, Cell Metab. 2005;1:329–30.
15. Hallscak JP, Beeman RW. Status of malathion resistance in five genera of beetle, Tribolium castaneum. BMC Genomics. 2020;21:1
16. Hanson RW, Reshef L. Regulation of phosphoenolpyruvate carboxykinase (GTP) gene expression. Annu Rev Biochem. 1997;66:581–611.
17. Hardie DG. AMP-activated/SNF1 protein kinases. Med diagn. 2007;8:774.
18. Hardie DG, Carling D, Halford N. Roles of the Snf1/RKin1/AMP-activated protein kinase family in the response to environmental and nutritional stress. Semin Cell Biol. 1994;5:409–16.
19. Hawley SA, Boudeau J, Reid JL, Mustard KJ, Udd L, Mäkelä TP, Alessi DR, Hardie DG. Complexes between the LKB1 tumor suppressor, STRAD–AMPK and their catalytic subunit are key regulators of AMPK activation. Cell Metab. 2005;2:19–19.
20. Herndon N, Shelton J, Gershen L, Ioannidis P, Ninova M, Dönitz J, et al. Enhanced genome assembly and a new official gene set for Tribolium castaneum. BMC Genomics. 2020;21:1–13.
21. Huang TJ, Verkhratsky A, Fernyhough P. Insulin enhances mitochondrial inner membrane potential and increases ATP levels through phosphoinositide 3-kinase in adult sensory neurons. Mol Cell Neurosci. 2005;28:42–54.
23. Hurley RL, Anderson KA, Franzone JM, Kemp BE, Means AR, Witter LS. The Ca\textsuperscript{2+} /calmodulin-dependent protein kinase kinases are AMP-activated protein kinase kinases. J Biol Chem. 2005;280:29060–6.

24. Jakobsen SN, Hardie DG, Moncic N, Tornqvist HE. 5′AMP-activated protein kinase phosphorylates IRS-1 on Ser–789 in mouse C2C12 myotubes in response to 5-aminooimidazole-4-carboxamide riboside. J Biol Chem. 2001; 276:46912–6.

25. Jiang H, Zhang N, Chen M, Meng X, Ji C, Ge H, Dong F, Mao L, Yang X, Xu Q, Khan W. Transcriptional and post-translational activation of AMPKα by oxidative, heat, and cold stresses in the red flour beetle, Tribolium castaneum. Cell Stress Chaperones. 2019;24:1079–89.

26. Jitrapakdee S, Sr Maurice M, Raymont J, Geldart GD, Wallace JC, Attwood PV. Structure, mechanism and regulation of pyruvate carboxylase. Biochimie. 2008;94:369–87.

27. Johnson EC, Kazgan N, Breit CA, Forsberg LJ, Hector CE, Worthen RJ, Onyenwoke R, Brennan JEM. Altered metabolism and persistent starvation behaviors caused by reduced AMPK function in Dro sophila. PloS One. 2010; 5:e12799.

28. Kawaguchi T, Otoskami K, Yamashita H, Kabashima T, Uyeda K. Mechanism for fatty acid “sparing” effect on glucose-induced transcription regulation of carbohydrate-responsive element-binding protein by AMP-activated protein kinase. J Biol Chem. 2002;277:3829–35.

29. Kawaguchi T, Takenoshita M, Kabashima T, Uyeda K. Glucose and cAMP regulate the L-type pyruvate kinase gene by phosphorylation/ dephosphorylation of the carbohydrate response element binding protein. Proc Natl Acad Sci U S A. 2001;98:13710–5.

30. Kerouz NJ, Hörsch D, Pons S, Kahn CR. Differential regulation of insulin receptor substrates-1 and -2 (IRS-1 and IRS-2) and phosphatidylinositol 3-kinase isoforms in liver and muscle of the obese diabetic (Ob/Ob) mouse. J Clin Invest. 1997;100:164–72.

31. Kohyama M, Higuchi N, Kato M, Koroh K, Yoshimoto T, Fujino T, Yada M, Yada R, Harada N, Enjoji M. SREBP-1c, regulated by the insulin and AMPK signaling pathways, plays a role in nonalcoholic fatty liver disease. Int J Mol Med. 2008;21:507–11.

32. Kuo JJ, Chang HH, Tsai TH, Lee TY. Curcumin ameliorates mitochondrial dysfunction associated with inhibition of gluconeogenesis in free fatty acid-mediated hepatic lipoproteinosis. Int J Mol Med. 2012;30:643–9.

33. Li B, Beeman RW, Park Y. Functions of duplicated genes encoding CCAP in C. elegans. Genetics. 2001;158:909–22.

34. Li B, Beeman RW, Park Y. Duplicated genes encoding CCAP in C. elegans. Genetics. 2001;158:909–22.

35. Liang SS, Hwang EK, Rhee SY, Wang J. Transcriptional and post-translational activation of AMPKα by oxidative, heat, and cold stresses in the red flour beetle, Tribolium castaneum. Cell Stress Chaperones. 2019;24:1079–89.

36. Madsen A, Bozickovic O, Bjune JI, Mellgren G, Sagen JV. Metformin inhibits AMPK kinase isoforms in liver and muscle of the obese diabetic (Ob/Ob) mouse. J Biol Chem. 2002;277:3829–35.

37. Marsin A, Bertrand L, Rider M, Deprez J, Beauloye C, Vincent MF, Van den Berghe G, Carling D, Hay E, Wüllner U, Wang J. Transcriptome analysis reveals global gene expression changes of Chlorella suppressalis in response to sublethal dose of chlorantraniliprole. Chemosphere. 2019; 234: 648–57.

38. Matsumoto Y, Sumiya E, Sugita T, Sekimizu K. An invertebrate hyperglycemic model for the identification of anti-diabetic drugs. PloS One. 2011;6:e18292.

39. Meng X, Dong F, Qian K, Mao L, Yang X, Ge H, Wu Z, Wang J. Transcriptome analysis reveals global gene expression changes of Chlorella suppressalis in response to sublethal dose of chlorantraniliprole. Chemosphere. 2019; 234: 648–57.

40. Musi N, Goodyear LJ. Targeting the AMP-activated protein kinase for the treatment of type 2 diabetes. Curr Drug Targets Immune Endocr Metabol Disord. 2002;2:119–27.

41. Musi N, Goodyear LJ. Targeting the AMP-activated protein kinase for the treatment of type 2 diabetes. Curr Drug Targets Immune Endocr Metabol Disord. 2002;2:119–27.

42. Nuttall RA, Dzamko N, Thomas WG, Rose-John S, Ernst M, Carling D, Kemp BE. Febraio MA, Steinberg GR. CNTF reverses obesity-induced insulin resistance by activating skeletal muscle AMPK. Nat Med. 2006;12:524.
65. Watts JL. Fat synthesis and adiposity regulation in Caenorhabditis elegans. Trends Endocrinol Metab. 2009;20:58–65.

66. Wendel AA, Lewin TM, Coleman RA. Glycerol-3-phosphate acyltransferases: rate limiting enzymes of triacylglycerol biosynthesis. Biochim Biophys Acta Mol Cell Biol Lipids. 2009;1791:501–6.

67. Wilson WA, Hawley SA, Hardie DG. Glucose repression/derepression in budding yeast: SNF1 protein kinase is activated by phosphorylation under derepressing conditions, and this correlates with a high AMP:ATP ratio. Curr Biol. 1996;6:1426–34.

68. Winder W, Hardie D. Inactivation of acetyl-CoA carboxylase and activation of AMP-activated protein kinase in muscle during exercise. Am J Physiol Endocrinol Metab. 1996;270:E299–304.

69. Woods A, Dickerson K, Heath R, Hong SP, Momcilovic M, Johnstone SR, Carlson M, Carling D. Ca++/calmodulin-dependent protein kinase kinase-β acts upstream of AMP-activated protein kinase in mammalian cells. Cell Metab. 2005;2:21–33.

70. Xu J, Sheng Z, Palli SR. Juvenile hormone and insulin regulate trehalose homeostasis in the red flour beetle, Tribolium castaneum. PLoS Genet. 2013;9:e1003535.

71. Yamauchi T, Kamon J, Minokoshi YA, Ito Y, Waki H, Uchida S, Yamashita S, Noda M, Kita S, Ueki K. Adiponectin stimulates glucose utilization and fatty-acid oxidation by activating AMP-activated protein kinase. Nat. Med. 2002;8:1288.

72. Yang CS, Lam CK, Chari M, Cheung GW, Kokorovic A, Gao S, Leclerc I, Rutter GA, Lam TK. Hypothalamic AMP-activated protein kinase regulates glucose production. Diabetes. 2010;59(2):2435–43.

73. Zhang T, Sawada K, Yamamoto N, Ashida H. 4-Hydroxyderricin and xanthoangelol from Ashitaba (Angelica keiskei) suppress differentiation of preadipocytes to adipocytes via AMPK and MAPK pathways. Mol Nutr Food Res. 2013;57:1729–40.

74. Zhou G, Myers R, Li Y, Chen Y, Shen X, Fenyk-Melody J, Wu M, Vintre J, Doebber T, Fujii N. Role of AMP-activated protein kinase in mechanism of metformin action. J Clin Invest. 2001;108:1167–74.

Publisher’s Note
Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.