Polymorphisms of the cryptochrome 2 and mitoguardin 2 genes are associated with the variation of lipid-related traits in Duroc pigs

Emilio Mármol-Sánchez1, Raquel Quintanilla2, Taina F. Cardoso3,1, Jordi Jordana Vidal4 & Marcel Amills1,4

The genetic factors determining the phenotypic variation of porcine fatness phenotypes are still largely unknown. We investigated whether the polymorphism of eight genes (MIGA2, CRY2, NPAS2, CIART, ARNTL2, PER1, PER2 and PCK1), which display differential expression in the skeletal muscle of fasted and fed sows, is associated with the variation of lipid and mRNA expression phenotypes in Duroc pigs. The performance of an association analysis with the GEMMA software demonstrated that the rs330779504 SNP in the MIGA2 gene is associated with LDL concentration at 190 days (LDL2, corrected P-value = 0.057). Moreover, the rs320439526 SNP of the CRY2 gene displayed a significant association with stearic acid content in the longissimus dorsi muscle (LD C18:0, corrected P-value = 0.015). Both SNPs were also associated with the mRNA levels of the corresponding genes in the gluteus medius skeletal muscle. From a biological perspective these results are meaningful because MIGA2 protein plays an essential role in mitochondrial fusion, a process tightly connected with the energy status of the cell, while CRY2 is a fundamental component of the circadian clock. However, inclusion of these two SNPs in chromosome-wide association analyses demonstrated that they are not located at the peaks of significance for the two traits under study (LDL2 for rs330779504 and LD C18:0 for rs320439526), thus implying that these two SNPs do not have causal effects.

The genome-wide analysis of gene expression data obtained from RNA-seq experiments can provide valuable information in order to understand the biology of production phenotypes and how they are genetically regulated. Cardoso et al.1 compared the muscle transcriptomic profiles of Duroc sows before and after feeding and, in doing so, they demonstrated that the ingestion of food is associated with changes in the mRNA levels of several circadian genes including the cryptochrome circadian regulator 2 (CRY2), neuronal PAS domain protein 2 (NPAS2), circadian associated repressor of transcription (CIART), aryl hydrocarbon receptor nuclear translocator like 2 (ARNTL2), period circadian regulator 1 (PER1) and period circadian regulator 2 (PER2). The identification of circadian clock regulator genes is particularly relevant because they have been broadly reported as major contributors to lipid metabolism and energy homeostasis2–7, driving changes in the expression of multiple transcripts and modulating cell response to different stimuli such as food intake5,6,7. Two other interesting genes identified by Cardoso et al.1 as differentially expressed before and after eating were mitoguardin 2 (MIGA2), which regulates mitochondrial fusion10, a process tightly connected with energy homeostasis11, and phosphoenolpyruvate carboxykinase 1 (PCK1), an enzyme fundamental for the maintenance of glucose and lipid levels12.
The expression of the eight genes mentioned above (ARNTL2, CIART, CRY2, NPAS2, PER1, PER2, PCK1 and MIGA2) is affected by food intake and there is ample evidence that they have a key role in carbohydrate and lipid metabolism^8,10,13-15^. The main hypothesis that we aim to test in the current work is whether the variability of concentration explained by rs330779504 genotype was 2.16% (SE = 0.03%).

The other association that remained significant after correction for multiple testing was that between LD and GM C18:3, and there was also an association between the CIART genotype and backfat thickness. Two SNPs in the PCK1 gene were associated with LD C17:0, and CRY2 and MIGA2 genotypes showed associations with several serum lipid and fatty acid composition traits. These results were consistent with the relevant role of the genes under study on metabolism and energy homeostasis. However, only two associations remained significant after correction for multiple testing.

Table 1. List of polymorphisms genotyped in a population of Duroc pigs (N = 345). SSC: pig chromosome.

| Gene | SSC | Start | End | Strand | SNP | Effect |
|------|-----|-------|-----|--------|-----|--------|
| MIGA2 | 1 | 269338125 | 269362328 | + | rs3225533788 | Splice region |
| | | | | | rs80923452 | Splice region |
| | | | | | rs80883236 | Splice region |
| | | | | | rs330779504 | Splice region |
| CRY2 | 2 | 16584431 | 16620699 | − | rs320439526 | 5′-UTR |
| NPAS2 | 3 | 53295744 | 53418480 | − | rs335603631 | Missense |
| CIART | 4 | 98797378 | 98814553 | − | rs322666984 | Missense |
| ARNTL2 | 5 | 46330522 | 46482280 | − | rs326158774 | Splice region |
| PER1 | 12 | 53341329 | 53376723 | − | rs344440225 | Splice region |
| | | | | | rs329662925 | Missense |
| | | | | | rs324793161 | Missense |
| | | | | | rs80910874 | Missense |
| | | | | | rs325502974 | Missense |
| PER2 | 15 | 137793421 | 137836268 | − | rs343196765 | Missense |
| | | | | | rs331782052 | Missense |
| | | | | | rs345064848 | Splice region |
| | | | | | rs320568163 | Missense |
| PCK1 | 17 | 57930356 | 57938817 | + | rs343196765 | Missense |

The other association that remained significant after correction for multiple testing was that between rs330779504 SNP (Table 2), a splice region variant located in the beginning of intron 14 (1:269.360 Mb) of the mitoguardin 2 gene (MIGA2). Pigs inheriting the A-allele showed an increased LDL cholesterol concentration (Fig. 1A), with homozygous AA animals having a higher median blood LDL concentration (69.35 mg/dL) than GA (61.75 mg/dL) and GG (58.40 mg/dL) individuals. Kruskal-Wallis ranking test for differences in median LDL concentrations yielded a P-value of 5.14E-03 (Supplementary Table 3), thus supporting the existence of significant differences among the three rs330779504 genotypes. Besides, this MIGA2 polymorphism also displayed an additive effect on palmitic acid content in LD muscle, total serum cholesterol concentration at ~190 days of age and the ratio between omega-6 and omega-3 desaturation in LD, but only at the nominal P-value level of significance (Table 2). The proportion of variance in LDL cholesterol concentration explained by rs330779504 genotype was 2.16% (SE = 0.03%).
Polymorphisms in the **MIGA2** and **CRY2** genes are associated with mRNA expression levels. To gain new insights into the molecular basis of the two significant associations found (Table 2), we investigated whether the rs330779504 and the rs320439526 SNPs are associated with the mRNA expression of the **MIGA2** and **CRY2** genes, respectively. Previously reported hepatic and GM muscle microarray data sets were employed for this purpose (Supplementary Table 2). Analysis with the GEMMA software revealed a significant association between the rs330779504 polymorphism and **MIGA2** mRNA expression levels in the GM muscle (Table 3). Pigs inheriting the **A**-allele of the rs330779504 polymorphism and **C**-allele of the rs320439526 polymorphism showed a reduced mRNA expression level compared to the **C**-allele of the rs330779504 and **T**-allele of the rs320439526. To achieve this goal, we merged the significant SNPs in a chromosome-wide association analysis.

### Table 2. Polymorphisms significantly associated with lipid-related traits.

| Gene  | SNP                      | Type                      | Trait                  | P-value | q-value | Δ (SE) | A1       | MAF  |
|-------|--------------------------|---------------------------|------------------------|---------|---------|--------|----------|------|
| MIGA2 | rs330779504 (1:269.360 Mb) | Splice region variant (G/A) | LDL<sub>2</sub>        | 2.71E-03 | 5.69E-02 | 6.26 (2.01) | A   | 0.236 |
|       |                          |                           | LDL (C16:0)           | 7.89E-03 | 1.66E-01 | ~0.43 (0.14) |     |       |
|       |                          |                           | TotalCholest<sub>2</sub> | 3.06E-02 | 2.73E-01 | 5.86 (2.53) |     |       |
|       |                          |                           | LDLFA/NA/FAn3         | 2.91E-02 | 6.10E-01 | ~1.00 (0.42) |     |       |
| CRY2  | rs320439526 (2:16.620 Mb) | 5′-UTR variant (C/T)      | LDL (C18:0)           | 7.04E-04 | 1.48E-02 | 0.39 (0.12) | T   | 0.353 |
|       |                          |                           | TotalCholest<sub>2</sub> | 4.22E-02 | 2.73E-01 | ~4.74 (2.23) |     |       |
|       |                          |                           | LDLFA                 | 3.10E-02 | 3.82E-01 | ~0.43 (0.20) |     |       |
|       |                          |                           | LSDFA                | 3.69E-02 | 3.75E-01 | 0.42 (0.20) |     |       |
| CRY2  | rs320439526 (2:16.620 Mb) | 5′-UTR variant (C/T)      | LD (C18:0)           | 7.04E-04 | 1.48E-02 | 0.39 (0.12) | T   | 0.353 |
|       |                          |                           | TotalCholest<sub>2</sub> | 4.22E-02 | 2.73E-01 | ~4.74 (2.23) |     |       |
|       |                          |                           | LDLFA                 | 3.10E-02 | 3.82E-01 | ~0.43 (0.20) |     |       |
|       |                          |                           | LSDFA                | 3.69E-02 | 3.75E-01 | 0.42 (0.20) |     |       |

**Inclusion of significant SNPs in a chromosome-wide association analysis.** After demonstrating that in the Lipgen population the rs330779504 (MIGA2) and rs320439526 (CRY2) SNPs are associated with serum LDL concentration at 190 days and LD C18:0, respectively, we aimed to investigate whether other SNP markers located in the vicinity of rs330779504 and rs320439526 display associations with these two traits with a higher level of significance than those observed for rs330779504 and rs320439526. To achieve this goal, we merged the rs330779504 SNP with 7,188 SNPs mapping to pig chromosome 1 (SSC1) and the rs320439526 SNP with 3,684 SNPs mapping to SSC2. The SSC1 and SSC2 SNP data were extracted from Porcine SNP60 BeadChip genotyping data reported by Manunza et al, and Gonzalez-Prendes et al. in the Lipgen population (Supplementary Table 4).
The associations between the markers rs330779504 (MIGA2) and rs320439526 (CRY2) with LDL serum concentration at ~190 days of age and with stearic acid content in LD, respectively, were only detected at the nominal level (Fig. 2). Indeed, we did not find any significant association at the chromosome-wide level when correcting for multiple testing with the false discovery rate (FDR) approach22 (Fig. 2).

Table 3. Associations between MIGA2 and CRY2 genotypes and the mRNA levels of the corresponding genes estimated with microarrays in gluteus medius skeletal muscle and liver samples from Duroc pigs; \(\delta\): estimated allele substitution effect and standard error (SE); \(A_1\): minor allele, MAF: Minor allele frequency; GM: gluteus medius skeletal muscle.
Discussion

One of the main goals of our study was to evaluate whether the polymorphisms of six genes with critical roles in the regulation of the circadian rhythms (CRY2, NPAS2, CIART, ARNTL2, PER1, PER2) are associated with the variation of lipid traits recorded in 345 Duroc pigs (Lipgen population). Indeed, circadian clock genes have been broadly reported as major contributors to the regulation of lipid metabolism and maintenance of energy homeostasis3,4,7, driving changes in the expression of multiple transcripts and thus causing differences in protein and enzymatic activity across the day-night cycle23,24. We have found multiple associations between the variability of the pig circadian genes and fatty acid composition traits, but the majority of them were only significant at the nominal level (Table 2). There are reports that indicate that there is a close relationship between the activity of circadian genes and fatty acid synthesis. For instance, fatty acid elongation is under circadian control because the cyclic acetylation of acetyl-CoA synthetase 1 by the SIRT1 deacetylase modulates the intracellular concentration of acetyl-CoA25. Moreover, alteration of circadian genes can potentially influence liver lipid metabolism in mice26. With regard to the circadian genes, the only association that remained significant after correction for multiple testing was that between the rs320439526 SNP of the CRY2 gene and C18:0 content in the longissimus dorsi muscle (Table 2, Supplementary Table 3). This association is particularly interesting because it has been demonstrated that the CRY1/2 genes can repress the peroxisome proliferator-activated receptor delta (PPARδ) transcription factor, which has a fundamental role in lipid metabolism15. Indeed, the inhibition of PPARδ by CRY1/2 is expected to decrease the rates of fatty acids transport and oxidation in the skeletal muscle15. Besides, the CRY2 gene has multiple effects on lipid metabolism. In response to a high-fat diet, CRY-deficient mice showed an increased body weight gain despite less feed consumption compared with wild-type animals, as a result of the activation of lipogenic pathways combined with increased insulin secretion and lipid storage27, thus leading to obesity propensity when CRY regulatory function was disrupted. We have also shown that the rs320439526 SNP of the CRY2 gene is associated with the expression of the CRY2 mRNA in the gluteus medius muscle (Table 3, Supplementary Table 4), suggesting that this polymorphism, or a nearby mutation, has regulatory effects on the transcriptional rate of the CRY2 gene. This polymorphism maps to the 5′-UTR of the CRY2 gene, a region that can have broad post-transcriptional effects on gene expression by interacting with RNA-binding proteins28. However, the Ensembl annotation of the rs320439526 SNP does not predict any functional effect, so we favour the hypothesis that this SNP is linked to another mutation with regulatory effects on CRY2 mRNA levels. The chromosome-wide (SSC2) association analysis depicted in Fig. 2 clearly shows that rs320439526 is not the marker displaying the most significant association with LD C18:0, thus indicating that the associations detected in our study have been produced by the existence of linkage disequilibrium between the rs320439526 SNP and a causal mutation yet to be found.

Figure 2. (A) Manhattan plot depicting the association of SNP rs330779504 and 7,188 additional SNPs mapping to pig chromosome 1 (SSC1) with serum low density lipoprotein concentration at ~190 days of age recorded in 345 Duroc pigs (Lipgen population). (B) Manhattan plot depicting the association of SNP rs320439526 and 3,684 additional SNPs mapping to pig chromosome 2 (SSC2) with stearic acid content in the longissimus dorsi muscle. The green line represents the nominal P-value of significance, while the blue line indicates the P-value of significance after correcting for multiple testing with an FDR test.
Another association that remained significant after correction for multiple testing was that between the MIGA2 rs330779504 SNP and serum LDL concentrations at ~190 days (Table 2, Supplementary Table 3). Moreover, this SNP was also associated with MIGA2 mRNA expression in the GM muscle and liver tissues (Table 3, Supplementary Table 4). The MIGA2 gene, also known as FAM73B, and its homolog MIGA1 (FAM73A) encode proteins localized to the outer membrane of mitochondria as membrane-integrated proteins and they have been previously associated with reduced body weight in mice and variations in backfat thickness in pigs. In a study performed by Zhang et al., it was reported that MIGA1/2 proteins stabilize the dimeric complex formed by active MitoPLD, thus facilitating mitochondrial fusion. Interestingly, the dynamics of mitochondrial fusion and fission is tightly related with the energy demand of cells. Indeed, nutrient abundance and starvation are associated with an increased frequency of fission and fusion events, respectively. Besides, the capacity to produce ATP in response to changes in energy demand and supply is modulated by mitochondrial morphology. A recent study reported that mitochondrial fusion induced by leptin could have important effects on the hepatic lipid accumulation, but to the best of our knowledge it is currently unknown whether mitochondrial fusion/fission has any effect on cholesterol and lipoprotein metabolism. Noteworthy, the chromosome-wide analysis pictured in Fig. 2 evidenced that the association observed between the MIGA2 rs330779504 marker and serum LDL levels at ~190 days is probably not causal, as there are some other neighboring SNPs that show more significant associations with this trait.

**Conclusions**

In this work, we wanted to test whether the variability of six circadian genes (ARNTL2, CIART, CRY2, NPS2, PER1 and PER2) and two additional genes (MIGA2 and PCK1) with key roles in energy homeostasis is associated with a set of lipid phenotypes recorded in Duroc pigs (Lipgen population). We have observed multiple associations between the variation of circadian genes and muscle fatty acid composition, but only that between the rs320439526 SNP of the CRY2 gene and LD C18:0 content remained significant after correction for multiple testing. We have also detected a significant association between the rs330779504 SNP of the MIGA2 gene and LDL concentration at 190 days. In the light of the results of the chromosome-wide analyses, we conclude that none of these two associations are causal.

**Methods**

**Ethics approval.** Animal care and management procedures were performed following Spanish national guidelines for the Good Experimental Practices and they were approved by the Ethical Committee of the Institut de Recerca i Tecnologia Agroalimentàries (IRTA).

**Animal material and phenotype recording.** As previously reported by Gallardo et al., a total of 345 Duroc barrows belonging to 5 half-sib families and distributed in 4 fattening batches were selected from a commercial pig line, devoted to high quality meat production. This line is characterized by its high content of intramuscular fat, a feature that results in the improvement of meat juiciness and taste, hence conferring a better consumer acceptance. Pigs were bred under intensive conditions of feeding and handling, and slaughtered when they reached 122 kg of live weight (~190 days of age). Phenotypic measures for different traits (Supplementary Table 1) were recorded during the productive cycle or after slaughtering: Triglycerides (TG), total cholesterol (TotalCholest), high-density lipoprotein (HDL) and low-density lipoprotein (LDL) serum concentrations at ~45 and ~190 days of age as reported by Gallardo et al., whereas intramuscular fat content in the LD and GM muscles and fatty acid composition for LD and GM were determined as described by Quintanilla et al.

**Selection and genotyping of twenty SNPs mapping to eight candidate genes.** Based on the results reported by Cardoso et al., we took into consideration eight genes that showed differential expression before and after feeding and that, moreover, play important roles in metabolism and circadian clock regulation (Table 1). The variability of these 8 genes was characterized by using, as a source of information, RNA-seq data results from 52 Duroc pigs retrieved from the same population analyzed herewith (Supplementary Table 2). Single nucleotide polymorphisms within selected genes were retrieved among variant calling results from sequences generated by Cardoso et al. Variant discovery analyses were performed by following the GATK Best Practices workflow for SNP calling (https://software.broadinstitute.org/gatk/documentation/article.php?id=3891) on RNA-seq data. Briefly, after read mapping, sequences were split into exon segments and intronic overhanging sequences hard-clipped. Mapping qualities were reassigned by using the SplitNCigarReads GATK tool (https://software.broadinstitute.org/gatk/), and the Haplotype Caller tool (https://software.broadinstitute.org/gatk) was used to detect SNPs for each analyzed sample (N = 52). Variant effect prediction on detected polymorphisms was estimated by using the SnpEff software and those that showed potential functional or regulatory effects (i.e. high impact, missense, splice site regions, 5′-UTR) within selected genes were kept for genotyping. Moreover, we also selected 3 SNPs showing potential functional or regulatory effects (rs322533788, rs335603631 and rs326158774) that were retrieved from the Ensembl database. A total of 20 selected SNPs and their flanking sequences (60 nucleotides upstream and downstream), were submitted to the Custom TaqMan Assay Design Tool website (https://www5.appliedbiosystems.com/tools/cdt/) (Life Technologies) to ascertain if they were amenable to genotyping in a TaqMan Open Array multiplex assay platform. Genotyping was performed at the Servei Veterinari de Genètica Molecular at the Universitat Autònoma de Barcelona (http://sct.uab.cat/svvm/en) by using a QuantStudio 12 K flex Real-Time PCR System (ThermoFisher Scientific).

**Association analyses between twenty selected SNPs and porcine lipid-related traits.** The PLINK software was used for processing genotyped data. Association analyses between genotyped polymorphisms and phenotypes were performed with the Genome wide efficient mixed-model association (GEMMA)
This package uses a mixed model approach to account for population stratification and relatedness by calculating a genomic kinship matrix with SNPs genotypes as random effects and provides an exact test of significance. We implemented a univariate mixed model as follows:

\[ y = W\alpha + x\delta + u + \varepsilon \]

where \( y \) is the vector of phenotypic observations for every individual; \( \alpha \) corresponds to a vector including the intercept plus the fixed effects, i.e. batch effect with 4 categories (all traits), farm origin effect with 3 categories (all traits), data of extraction with 2 categories within batch (only for TotalCholest, TG, HDL and LDL serum concentration, that were measured at approximately 45 and 190 days). The \( \alpha \) vector also contains the regression coefficients of the following covariates: live weight at slaughterhouse for TotalCholest, TG, HDL and LDL serum concentrations, and IMF content in LD and GM for LD and GM fatty acid composition respectively. \( W \) is the incidence matrix relating phenotypes with the corresponding effects; \( x \) is the vector of the genotypes corresponding to the set of selected polymorphisms; \( \delta \) is the allele substitution effect for each polymorphism; \( u \) is a vector of random individual effects with a \( \delta \)-dimensional multivariate normal distribution \( \text{MVN}_\delta (0, \lambda^{-1} \Omega) \), where \( \lambda^{-1} \) is the variance of the residual errors, \( \lambda \) is the ratio between the two variance components and \( \Omega \) is a known relatedness matrix derived from the SNPs; and \( \varepsilon \) is the vector of residual errors.

The association between lipid-related traits and the twenty analysed polymorphisms was assessed on the basis of the estimated allele substitution effects. The significance of these effects was established by implementing a correction for multiple testing using the FDR method reported by Benjamini and Hochberg. Moreover, we compared the phenotypic medians corresponding to each one of the three possible genotypes by applying the non-parametric Kruskal–Wallis test, due to the non-normal distribution of lipid phenotypes under study.

**Association analyses between the rs330779504 and rs320439526 polymorphisms and the expression of the genes that contain them.** Gluteus medius skeletal muscle and liver samples were collected from 103 Duroc pigs belonging to the Lipgen population. Samples were retrieved after slaughtering, and immediately frozen at \(-80^\circ\text{C}\) in liquid nitrogen. Total RNA was isolated from GM samples by using the TRIzol method and the RiboPure kit (Ambion, Austin, TX) following manufacturer’s recommendations. Transcriptomic mRNA expression profiles were then assessed by hybridization to the GeneChip Porcine arrays (Affymetrix Inc., Santa Clara, CA), as previously reported by Cânovas et al. Expression data corresponding to GM muscle and liver samples are deposited in NCBI’s Gene Expression Omnibus and can be accessed through GEO Series accession number GSE115484 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE115484). Data pre-processing, background correction, normalization and log-transformation of expression values between samples were carried out by computing a Robust Multi-array Average (RMA) as described by Irizarry et al.

The correspondence between genes and microarray expressed probes was assessed with the Biomart database available at Ensembl repositories. Expression levels for selected genes were then extracted from microarray samples for both GM muscle and liver tissues and used as continuous variables in association analyses, following the same statistical model previously described for phenotype records and correcting for batch (4 categories), farm of origin (3 categories) and laboratory (2 categories) as fixed effects. Moreover, we compared the phenotypic means corresponding to each one of the three possible genotypes by applying an ANOVA test.

**Inclusion of the MIGA2 rs330779504 and CRY2 rs320439526 SNPs in a chromosome-wide association analysis.** As previously described by Manunza et al. and González-Prendes et al., the population employed in the current experiment was typed with the Porcine SNP60 BeadChip (Illumina, San Diego, CA) which contains probes for 62,163 SNPs (Supplementary Table 2). The GenomeStudio software (Illumina) was used for quality control analyses, as reported by Manunza et al. The PLINK software was used for removing SNPs that (a) did not map to autosomal chromosomes, (b) had minor allele frequency (MAF) < 0.05, (c) with rate of missing genotypes > 0.05, (d) major departures from the Hardy-Weinberg equilibrium (P-value = 0.001), (e) had a GenCall score < 0.15, (f) had a call rate < 0.95, or (g) that could not be mapped to the pig genome reference. A total of 36,710 SNPs were finally retrieved after filtering and merged with genotyping data corresponding to the rs330779504 and the rs320439526 SNPs. Association analyses were performed with the GEMMA software as described before, and multiple testing correction was implemented with the FDR method by establishing a chromosome-wide threshold of significance.

**Data Availability**

Expression data corresponding to GM muscle and liver samples are deposited at NCBI’s Gene Expression Omnibus and are accessible through GEO Series accession number GSE115484 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE115484). Genotypes and phenotypes for the 345 Duroc pigs (Lipgen population) have been deposited in the Figshare public repository (https://figshare.com/s/2e636697009360986794).

**References**

1. Cardoso, T. F. et al. Nutrient supply affects the mRNA expression profile of the porcine skeletal muscle. BMC Genomics 18, 603 (2017).
2. Froy, O. The relationship between nutrition and circadian rhythms in mammals. Front. Neuroendocrinol. 28, 61–71 (2007).
3. Green, C. R., Takahashi, J. S. & Bass, J. The meter of metabolism. Cell 134, 728–742 (2008).
4. Laposky, A. D., Bass, J., Kohsaka, A. & Turek, F. W. Sleep and circadian rhythms: Key components in the regulation of energy metabolism. FEBS Lett. 582, 142–151 (2008).
5. Froy, O. & Miskin, R. Effect of feeding regimens on circadian rhythms: implications for aging and longevity. Aging 2, 7–27 (2010).
6. Paschos, G. K. Circadian clocks, feeding time, and metabolic homeostasis. Front. Pharmacol. 6, 112 (2015).
42. Edgar, R., Domrachev, M. & Lash, A. E. Gene Expression Omnibus: NCBI gene expression and hybridization array data repository.

39. Purcell, S.

31. Choi, S.-Y.

27. Putti, R., Sica, R., Migliaccio, V. & Lionetti, L. Diet impact on mitochondrial bioenergetics and dynamics.

32. Vamecq, J.

29. Bassett, J. H. D.

26. Green, C. B.

34. Hsu, W.-H., Lee, B.-H. & Pan, T.-M. Leptin-induced mitochondrial fusion mediates hepatic lipid accumulation.

24. Kumar Jha, P., Challet, E. & Kalsbeek, A. Circadian rhythms in glucose and lipid metabolism in nocturnal and diurnal mammals.

25. Sahar, S.

23. Ruiter, M.

22. Benjamini, Y. & Hochberg, Y. Controlling the false discovery rate: a practical and powerful approach to multiple testing.

19. Quintanilla, R.

18. Manunza, A.

a FPU pre-doctoral fellowship from the Spanish Ministry of Education (FPU15/01733).

7. McGinnis, G. R. & Young, M. E. Circadian regulation of metabolic homeostasis: causes and consequences. Nat. Sci. Sleep 8, 163–80 (2016).

8. Patel, S. A., Velingkaar, N., Makwana, K., Chaudhari, A. & Kondratov, R. Calorie restriction regulates circadian clock gene expression through BMAL1 dependent and independent mechanisms. Sci. Rep. 6, 25970 (2016).

9. Chaudhari, A., Gupta, R., Makwana, K. & Kondratov, R. Circadian clocks, diets and aging. Nutr. Healthy Aging 4, 101–112 (2017).

10. Zhang, Y. et al. Mitochondria regulates mitochondrial fusion through MitoPLD and is required for neuronal homeostasis. Mol. Cell 61, 111–24 (2016).

11. Westermann, B. Bioenergetic role of mitochondrial fusion and fission. Biochim. Biophys. Acta - Bioenerg. 1817, 1833–1838 (2012).

12. Millward, C. A. et al. Phosphoenolpyruvate carboxykinase (Pck1) helps regulate the triglyceride/fatty acid cycle and development of insulin resistance in mice. J. Lipid Res. 51, 1452–1463 (2010).

13. Grimaldi, B. et al. PER2 controls lipid metabolism by direct regulation of PPARγ. Cell Metab. 12, 509–20 (2010).

14. Machicao, E. et al. Glucose-raising polymorphisms in the human clock gene cryptochrome 2 (CRY2) affect hepatic lipid content. PLoS One 11, e0145563 (2016).

15. Jordan, S. D. et al. CRY1/2 selectively repress PPARα and limit exercise capacity. Cell Metab. 26, 243–255 (2017).

16. Cardoso, T. F. et al. RNA-seq based detection of differentially expressed genes in the skeletal muscle of Duroc pigs with distinct lipid profiles. Sci. Rep. 7, 40005 (2017).

17. Gallardo, D. et al. Mapping of quantitative trait loci for cholesterol, LDL, HDL, and triglyceride serum concentrations in pigs. Physiol. Genomics 35, 199–209 (2008).

18. Manunza, A. et al. A genome-wide association analysis for porcine serum lipid traits reveals the existence of age-specific genetic determinants. BMC Genomics 15, 758 (2014).

19. Quintanilla, R. et al. Porcine intramuscular fat content and composition are regulated by quantitative trait loci with muscle-specific effects. J. Anim. Sci. 89, 7963–71 (2011).

20. Canovas, A. et al. Segregation of regulatory polymorphisms with effects on the gluteus medius transcriptome in a purebred pig population. PLoS One 7, e35583 (2012).

21. González-Prendes, R. et al. Joint QTL mapping and gene expression analysis identify positional candidate genes influencing pork quality traits. Sci. Rep. 7, 39830 (2017).

22. Benjamini, Y. & Hochberg, Y. Controlling the false discovery rate: a practical and powerful approach to multiple testing. Journal of the Royal Statistical Society. Series B (Methodological) 57, 289–300 (1995).

23. Ruiter, M. et al. The daily rhythm in plasma glucagon concentrations in the rat is modulated by the biological clock and by feeding behavior. Diabetes 52, 1709–15 (2003).

24. Kumar Iha, P., Challet, E. & Rabska, A. Circadian rhythms in glucose and lipid metabolism in nocturnal and diurnal mammals. Mol. Cell Endocrinol. 418, 74–88 (2015).

25. Sahar, S. et al. Circadian control of fatty acid elongation by SIRT1 protein-mediated decacylation of acetyl-coenzyme A synthetase 1. J. Biol. Chem. 289, 6091–6097 (2014).

26. Green, C. B. et al. Loss of Nocturnin, a circadian deadenylase, confers resistance to hepatic steatosis and diet-induced obesity. Proc. Natl. Acad. Sci. USA 104, 9888–9893 (2007).

27. Putti, R., Sica, R., Migliaccio, V. & Lionetti, L. Diet impact on mitochondrial bioenergetics and dynamics. Front. Physiol. 6, 109 (2015).

28. Mignone, F., Gissi, C., Liuni, S. & Pesole, G. Untranslated regions of mRNAs. Genome Biol. 3, REVIEWS0004 (2002).

29. Bassett, J. H. D. et al. Rapid-throughput skeletal phenotyping of 100 knockout mice identifies 9 new genes that determine bone strength. PLoS Genet. 8, e1002858 (2012).

30. Lee, K. T. et al. Neuronal genes for subcutaneous fat thickness in human and pig are identified by local genomic sequencing and combined SNP association study. PLoS One 6, e16356 (2011).

31. Choi, S.-Y. et al. A common lipid link Mfn-mediated mitochondrial fusion and SNARE-regulated exocytosis. Nat. Cell Biol. 8, 1255–1262 (2006).

32. Vaneecq, J. et al. Mitochondrial dysfunction and lipid homeostasis. Curr. Drug Metab. 13, 1388–1400 (2012).

33. Schreper, E. & Scarzanella, L. Mitofusins, from mitochondria to metabolism. Mol. Cell 61, 683–694 (2016).

34. Hsu, W.-H., Lee, B.-H. & Pan, T.-M. Leptin-induced mitochondrial fusion mediates hepatic lipid accumulation. Int. J. Obes. 39, 1750–6 (2015).

35. Gallardo, D. et al. Alternative splicing at exon 28 of the acetyl-coenzyme A carboxylase α gene in adult pigs and embryos. Anim. Genet. 39, 205–206 (2008).

36. Gallardo, D. et al. Polymorphism of the pig acetyl-coenzyme A carboxylase α gene is associated with fatty acid composition in a Duroc commercial line. Anim. Genet. 40, 410–417 (2009).

37. Wood, J. D. et al. Fat deposition, fatty acid composition and meat quality: A review. Meat Sci. 78, 343–358 (2008).

38. Cingolani, P. et al. A program for annotating and predicting the effects of single nucleotide polymorphisms, Snpeff. Fly 6, 80–92 (2012).

39. Purcell, S. et al. PLINK: A tool set for whole-genome association and population-based linkage analyses. Am. J. Hum. Genet. 81, 559–575 (2007).

40. Zhou, X. & Stephens, M. Genome-wide efficient mixed-model analysis for association studies. Nat. Genet. 44, 821–824 (2012).

41. Chomczynski, P. & Sacchi, N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal. Biochem. 162, 156–9 (1987).

42. Edgar, R., Domrachev, M. & Lash, A. E. Gene Expression Omnibus: NCBI gene expression and hybridization array data repository. Nucleic Acids Res. 30, 207–210 (2002).

43. Irizarry, R. A. et al. Exploration, normalization, and summaries of high density oligonucleotide array probe level data. Biostatistics 4, 249–264 (2003).

Acknowledgements The authors are indebted to Selección Batallé S.A. for providing the animal material. We gratefully acknowledge to J. Reixach (Selecciò Batallé), J. Soler (IRTA), C. Millan (IRTA), A. Quintana (IRTA) and A. Rossell (IRTA) for their collaboration in the experimental protocols and pig management. We also want to thank MINECO for the Center of Excellence Severo Ochoa 2016–2019 (SEV-2015-0533) grant awarded to the Centre for Research in Agricultural Genomics (CRAG), and the CERCA Programme of the Generalitat de Catalunya for their support. Part of the research presented in this publication was funded by grants AGL2013–48742-C2–1-R and AGL2013–48742-C2–2–R awarded by the Spanish Ministry of Economy and Competitivity, and grant 2014 SGR 1528 from the Agency for Management of University and Research Grants of the Generalitat de Catalunya. Tainá Figueiredo Cardoso was funded with a fellowship from the CAPES Foundation of Coordination of Improvement of Higher Education, Ministry of Education of the Federal Government of Brazil. Emilio Mármol-Sánchez was funded with a FPU pre-doctoral fellowship from the Spanish Ministry of Education (FPU15/01733).
Author Contributions
M.A., R.Q. and J.J. designed the experiment. R.Q. generated the animal material and collected the phenotypic and microarray data. E.M.S. and M.A. selected the SNPs to be genotyped. E.M.S. did all bioinformatic and statistical analyses. T.F.C. contributed to the analysis of gene expression data. E.M.S. and M.A. wrote the paper. All authors read and approved the content of the manuscript.

Additional Information
Supplementary information accompanies this paper at https://doi.org/10.1038/s41598-019-45108-z.

Competing Interests: The authors declare no competing interests.

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

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 license, and indicate if changes were made. The images or other third party material in this article are included in the article’s Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the article’s Creative Commons license 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 license, visit http://creativecommons.org/licenses/by/4.0/.

© The Author(s) 2019