The AMPK signaling pathway is associated with the intramuscular fat trait in pigs

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Background. Intramuscular fat (IMF) is an important economic trait for pork quality and a complex quantitative trait regulated by multiple genes. The objective of this work was to investigate the novel transcriptional effects of a multigene pathways on IMF deposition in the longissimus dorsi(LD) muscles of pigs.

Methods. Potential signaling pathways were screened by mining data from three gene expression profiles in the GEO database. We designed quantitative real-time reverse transcription-polymerase chain reaction (qRT-PCR) arrays for the candidate signaling pathways to verify the results in the LD muscles of three pig breeds with different IMF contents(Large White, Berkshire and Min).

Results. The AMPK signaling pathway was screened via bioinformatic analysis. Ten key hub genes of this signaling pathway(*AMPK, ADIPOR1, ADIPOR2, LKB1, CAMKKβ, CPT1A, CPT1B, PGC-1α, CD36* and *ACC1*) were differentially expressed. Statistical analyses revealed that AMPK pathway activity clearly varied among the three pig breeds.

Conclusion. Based on these results, we concluded that the activation of the AMPK signaling pathway plays a positive role in reducing IMF deposition in pigs.

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Abstract

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Introduction

As one of the most important domesticated animals for agricultural production, pigs provide many meat products for humans(Puig-Oliveras et al. 2014). In modern society, pork quality has had an increasing influence on consumer acceptance and initial purchasing decisions. Consumers are interested in several major pork quality traits, including meat color, pH value, water holding capacity and intramuscular fat (IMF) content, which are becoming increasingly important economically(Font-i-Furnols et al. 2012; Nonneman et al. 2013). Skeletal muscle is a heterogeneous tissue comprising different types of myofibers, connective tissue, vascular tissue, nervous tissue, and IMF(Larzul et al. 1997). IMF is a major meat quality trait in pigs, and its content is directly associated with the sensory qualities, flavor, juiciness, tenderness and nutritional quality of pork(Wang et al. 2017a; Won et al. 2018). In recent decades, several studies have focused on the relationship between IMF and pork quality(Brewer et al. 2001; Lim

et al. 2017; Suzuki et al. 2005; van Laack et al. 2001).

Famous lean pig breeds, such as Large White, Landrace and Duroc, have lower IMF contents and reduced meat quality due to the intensive selection processes used to improve pork productivity. However, many excellent indigenous breeds are distributed in China, such as the Jinhua, Laiwu, Meishan and Min, that have higher IMF contents and better meat quality than the lean breeds (Dai et al. 2009; Gao et al. 2011; Wang et al. 2017b; Wu et al. 2013; Xiong et al. 2015; Yang et al. 2014). Thus, it will be beneficial to reveal the molecular mechanisms of IMF deposition by comparing gene expression between lean and indigenous Chinese pig breeds.

With the rapid development of microarray and RNA-seq technologies in the last few decades, researchers are now able to study many differentially expressed genes (DEGs) simultaneously in a given tissue. To date, many studies concerning meat quality traits and gene expression in pigs have been reported(Li et al. 2018; Pena et al. 2014; Tao et al. 2017). Fortunately, the relevant datasets have been deposited and stored in the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (GEO) database and are freely accessible to researchers worldwide. However, few studies have focused on integrating and reanalyzing these datasets, which contain valuable clues regarding important porcine economic traits. Thus, by integrating and reanalyzing these datasets, we can provide significant insights into the molecular changes associated with IMF deposition.

In this study, we integrated and reanalyzed three original expression profiles from the GEO database based on a current popular differential gene expression analysis method. We found that the AMPK pathway plays a critical role in IMF deposition. We further validated this pathway through the use of quantitative real-time reverse transcription-polymerase chain reaction (qRT-PCR) arrays in the Large White, Berkshire and Min pig breeds.

Methods

GEO data collection

The gene expression profiles *GSE24192*, *GSE75045* and *GSE99092*(Gao et al. 2011; Li et al. 2016; Xu et al. 2018) were downloaded from the GEO database. The *GSE24192* dataset contained six samples, which included three Large White longissimus dorsi (LD) samples and three Northeastern Indigenous LD samples. The *GSE75045*dataset contained six samples, which included three Large White LD samples and three Wannanhua LD samples. The *GSE*99092 dataset contained six samples, which included three Large White LD samples and three Wei LD samples.

Identification of DEGs

The limma package from Bioconductor and the online tool iDEP(https://github.com/gexijin/iDEP) were used to identify DEGs for the selected gene expression profile datasets(Smyth 2005). A p value less than 0.05 and $|\log_{\text{Fold Change(FC)}}| \geq 1$ were regarded as the cutoff thresholds for DEGs. The online tool ClustVis was used to draw heatmaps(Metsalu & Vilo 2015).

Signaling pathway enrichment analysis of DEGs

To analyze the functions of the DEGs, we performed Kyoto Encyclopedia of Genes and

Genomes (KEGG) pathway analysis of the DEGs using the online tool Kobas 3.0(Xie et al. 2011). A p value less than 0.05 was considered statistically significant. ClusterProfiler was used for statistical analysis and visualization of the functional profiles of the DEGs in the GEO datasets and qRT-PCR arrays(Yu et al. 2012).

Animals and tissue collection

Three sows each from the Min, Large White and Berkshire breeds were used in this study. The Min pig is an excellent indigenous breed from northeastern the China with an IMF content higher than that in Large White and Berkshire pigs(Gao et al. 2011). Moreover, the Berkshire has a higher IMF content than the Large White(Jung et al. 2015). The pigs were raised for 180 days under the same conditions. When the pigs were slaughtered, the LD muscle was collected from between the seventh and ninth ribs from the carcasses. All tissue samples were quickly frozen immediately after collection and stored at -80°C until use in qRT-PCR arrays.

RNA extraction and quantitative real-time reverse transcription-polymerase chain reaction (qRT-PCR) arrays

Total RNA from LD was isolated from approximately 200 mg frozen tissue using TRIzol-A$^+$ Reagent (TIANGEN, Beijing, China) following the manufacturer's instructions. A BioRT cDNA First Strand Synthesis Kit (Bioer Technology, Hangzhou, China) was used to synthesize first-strand cDNA. Subsequently, the expression levels of the target genes were analyzed on an iQ™5 real-time PCR detection system (Bio-Rad). A BioEasy SYBR Green I Real Time PCR kit (Bioer Technology) was used according to the manual to detect each sample in triplicate. The primers used for qRT-PCR arrays are listed in Table S1. The gene IDs from the selected pathways were obtained from the KEGG database(Table S2). The $2^{-\Delta CT}$ method was used to analyze our results.

Animal ethics

All animal procedures were performed according to the University Committee on the Use and Care of Animals at Jilin University(approval ID: 201706030).

Results

Identification of DEGs in GEO datasets

According to the cutoff threshold($p < 0.05$ and $|\log_{FC}| \geq 1$), in *GSE24192*, 1237 DEGs were identified in the LD of Large White pigs when compared with the indigenous Chinese breeds, including 877 upregulated genes and 360 down-regulated genes. In *GSE75045*, a total of 2582 DEGs were identified in the LD of Large White pigs, including 1096 upregulated genes and 1486 down-regulated genes. Finally, in *GSE99092*, a total of 1822 DEGs were identified in the LD of Large White pigs, including 809 upregulated genes and 1013 down-regulated genes.

Pathway enrichment of DEGs in GEO datasets

The KEGG pathway enrichment results shown(Fig. 1B, Fig. 1C, Table 1, Table 2, Table 3)yielded no shared pathways among the down-regulated DEGs of LWs in the three GEO datasets. By contrast, the AMPK signaling pathway (ssc04152),the peroxisome proliferator-activated receptor (PPAR) signaling pathway(ssc03320),fat digestion and absorption(ssc04975), fatty acid metabolism(ssc01212), metabolic pathways(ssc01100) and biosynthesis of amino acids(ssc01230) were among the upregulated DEGs in LWs in the three GEO datasets. The

AMPK signaling pathway maybe a potentially novel pathway for regulating IMF deposition in pigs.

Validation of the AMPK signaling pathway in LD muscles of the three pig breeds

The AMPK signaling pathway (ssc04152) consists of 117genes(Table S2).In this study, 114 of these genes were validated via qRT-PCR array. Of them, 40 genes were differentially expressed in the Large White LD, with 22 upregulated and 18 down-regulated. The qRT-PCR results for the AMPK signaling pathway are shown in Fig. 2, Fig. 3, Fig. 4 and Table 4. A heatmap of the AMPK signaling pathway is presented in Fig. 2B, and the expression patterns of the DEGs in the AMPK signaling pathway among the Large White, Berkshire and Min breeds are displayed in Fig. 2C.The expression patterns often keyhub genes in the AMPK signaling pathway(*AMPK, ADIPOR1, ADIPOR2, LKB1, CAMKKβ, CPT1A, CPT1B, PGC-1α, CD36 and ACC1*) are presented in Fig. 3. Fig. 4 shows a colored map of the AMPK signaling pathway in the LD of Large White pigs. Taken together, these results show that the AMPK signaling pathway is more active in the Large White breed than in the Berkshire and Min breeds.

GO enrichment of DEGs in qRT-PCR arrays

The biological processes encoded by upregulated genes were involved in fatty acid oxidation, lipid oxidation and fatty acid metabolic processes, while the down-regulated genes mainly targeted carbohydrate metabolic processes, including glucose, hexose, and monosaccharide metabolism, as well as hexose and monosaccharide catabolism (Fig. 5). These results indicated that compared with the LD of Min pigs, the LD of Large White pigs consumes more fat in energy metabolism rather than carbohydrates.

Discussion

In the modern pork industry, IMF content is an important trait that is positively associated with pork quality and demanded by consumers. As a complex meat trait, IMF deposition in the LD muscle is regulated by multiple genes and pathways. In this study, by integrating and reanalyzing three gene expression profiles, we compared the pathways related to IMF deposition in the LD muscle of Large White pigs with those of indigenous breeds. Several candidate signaling pathways were found, and the expression patterns of genes in the AMPK pathway in pigs were validated by qRT-PCR array in subsequent experiments.

The AMPK signaling pathway plays critical roles in controlling both glucose and lipid metabolism. *AMPK* is the central gene of the AMPK signaling pathway and a heterotrimeric enzyme with α, β, and γ subunits. Once activated, *AMPK* promotes lipid oxidation and glucose uptake, inhibits lipid synthesis and decreases IMF content(Carling 2004). Accordingly, the activity of AMPK is inversely correlated with IMF accumulation. In the present study, *AMPK* was highly expressed in the LD muscle of the Large White pig breed, which has a lower IMF content. This result is consistent with several previous reports demonstrating that the expression levels of *AMPK* are higher in low-IMF-content skeletal muscle(Tong et al. 2008; Underwood et al. 2008; Underwood et al. 2007).

ADIPOR1 and *ADIPOR2* are two major receptors for adiponectin and play key roles in metabolic pathways that regulate glucose and lipid metabolism, inflammation and oxidative

stress(Yamauchi et al. 2014). *ADIPOR1* and *ADIPOR2* mediate the metabolic actions of adiponectin by activating *AMPK* and *PPARα*, respectively. This activation leads to increased fatty acid oxidation and glucose uptake(Yamauchi et al. 2002; Yamauchi et al. 2007; Yoon et al. 2006). Moreover, these genes were both upregulated in the low-IMF-content LD muscle in the present study. This observation may account for the low IMF accumulation in Large White pigs. Interestingly, two upstream kinases of *AMPK*, the tumor suppressor *LKB1* and Ca^{2+}/calmodulin-dependent kinase kinase β (*CaMKKβ*), were simultaneously highly expressed in our study. AMPK can be activated via two distinct mechanisms, a *LKB1*-dependent pathway and a Ca^{2+}/*CaMKKβ*-dependent pathway(Abbott et al. 2009; Green et al. 2011; Sakamoto et al. 2005; Zhou et al. 2009). Furthermore, carnitine palmitoyltransferase-1 (*CPT1*), a rate-limiting enzyme of mitochondrial fatty acid β-oxidation, is closely associated with fat deposition. Additionally, *CPT1A* and *CPT1B*, two common *CPT1* subtypes in mammals, play prominent roles in fatty acid oxidation and lipid accumulation(Qiu et al. 2017; Zhang et al. 2014). Moreover, according to the KEGG database, *CPT1A* and *CPT1B* are involved in the AMPK signaling pathway, implying that these genes participate in the mediation of fatty acid oxidation. In agreement with these previous results, both *CPT1A* and *CPT1B* were highly expressed in the LD we studied, suggesting that they might be associated with IFM deposition.

Likewise, the fatty acid transporter fatty acid translocase/cluster of differentiation 36 (*FAT/CD36*)has been identified as contributing to fatty acid transport and oxidation(Bonen et al. 2004; Campbell et al. 2004; Ibrahimi et al. 1999). *FAT/CD36*, along with *CPT1*, has also been found to regulate FA oxidation in skeletal muscle(Schenk & Horowitz 2006; Smith et al. 2011). In our results, *FAT/CD36* was highly expressed, suggesting that the expression of this gene has a negative effect on IMF deposition in Large White pigs. The *ACC1* gene encodes acetyl-CoA carboxylase(*ACC*), which is the rate-limiting enzyme responsible for the de novo synthesis of fatty acids(Jones et al. 2017). As a target gene of the AMPK signaling pathway, the activity of *ACC* is inhibited by *AMPK*. Likewise, an increase in activity can also inhibit the expression and fatty acid oxidation(Alam & Saggerson 1998; Saha et al. 2000; Scaglia et al. 2009). Here, *ACC1* was significantly down-regulated in the Large White LD.

Additionally, the peroxisome proliferator-activated receptor gamma coactivator 1-alpha (*PPARGC1A, PGC-1α*) gene, which plays an important role in glucose and fatty acid metabolism and has a negative relationship with IMF content, was highly expressed(Beeson et al. 2012; Li et al. 2014; Yu et al. 2013). Moreover, *PGC-1α* was involved in the activation of the CPT1 gene(Louet et al. 2002). Our results suggest that the activation of the AMPK signaling pathway may reduce the IMF content in the LD of Large White pigs.

Conclusions

In conclusion, this study illustrates that the accumulation of IMF in Large White pigs is related to the activation of the AMPK signaling pathway. The relatively high expression of genes in the AMPK pathway maybe one of the more significant features of pigs with artificially lean meat. Our results are also helpful for interpreting the different molecular mechanisms of IMF deposition between lean and fat pig breeds.

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References

Abbott MJ, Edelman AM, and Turcotte LP. 2009. CaMKK is an upstream signal of AMP-activated protein kinase in regulation of substrate metabolism in contracting skeletal muscle. *American Journal of Physiology-Regulatory Integrative and Comparative Physiology* 297:R1724-R1732. 10.1152/ajpregu.00179.2009

Alam N, and Saggerson ED. 1998. Malonyl-CoA and the regulation of fatty acid oxidation in soleus muscle. *Biochemical Journal* 334 (Pt 1):233-241.

Beeson CC, Beeson GC, Buff H, Eldridge J, Zhang A, Seth A, Demcheva M, Vournakis JN, and Muise-Helmericks RC. 2012. Integrin-dependent Akt1 activation regulates PGC-1 expression and fatty acid oxidation. *J Vasc Res* 49:89-100. 10.1159/000332326

Bonen A, Campbell SE, Benton CR, Chabowski A, Coort SLM, Han XX, Koonen DPY, Glatz JFC, and Luiken JJFP. 2004. Regulation of fatty acid transport by fatty acid translocase/CD36. *Proceedings of the Nutrition Society* 63:245-249. Doi 10.1079/Pns2004331

Brewer MS, Zhu LG, and McKeith FK. 2001. Marbling effects on quality characteristics of pork loin chops: consumer purchase intent, visual and sensory characteristics. *Meat Science* 59:153-163. Doi 10.1016/S0309-1740(01)00065-1

Campbell SE, Tandon NN, Woldegiorgis G, Luiken JJFP, Glatz JFC, and Bonen A. 2004. A novel function for fatty acid translocase (FAT)/CD36 - Involvement in long chain fatty acid transfer into the mitochondria. *Journal of Biological Chemistry* 279:36235-36241. 10.1074/jbc.M400566200

Carling D. 2004. The AMP-activated protein kinase cascade - a unifying system for energy control. *Trends in Biochemical Sciences* 29:18-24. 10.1016/j.tibs.2003.11.005

Dai FW, Feng DY, Cao QY, Ye H, Zhang CM, Xia WG, and Zuo JJ. 2009. Developmental differences in carcass, meat quality and muscle fibre characteristics between the Landrace and a Chinese native pig. *South African Journal of Animal Science* 39:267-273.

Font-i-Furnols M, Tous N, Esteve-Garcia E, and Gispert M. 2012. Do all the consumers accept marbling in the same way? The relationship between eating and visual acceptability of pork with different intramuscular fat content. *Meat Science* 91:448-453. 10.1016/j.meatsci.2012.02.030

Gao Y, Zhang YH, Jiang H, Xiao SQ, Wang S, Ma Q, Sun GJ, Li FJ, Deng Q, Dai LS, Zhao ZH, Cui XS, Zhang SM, Liu DF, and Zhang JB. 2011. Detection of differentially expressed genes in the longissimus dorsi of Northeastern Indigenous and Large White pigs. *Genetics and Molecular Research* 10:779-791. 10.4238/vol10-2gmr1170

Green MF, Anderson KA, and Means AR. 2011. Characterization of the CaMKK beta-AMPK

signaling complex. *Cellular Signalling* 23:2005-2012. 10.1016/j.cellsig.2011.07.014

Ibrahimi A, Bonen A, Blinn WD, Hajri T, Li X, Zhong K, Cameron R, and Abumrad NA. 1999. Muscle-specific overexpression of FAT/CD36 enhances fatty acid oxidation by contracting muscle, reduces plasma triglycerides and fatty acids, and increases plasma glucose and insulin. *Journal of Biological Chemistry* 274:26761-26766. DOI 10.1074/jbc.274.38.26761

Jones JEC, Esler WP, Patel R, Lanba A, Vera NB, Pfefferkorn JA, and Vernochet C. 2017. Inhibition of Acetyl-CoA Carboxylase 1 (ACC1) and 2 (ACC2) Reduces Proliferation and De Novo Lipogenesis of EGFRvIII Human Glioblastoma Cells. *PLoS One* 12. ARTN e0169566

10.1371/journal.pone.0169566

Jung JH, Shim KS, Na CS, and Choe HS. 2015. Studies on Intramuscular Fat Percentage in Live Swine Using Real-time Ultrasound to Determine Pork Quality. *Asian-Australasian Journal of Animal Sciences* 28:318-322. 10.5713/ajas.14.0927

Larzul C, Lefaucheur L, Ecolan P, Gogue J, Talmant A, Sellier P, Le Roy P, and Monin G. 1997. Phenotypic and genetic parameters for longissimus muscle fiber characteristics in relation to growth, carcass, and meat quality traits in large white pigs. *J Anim Sci* 75:3126-3137.

Li B, Weng Q, Dong C, Zhang Z, Li R, Liu J, Jiang A, Li Q, Jia C, Wu W, and Liu H. 2018. A Key Gene, PLIN1, Can Affect Porcine Intramuscular Fat Content Based on Transcriptome Analysis. *Genes (Basel)* 9. 10.3390/genes9040194

Li Q, Wang Z, Zhang B, Lu Y, Yang Y, Ban D, Wu C, and Zhang H. 2014. Single nucleotide polymorphism scanning and expression of the pig PPARGC1A gene in different breeds. *Lipids* 49:1047-1055. 10.1007/s11745-014-3928-1

Li XJ, Zhou J, Liu LQ, Qian K, and Wang CL. 2016. Identification of genes in longissimus dorsi muscle differentially expressed between Wannanhua and Yorkshire pigs using RNA-sequencing. *Anim Genet* 47:324-333. 10.1111/age.12421

Lim KS, Lee KT, Park JE, Chung WH, Jang GW, Choi BH, Hong KC, and Kim TH. 2017. Identification of differentially expressed genes in longissimus muscle of pigs with high and low intramuscular fat content using RNA sequencing. *Anim Genet* 48:166-174. 10.1111/age.12518

Louet JF, Hayhurst G, Gonzalez FJ, Girard J, and Decaux JF. 2002. The coactivator PGC-1 is involved in the regulation of the liver carnitine palmitoyltransferase I gene expression by cAMP in combination with HNF4 alpha and cAMP-response element-binding protein (CREB). *Journal of Biological Chemistry* 277:37991-38000.

Metsalu T, and Vilo J. 2015. ClustVis: a web tool for visualizing clustering of multivariate data using Principal Component Analysis and heatmap. *Nucleic Acids Res* 43:W566-W570. 10.1093/nar/gkv468

Nonneman DJ, Shackelford SD, King DA, Wheeler TL, Wiedmann RT, Snelling WM, and Rohrer GA. 2013. Genome-wide association of meat quality traits and tenderness in swine. *J Anim Sci* 91:4043-4050. 10.2527/jas.2013-6255

Pena RN, Quintanilla R, Manunza A, Gallardo D, Casellas J, and Amills M. 2014. Application of

the microarray technology to the transcriptional analysis of muscle phenotypes in pigs. *Anim Genet* 45:311-321. 10.1111/age.12146

Puig-Oliveras A, Ramayo-Caldas Y, Corominas J, Estelle J, Perez-Montarelo D, Hudson NJ, Casellas J, Folch JM, and Ballester M. 2014. Differences in Muscle Transcriptome among Pigs Phenotypically Extreme for Fatty Acid Composition. *PLoS One* 9. ARTN e99720
10.1371/journal.pone.0099720

Qiu FF, Xie L, Ma JE, Luo W, Zhang L, Chao Z, Chen SH, Nie QH, Lin ZM, and Zhang XQ. 2017. Lower Expression of SLC27A1 Enhances Intramuscular Fat Deposition in Chicken via Down-Regulated Fatty Acid Oxidation Mediated by CPT1A. *Frontiers in Physiology* 8. Artn 449
10.3389/Fphys.2017.00449

Saha AK, Schwarsin AJ, Roduit R, Masse F, Kaushik V, Tornheim K, Prentki M, and Ruderman NB. 2000. Activation of malonyl-CoA decarboxylase in rat skeletal muscle by contraction and the AMP-activated protein kinase activator 5-aminoimidazole-4-carboxamide-1-beta -D-ribofuranoside. *J Biol Chem* 275:24279-24283. 10.1074/jbc.C000291200

Sakamoto K, McCarthy A, Smith D, Green KA, Hardie DG, Ashworth A, and Alessi DR. 2005. Deficiency of LKB1 in skeletal muscle prevents AMPK activation and glucose uptake during contraction. *Embo Journal* 24:1810-1820. 10.1038/sj.emboj.7600667

Scaglia N, Chisholm JW, and Igal RA. 2009. Inhibition of stearoylCoA desaturase-1 inactivates acetyl-CoA carboxylase and impairs proliferation in cancer cells: role of AMPK. *PLoS One* 4:e6812. 10.1371/journal.pone.0006812

Schenk S, and Horowitz JF. 2006. Coimmunoprecipitation of FAT/CD36 and CPT I in skeletal muscle increases proportionally with fat oxidation after endurance exercise training. *American Journal of Physiology-Endocrinology and Metabolism* 291:E254-E260. 10.1152/ajpendo.00051.2006

Smith BK, Jain SS, Rimbaud S, Dam A, Quadrilatero J, Ventura-Clapier R, Bonen A, and Holloway GP. 2011. FAT/CD36 is located on the outer mitochondrial membrane, upstream of long-chain acyl-CoA synthetase, and regulates palmitate oxidation. *Biochemical Journal* 437:125-134. 10.1042/Bj20101861

Smyth GK. 2005. limma: Linear Models for Microarray Data. *Bioinformatics & Computational Biology Solutions Using R & Bioconductor*:397--420.

Suzuki K, Irie M, Kadowaki H, Shibata T, Kumagai M, and Nishida A. 2005. Genetic parameter estimates of meat quality traits in Duroc pigs selected for average daily gain, longissimus muscle area, backfat thickness, and intramuscular fat content. *J Anim Sci* 83:2058-2065.

Tao X, Liang Y, Yang XM, Pang JH, Zhong ZJ, Chen XH, Yang YK, Zeng K, Kang RM, Lei YF, Ying SC, Gong JJ, Gu YR, and Lv XB. 2017. Transcriptomic profiling in muscle and adipose tissue identifies genes related to growth and lipid deposition. *PLoS One* 12. ARTN e0184120
10.1371/journal.pone.0184120

Tong J, Zhu MJ, Underwood KR, Hess BW, Ford SP, and Du M. 2008. AMP-activated protein kinase and adipogenesis in sheep fetal skeletal muscle and 3T3-L1 cells. *J Anim Sci* 86:1296-1305. 10.2527/jas.2007-0794

Underwood KR, Means WJ, Zhu MJ, Ford SP, Hess BW, and Du M. 2008. AMP-activated protein kinase is negatively associated with intramuscular fat content in longissimus dorsi muscle of beef cattle. *Meat Science* 79:394-402. 10.1016/j.meatsci.2007.10.025

Underwood KR, Tong J, Zhu MJ, Shen QW, Means WJ, Ford SP, Paisley SI, Hess BW, and Du M. 2007. Relationship between kinase phosphorylation, muscle fiber typing, and glycogen accumulation in Longissimus muscle of beef cattle with high and low intramuscular fat. *Journal of Agricultural and Food Chemistry* 55:9698-9703. 10.1021/jf071573z

van Laack RLJM, Stevens SG, and Stalder KJ. 2001. The influence of ultimate pH and intramuscular fat content on pork tenderness and tenderization. *J Anim Sci* 79:392-397.

Wang XW, Ding RR, Quan JP, Yang LX, Yang M, Zheng EQ, Liu DW, Cai GY, Wu ZF, and Yang J. 2017a. Genome-wide association analysis reveals genetic loci and candidate genes associated with intramuscular fat in Duroc pigs. *Frontiers of Agricultural Science and Engineering* 4:335-341. 10.15302/J-Fase-2017152

Wang YD, Ma C, Sun Y, Li Y, Kang L, and Jiang YL. 2017b. Dynamic transcriptome and DNA methylome analyses on longissimus dorsi to identify genes underlying intramuscular fat content in pigs. *BMC Genomics* 18. Artn 780
10.1186/S12864-017-4201-9

Won S, Jung J, Park E, and Kim H. 2018. Identification of genes related to intramuscular fat content of pigs using genome-wide association study. *Asian-Australasian Journal of Animal Sciences* 31:157-162. 10.5713/ajas.17.0218

Wu T, Zhang ZH, Yuan ZQ, Lo LJ, Chen J, Wang YZ, and Peng JR. 2013. Distinctive Genes Determine Different Intramuscular Fat and Muscle Fiber Ratios of the longissimus dorsi Muscles in Jinhua and Landrace Pigs. *PLoS One* 8. ARTN e53181
10.1371/journal.pone.0053181

Xie C, Mao XZ, Huang JJ, Ding Y, Wu JM, Dong S, Kong L, Gao G, Li CY, and Wei LP. 2011. KOBAS 2.0: a web server for annotation and identification of enriched pathways and diseases. *Nucleic Acids Res* 39:W316-W322. 10.1093/nar/gkr483

Xiong XW, Liu XX, Zhou LS, Yang J, Yang B, Ma HB, Xie XH, Huang YX, Fang SM, Xiao SJ, Ren J, Chen CY, Ma JW, and Huang LS. 2015. Genome-wide association analysis reveals genetic loci and candidate genes for meat quality traits in Chinese Laiwu pigs. *Mammalian Genome* 26:181-190. 10.1007/s00335-015-9558-y

Xu JG, Wang CL, Jin EH, Gu YF, Li SH, and Li QG. 2018. Identification of differentially expressed genes in longissimus dorsi muscle between Wei and Yorkshire pigs using RNA sequencing. *Genes & Genomics* 40:413-421. 10.1007/s13258-017-0643-3

Yamauchi T, Iwabu M, Okada-Iwabu M, and Kadowaki T. 2014. Adiponectin receptors: A review of their structure, function and how they work. *Best Practice & Research Clinical Endocrinology & Metabolism* 28:15-23. 10.1016/j.beern.2013.09.003

Yamauchi T, Kamon J, Minokoshi Y, Ito Y, Waki H, Uchida S, Yamashita S, Noda M, Kita S, Ueki K, Eto K, Akanuma Y, Froguel P, Foufelle F, Ferre P, Carling D, Kimura S, Nagai R, Kahn BB, and Kadowaki T. 2002. Adiponectin stimulates glucose utilization and fatty-acid oxidation by activating AMP-activated protein kinase. *Nature Medicine* 8:1288-1295. 10.1038/nm788

Yamauchi T, Nio Y, Maki T, Kobayashi M, Takazawa T, Iwabu M, Okada-Iwabu M, Kawamoto S, Kubota N, Kubota T, Ito Y, Kamon J, Tsuchida A, Kumagai K, Kozono H, Hada Y, Ogata H, Tokuyama K, Tsunoda M, Ide T, Murakami K, Awazawa M, Takamoto I, Froguel P, Hara K, Tobe K, Nagai R, Ueki K, and Kadowaki T. 2007. Targeted disruption of AdipoR1 and AdipoR2 causes abrogation of adiponectin binding and metabolic actions. *Nature Medicine* 13:332-339. 10.1038/nm1557

Yang SB, Li XL, Li K, Fan B, and Tang ZL. 2014. A genome-wide scan for signatures of selection in Chinese indigenous and commercial pig breeds. *BMC Genet* 15. Artn 7 10.1186/1471-2156-15-7

Yoon MJ, Lee GY, Chung JJ, Ahn YH, Hong SH, and Kim JB. 2006. Adiponectin increases fatty acid oxidation in skeletal muscle cells by sequential activation of AMP-activated protein kinase, p38 mitogen-activated protein kinase, and peroxisome proliferator-activated receptor alpha. *Diabetes* 55:2562-2570. 10.2337/db05-1322

Yu GC, Wang LG, Han YY, and He QY. 2012. clusterProfiler: an R Package for Comparing Biological Themes Among Gene Clusters. *Omics-a Journal of Integrative Biology* 16:284-287. 10.1089/omi.2011.0118

Yu KF, Shu G, Yuan FF, Zhu XT, Gao P, Wang SB, Wang LN, Xi QY, Zhang SQ, Zhang YL, Li Y, Wu TS, Yuan L, and Jiang QY. 2013. Fatty Acid and Transcriptome Profiling of Longissimus Dorsi Muscles between Pig Breeds Differing in Meat Quality. *International Journal of Biological Sciences* 9:108-118. 10.7150/ijbs.5306

Zhang YF, Yuan ZQ, Song DG, Zhou XH, and Wang YZ. 2014. Effects of cannabinoid receptor 1 (brain) on lipid accumulation by transcriptional control of CPT1A and CPT1B. *Anim Genet* 45:38-47. 10.1111/age.12078

Zhou L, Deepa SS, Etzler JC, Ryu J, Mao X, Fang Q, Liu DD, Torres JM, Jia W, and Lechleiter JD. 2009. Adiponectin activates AMPK in muscle cells via APPL1/LKB1- and PLC/Ca2+/CaMKK-dependent pathways. *Journal of Biological Chemistry* 284.

Figure 1

Scatter plot (A) and KEGG enrichment of DEGs (B) in *GES24192*, *GSE75045* and *GSE99092*.

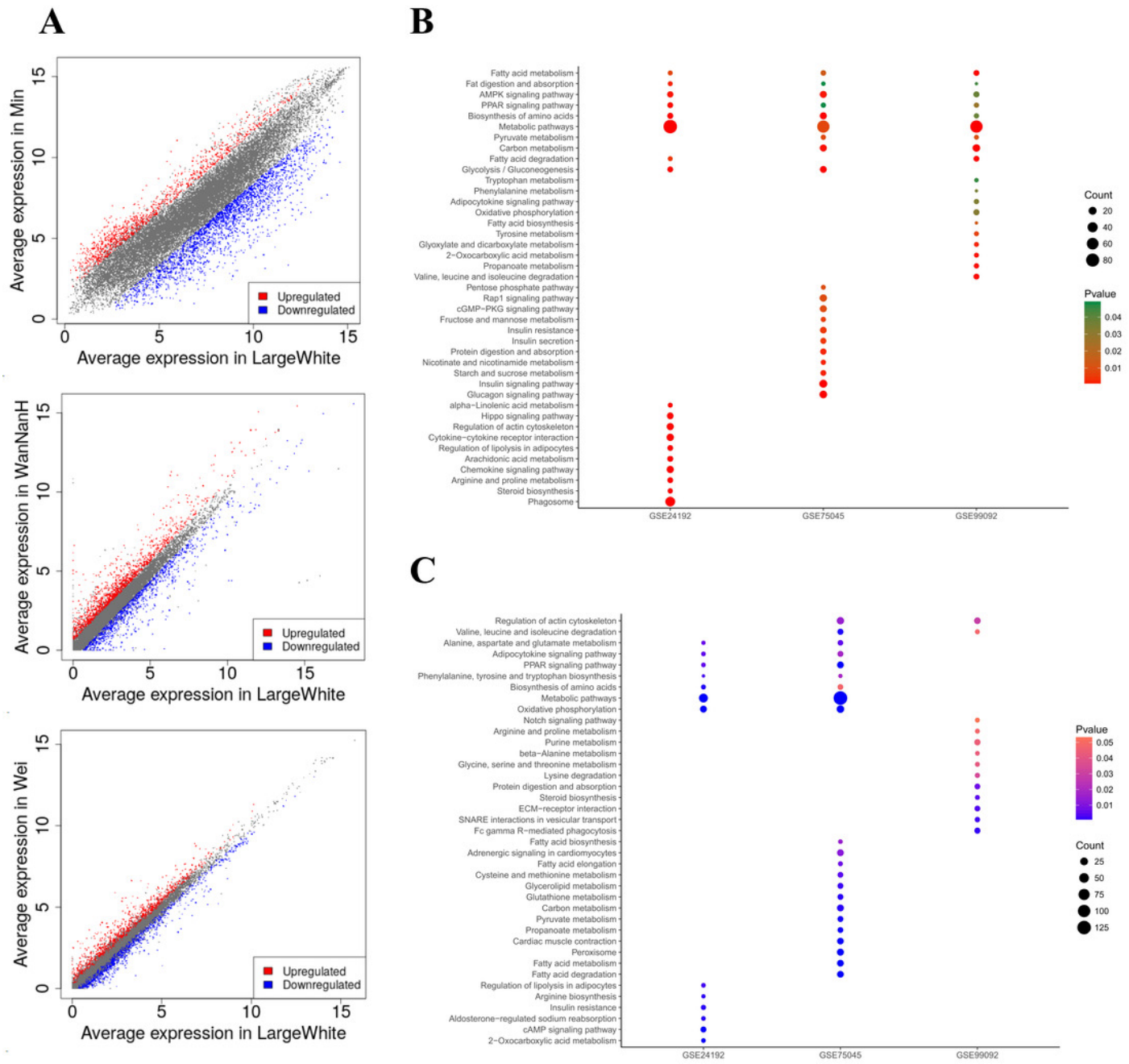

Figure 2

qRT-PCR array results in three pig breeds.

(A) The number of DEGs. (B) A heatmap of all array genes. (C) Parallel coordinate plots and heatmap illustrations of DEGs. Absolute (abs) values of log fold change (FC) value > 1, false discovery rate (FDR) cutoff value < 0.05.

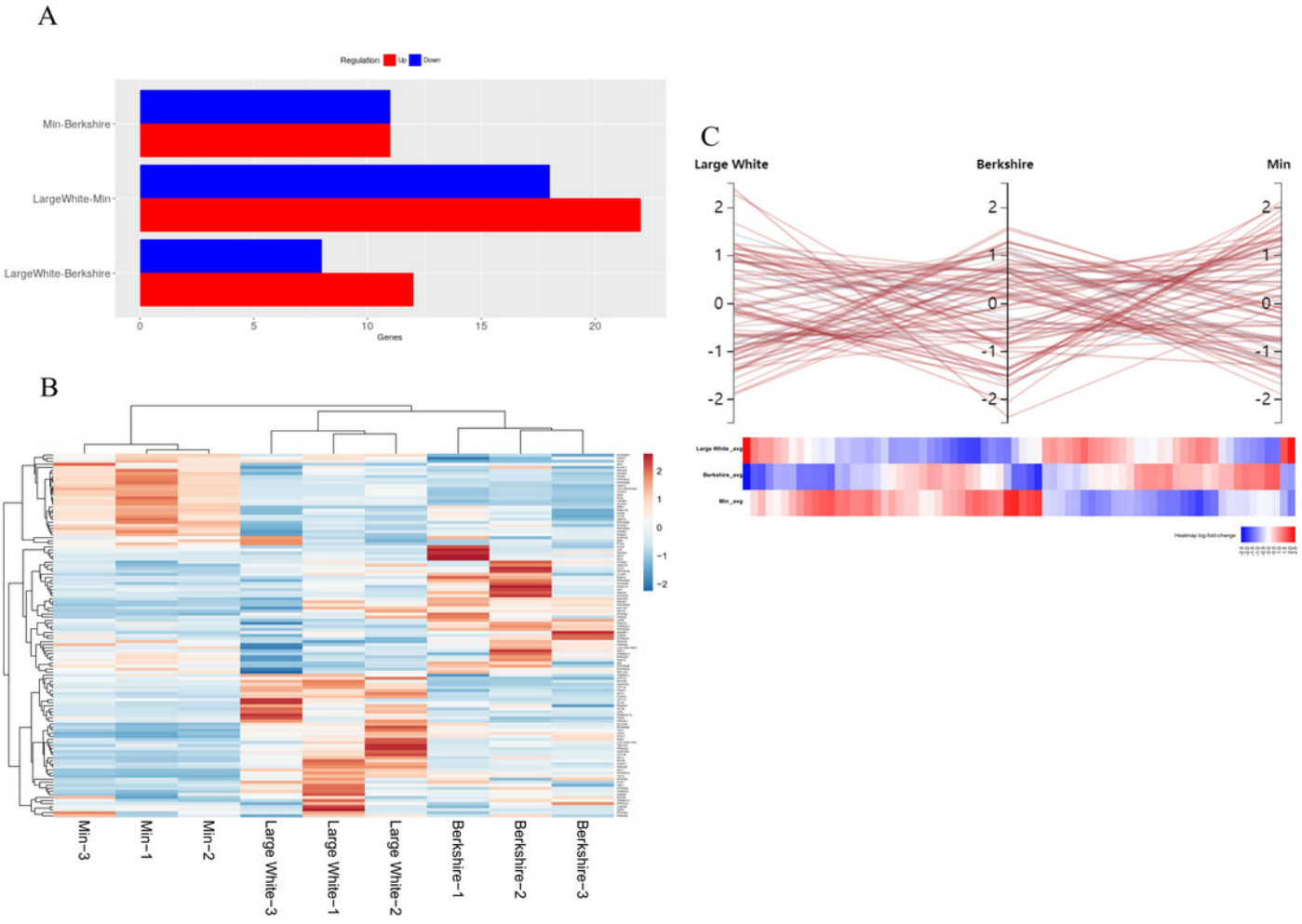

Figure 3

Comparisons of the expression of ten key hub genes in the AMPK signaling pathway in three pig breeds.

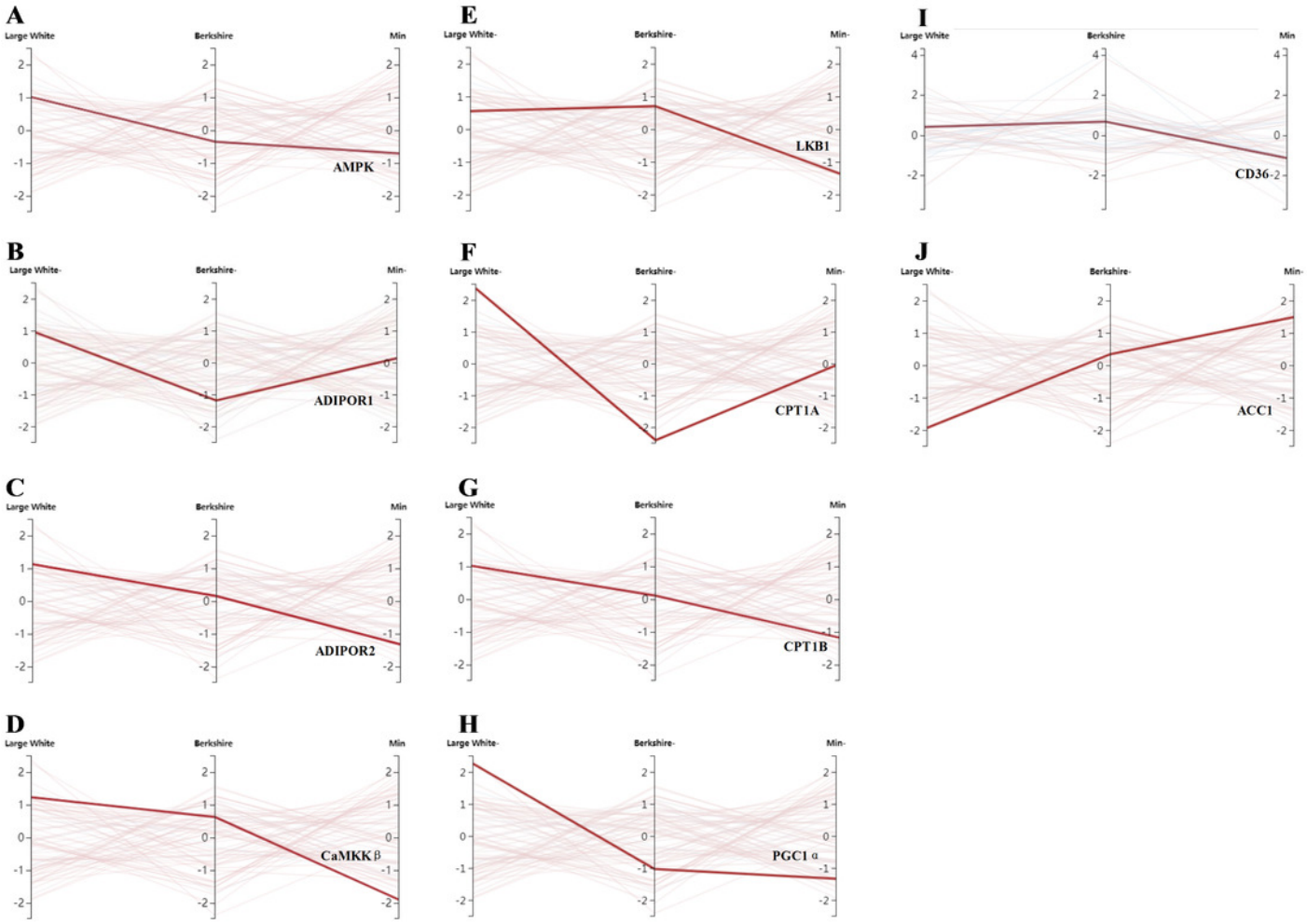

Figure 4

Colored map of the AMPK signaling pathway.

Upregulated and down-regulated genes are colored in red and blue, respectively.

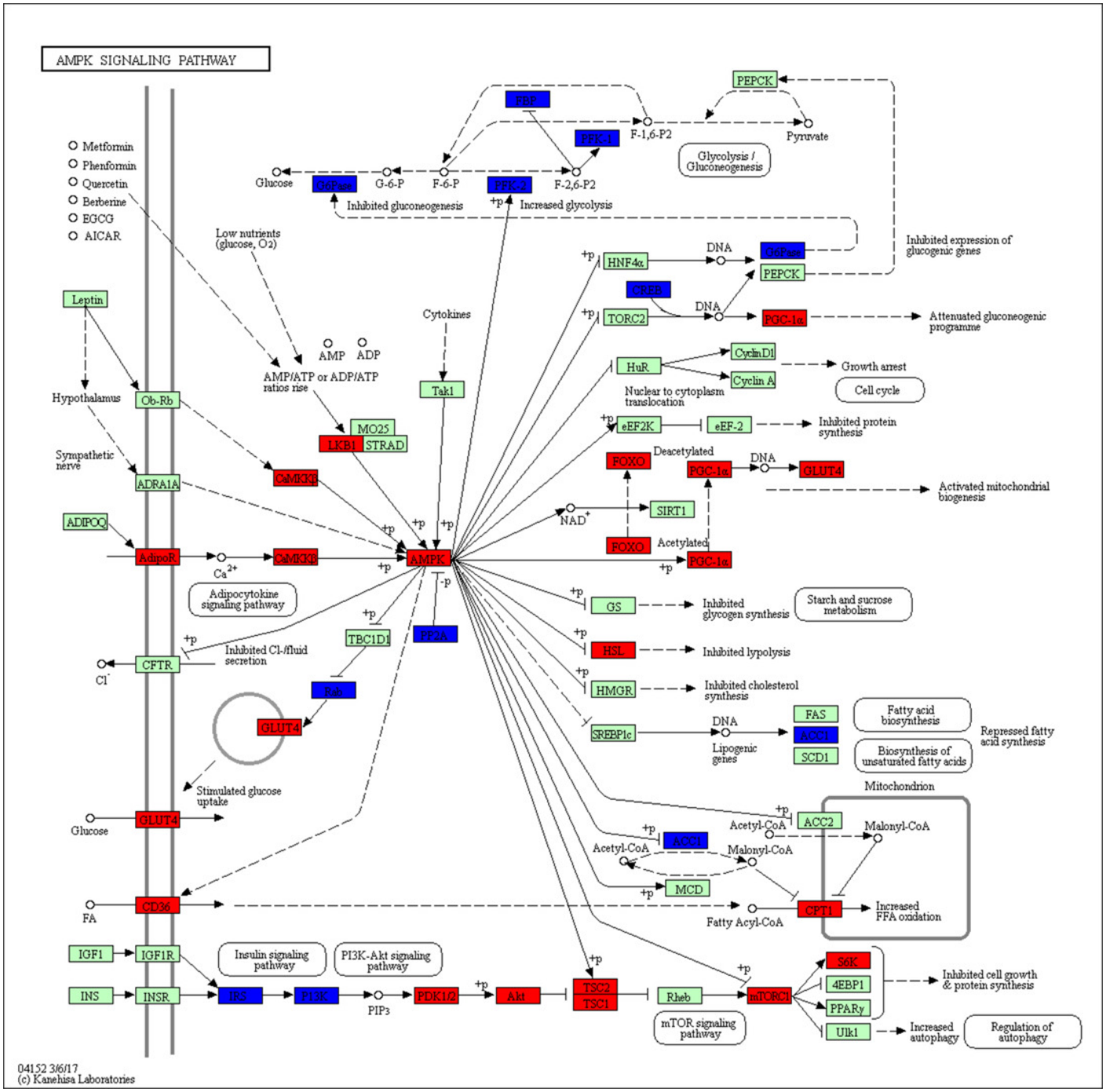

Figure 5

GO includes three complementary biological roles, Biological Process (BP), Molecular Function(MF) and Cellular Component (CC), of the DEGs in the qRT-PCR array.

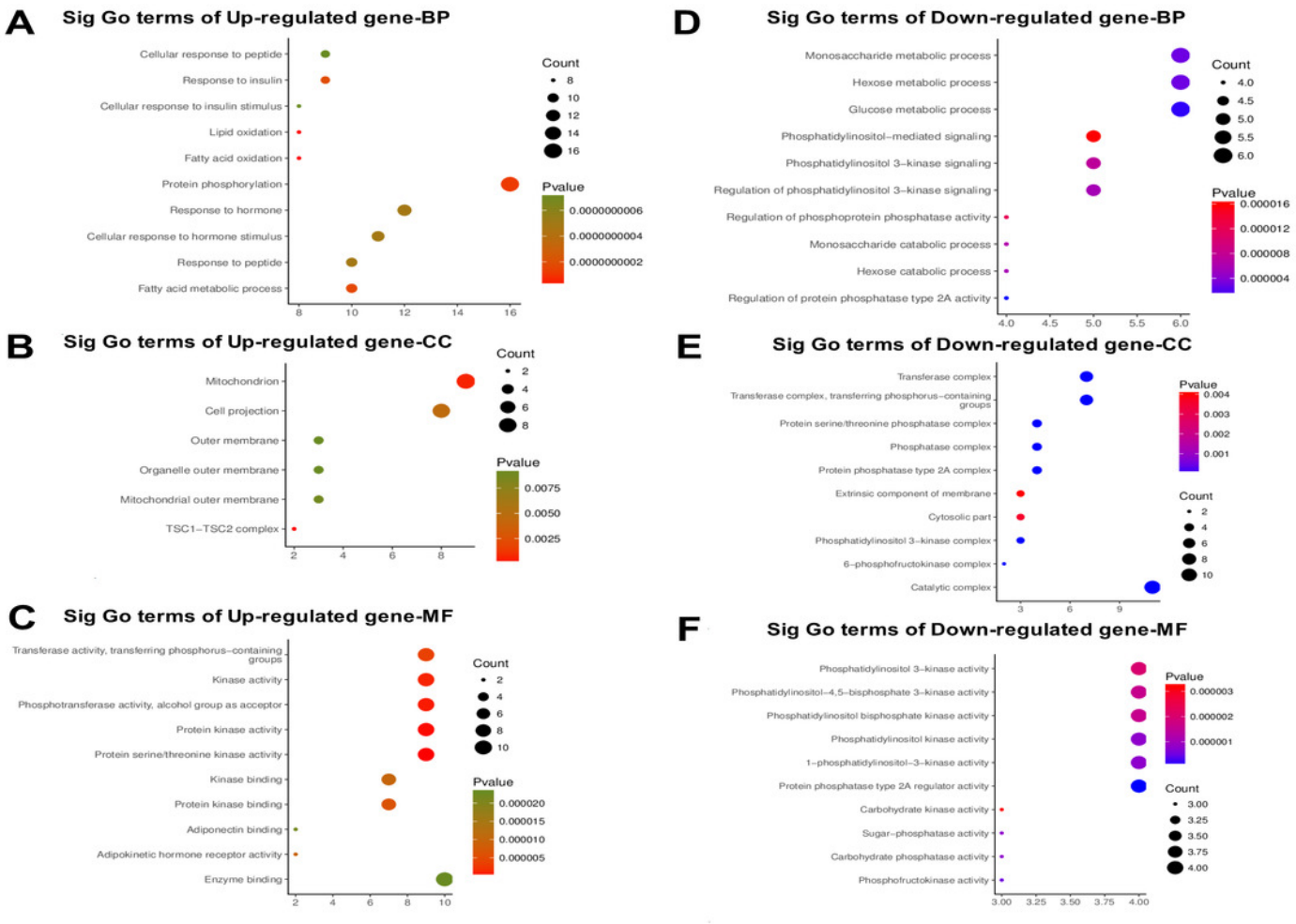

Table 1(on next page)

KEGG pathway enrichment of the DEGs in the Large White pigs from *GSE24912.*

Pathway ID	Name	Gene count	P-Value
Up-regulated DEGs			
ssc04145	Phagosome	36	1.66E-22
ssc01100	Metabolic pathways	87	2.06E-18
ssc00100	Steroid biosynthesis	6	3.08E-05
ssc00330	Arginine and proline metabolism	8	4.48E-05
ssc04062	Chemokine signaling pathway	15	5.27E-05
ssc00590	Arachidonic acid metabolism	9	5.59E-05
ssc01230	Biosynthesis of amino acids	9	8.49E-05
ssc04923	Regulation of lipolysis in adipocytes	8	0.000132401
ssc04060	Cytokine-cytokine receptor interaction	16	0.000192146
ssc00010	Glycolysis/Gluconeogenesis	8	0.000223585
ssc04810	Regulation of actin cytoskeleton	15	0.000274584
ssc04390	Hippo signaling pathway	12	0.000297981
ssc00592	alpha-Linolenic acid metabolism	5	0.000429579
ssc03320	PPAR signaling pathway	8	0.000471729
ssc04152	AMPK signaling pathway	10	0.000975992
ssc04975	Fat digestion and absorption	5	0.002284353
ssc00071	Fatty acid degradation	5	0.004659421
ssc01212	Fatty acid metabolism	5	0.007777676
Down-regulated DEGs			
ssc00190	Oxidative phosphorylation	17	1.67E-13
ssc01100	Metabolic pathways	38	5.02E-10
ssc01210	2-Oxocarboxylic acid metabolism	4	5.24E-05
ssc04024	cAMP signaling pathway	10	5.68E-05
ssc01230	Biosynthesis of amino acids	5	0.000728512
ssc04960	Aldosterone-regulated sodium reabsorption	4	0.000976159
ssc04931	Insulin resistance	6	0.001025991
ssc00220	Arginine biosynthesis	3	0.001331024
ssc04923	Regulation of lipolysis in adipocytes	4	0.00279057
ssc00400	Phenylalanine, tyrosine and tryptophan biosynthesis	2	0.003138276
ssc03320	PPAR signaling pathway	4	0.005585597
ssc04920	Adipocytokine signaling pathway	4	0.005856644
ssc00250	Alanine, aspartate and glutamate metabolism	3	0.006587795

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Table 2(on next page)

KEGG pathway enrichment of the DEGs in the Large White pigs from *GSE75045.*

Pathway ID	Name	Gene count	P-Value
Up-regulated DEGs			
ssc04922	Glucagon signaling pathway	18	2.68E-07
ssc04910	Insulin signaling pathway	20	5.21E-06
ssc00010	Glycolysis / Gluconeogenesis	13	9.46E-06
ssc01230	Biosynthesis of amino acids	14	1.18E-05
ssc01200	Carbon metabolism	14	0.000535156
ssc04152	AMPK signaling pathway	14	0.001013445
ssc00500	Starch and sucrose metabolism	8	0.002006183
ssc00760	Nicotinate and nicotinamide metabolism	6	0.002099766
ssc04974	Protein digestion and absorption	10	0.002287531
ssc04911	Insulin secretion	9	0.004713676
ssc04931	Insulin resistance	12	0.004816387
ssc00051	Fructose and mannose metabolism	6	0.006495029
ssc01100	Metabolic pathways	67	0.007996755
ssc00620	Pyruvate metabolism	6	0.008496652
ssc04022	cGMP-PKG signaling pathway	14	0.009206175
ssc04015	Rap1 signaling pathway	16	0.009321609
ssc00030	Pentose phosphate pathway	5	0.009465659
ssc01212	Fatty acid metabolism	7	0.013747689
ssc03320	PPAR signaling pathway	6	0.048074571
ssc04975	Fat digestion and absorption	4	0.048305332
Down-regulated DEGs			
ssc01100	Metabolic pathways	127	1.26E-09
ssc00071	Fatty acid degradation	16	1.62E-07
ssc01212	Fatty acid metabolism	16	2.91E-07
ssc03320	PPAR signaling pathway	16	2.16E-05
ssc00190	Oxidative phosphorylation	23	2.84E-05
ssc04146	Peroxisome	18	5.44E-05
ssc04260	Cardiac muscle contraction	15	0.000118319
ssc00640	Propanoate metabolism	9	0.000186267
ssc00620	Pyruvate metabolism	10	0.000368986
ssc00280	Valine, leucine and isoleucine degradation	11	0.000568137
ssc01200	Carbon metabolism	17	0.001051103
ssc00480	Glutathione metabolism	10	0.001135691
ssc00561	Glycerolipid metabolism	10	0.002151854
ssc00270	Cysteine and methionine metabolism	9	0.00487234
ssc00250	Alanine, aspartate and glutamate metabolism	8	0.005328411
ssc00062	Fatty acid elongation	5	0.00716945
ssc04261	Adrenergic signaling in cardiomyocytes	16	0.011976354

ssc04810	Regulation of actin cytoskeleton	21	0.015272497
ssc00061	Fatty acid biosynthesis	4	0.015273268
ssc04920	Adipocytokine signaling pathway	10	0.018163816
ssc00400	Phenylalanine, tyrosine and tryptophan biosynthesis	3	0.018757614
ssc01230	Biosynthesis of amino acids	9	0.047769246

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Table 3(on next page)

KEGG pathway enrichment of the DEGs in the Large White pigs from *GSE99092.*

Pathway ID	Name	Gene count	P-Value
Up-regulated DEGs			
ssc01200	Carbon metabolism	17	5.04E-07
ssc01100	Metabolic pathways	68	3.05E-06
ssc00071	Fatty acid degradation	8	0.000172009
ssc01212	Fatty acid metabolism	8	0.000237445
ssc00280	Valine, leucine and isoleucine degradation	8	0.000372631
ssc00640	Propanoate metabolism	6	0.000547718
ssc01210	2-Oxocarboxylic acid metabolism	5	0.000814208
ssc00630	Glyoxylate and dicarboxylate metabolism	5	0.002262259
ssc00350	Tyrosine metabolism	5	0.006611807
ssc00620	Pyruvate metabolism	5	0.010773515
ssc00061	Fatty acid biosynthesis	3	0.013205423
ssc03320	PPAR signaling pathway	6	0.02756666
ssc00190	Oxidative phosphorylation	9	0.032446069
ssc04920	Adipocytokine signaling pathway	6	0.033667129
ssc00360	Phenylalanine metabolism	3	0.03516625
ssc01230	Biosynthesis of amino acids	6	0.038183418
ssc04152	AMPK signaling pathway	9	0.038320574
ssc04975	Fat digestion and absorption	3	0.043808805
ssc00380	Tryptophan metabolism	4	0.045494658
Down-regulated DEGs			
ssc04666	Fc gamma R-mediated phagocytosis	11	0.001605346
ssc04130	SNARE interactions in vesicular transport	7	0.002628667
ssc04512	ECM-receptor interaction	9	0.004045914
ssc00100	Steroid biosynthesis	5	0.004943052
ssc04974	Protein digestion and absorption	9	0.007220933
ssc04810	Regulation of actin cytoskeleton	15	0.028724952
ssc00310	Lysine degradation	6	0.034132307
ssc00260	Glycine, serine and threonine metabolism	5	0.040945889
ssc00410	beta-Alanine metabolism	4	0.042853667
ssc04330	Notch signaling pathway	5	0.053363183
ssc00230	Purine metabolism	12	0.043788648
ssc00280	Valine, leucine and isoleucine degradation	5	0.047736312
ssc00330	Arginine and proline metabolism	5	0.047736312

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Table 4(on next page)

qRT-PCR array results for the AMPK signaling pathway (Large White-Min).

Up-regulated genes
CD36,PPARGC1A,AKT2,AKT1,CPT1B,ADIPOR2,PPP2R1A,CPT1A,FOXO1,LIPE,STK11,FOXO3, MTOR,ADIPOR1,RPS6KB2,AKT3,PRKAB1,PDPK1,TSC2,SLC2A4,CAMKK2,TSC1
Down-regulated genes
PFKM,FBP2,ACACA,RAB2A,PFKFB1,PPP2R2A,IRS1,CREB3,PPP2R5A,PPP2R5B, PFKL,PIK3R2,PPP2R5E,CREB5,G6PC3,RAB11B,PIK3CB,PIK3CD

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