# Modeling miRNA-driven post-transcriptional regulation by using exon-intron split analysis (EISA) in pigs

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# Abstract

Bulk sequencing of RNA transcripts has typically been used to quantify gene expression levels in different experimental systems. However, linking differentially expressed (DE) mRNA transcripts to gene expression regulators, such as miRNAs, remains challenging, as miRNA-mRNA interactions are commonly identified *post hoc* after selecting sets of genes of interest, thus biasing the interpretation of underlying gene regulatory networks. In this study, we aimed at disentangling miRNA-driven post-transcriptional signals linked to porcine muscle and adipose tissue energy homeostasis. For this purpose, we performed an exon-intron split analysis (EISA) on muscle and fat RNA-seq data from two independent pig populations. One of these populations was subjected to fasting-feeding conditions, while the other represented divergent fatness profiles. After running EISA, protein-coding mRNA genes with downregulated exonic fractions and high post-transcriptional signals were significantly enriched for binding sites of DE upregulated miRNAs. Moreover, these downregulated genes showed an increased expression covariation for the exonic fraction compared to the intronic fraction. On the contrary, they did not show enrichment for binding sites of non-DE highly expressed or downregulated DE miRNAs. Among the set of loci displaying miRNA-driven post-transcriptional regulatory signals, we observed genes related to glucose homeostasis (*DKK2*, *PDK4*, *IL18*, *NR4A3*, *CHRNA1*, *TET2*), cell differentiation (*PBX1*, *BACH2*) and adipocytes metabolism (*SESN3*, *ESRRG*, *SAMD4*, *LEP*, *PTGFR*, *SERPINE2*, *RNF157*, *GPLD1*, *NCF2*, *OSBPL10*, *PRSS23*). Our results highlighted mRNA genes showing post-transcriptional miRNA-driven downregulation using the exonic and intronic fractions of RNA-seq datasets from muscle and adipose tissues in pigs.


**Keywords:** Exon-intron split analysis, microRNA, pigs, energy homeostasis.

# 1. Introduction

Messenger RNA (mRNA) expression and turnover in metabolic processes are subjected to complex yet poorly characterized regulatory mechanisms that contribute to shaping fine-tuned biological responses to different stimuli [1]. Cellular metabolic changes are hence a direct manifestation of intricate interactions between expressed transcripts and other regulatory elements that modify their abundance, localization, fate and degradation rate. MicroRNAs (miRNAs) are primarily engaged in the post-transcriptional control of gene expression through inhibition of translation and/or destabilization of target mRNAs by poly(A) shortening and subsequent degradation [2].

Changes in the abundance of mRNAs targeted by miRNAs can be inferred through covariation analysis. Such approach can help to unravel direct or indirect molecular interactions connecting and regulating biological networks.

In order to disentangle regulatory functions driven by miRNAs, research studies typically focus on specific sets of genes of interest, showing significant expression changes and harboring binding sites for DE miRNAs [3–9]. This approach, however, is biased by the fact that genes are selected after differential expression analysis and by the performance of an *ad hoc* search of predicted interactions between the 3'-UTRs of mRNAs and the seed regions of miRNAs. Besides, one of the main limitations of differential expression analysis is that it does not discriminate whether changes in expression take place either at the transcriptional or post-transcriptional levels. Such distinction is essential to understand at which level of the mRNA life-cycle the regulation is taking place.

To address this issue, Gaidatzis *et al*. [10] proposed that the magnitude of the post-transcriptional component can be deduced by comparing the amounts of exonic and intronic reads. Based on this, these authors devised a methodology denoted *exon-intron split analysis* (EISA), which separates the transcriptional and post-transcriptional

components of gene regulation. By considering that intronic reads are mainly derived from heterogeneous nuclear RNAs (unprocessed mRNAs or pre-mRNAs), they assessed the magnitude of the transcriptional regulation. Such assumption is based on early reports describing intronic expression as a proxy of nascent transcription and co-transcriptional splicing events [11–13]. In this way, a gene showing similar levels of intronic reads in two different states but a strong downregulation of exonic reads after applying a certain treatment or challenge (nutrition, infection, temperature etc.), could be indicative of an induced inhibition at the post-transcriptional level [10,14,15].

A high number of differential expression studies have been performed in pigs during the last decade [16–24], but to the best of our knowledge, in none of these studies the transcriptional and post-transcriptional components of gene regulation have been independently analyzed. In the present study, we aimed to dissect the contribution of miRNAs to post-transcriptional regulation in pigs by applying the EISA methodology using two independent porcine experimental models.

# 2. Materials and methods

## 2.1. Experimental design, sampling and processing

In this study, we have used two independent experimental systems:

(i) Duroc pigs: Twenty-three gilts divided in two fasting/feeding regimes, i.e., 11 gilts (*AL-T0*) slaughtered in fasting conditions and 12 gilts (*AL-T2*) slaughtered immediately after 7 h with access to *ad libitum* feed intake [7,22,25]. Immediately after slaughtering, *gluteus medius* (GM) skeletal muscle samples were collected and snap-frozen at -80°C.

(ii) Duroc-Göttingen minipig $F_2$ inter-cross: Ten individuals with divergent fatness profiles for their body mass index (BMI) metric (5 *lean* and 5 *obese*) were selected from

the UNIK resource population [26,27], as described in Jacobsen et al. 2019 [28]. Retroperitoneal adipose tissue was collected at slaughter and mature adipocytes were subsequently isolated following the protocol of Decaunes et al. 2011 [29] with modifications reported in [28].

Further details about RNA-seq and small RNA-seq expression data generated from both experimental designs have been previously described [7,22,28]. Sequencing reads generated in the RNA-Seq and small RNA-Seq datasets from both pig resources were trimmed with the Cutadapt software [30]. Reads were mapped against the Sscrofa11.1 porcine assembly [31] with the HISAT2 aligner [32] and default parameters for RNA-Seq reads. In contrast, the Bowtie Alignment v.1.2.1.1 software [33] with small sequence reads specifications (*bowtie -n 0 -l 25 -m 20 -k 1 --best --strata*) was used to align small RNA-Seq reads to the Sscrofa11.1 porcine reference assembly [31].

### 2.2. Exon/Intron quantification

We generated exonic and intronic-specific annotations spanning all genes available using the gtf formatted Sscrofa.11.1 v.103 gene annotation file (Ensembl repositories: http://ftp.ensembl.org/pub/release-103/gtf/sus_scrofa/). Overlapping intronic/exonic regions, as well as singleton positions were removed [34]. Each intronic region was trimmed by removing 10 nucleotides on both ends to avoid exonic reads mapping close to exon/intron junctions. We then used the featureCounts function included in the Rsubread package [35] to quantify gene expression profiles based on exonic and intron expression patterns for each gene, independently. MiRNA expression profiles were estimated using the Sscrofa11.1 v.103 mature miRNA annotation with the featureCounts software tool [36] in single-end mode and with default parameters.

## 2.3. Exon/intron split analysis (EISA).

We applied EISA to infer post-transcriptional gene regulation in our two independent porcine datasets. For this purpose, we separately estimated the exonic and intronic abundance of each annotated mRNA gene using the Sscrofa11.1 v.103 exon/intron custom annotation generated as described above. Only genes showing average expression values above 1 count-per-million in at least 50% of animals were retained for further analyses. Normalization was performed independently for exon and intron counts by multiplying each $i^{th}$ gene expression in each $j^{th}$ sample by the corresponding mean gene expression and dividing by the total number of quantified counts per sample [10]. Exonic and intronic gene abundances were subsequently transformed to a $\log_2$ scale, adding a pseudo-count of 1 and averaged within each considered treatment groups (*AL-T0* and *AL-T2* for GM tissues and *lean* and *obese* for adipocyte isolates).

Only genes with successful exonic and intronic quantified read counts were considered in our analyses. The transcriptional component (Tc) contribution to the observed differences in each $i^{th}$ gene was expressed as the increment of intronic counts in fed (*AL-T2*) and *obese* animals with respect to fasting (*AL-T0*) and *lean* animals ($\Delta Int = Int_{2i} - Int_{1i}$), respectively. The increment of exonic counts ($\Delta Ex$) was also calculated, and the post-transcriptional component (PTc) effect was expressed as $\Delta Ex - \Delta Int = (Ex_{2i} - Ex_{1i}) - (Int_{2i} - Int_{1i})$. Both components were z-scored to represent comparable ranges between $\Delta Ex$ and $\Delta Int$ estimates. All implemented analyses have been summarized in **Fig. S1**. A ready-to-use modular pipeline for running EISA is publicly available at https://github.com/emarmolsanchez/EISACompR

## 2.4. Post-transcriptional signal prioritization

In order to obtain a prioritized list of genes showing relevant signals of post-transcriptional regulation, the top 5% genes with the highest negative PTc scores were retrieved. We only focused on genes showing strongly reduced exonic fraction (ΔEx) values of at least 2-folds for post-transcriptional signals in both experimental systems.

### 2.5. Differential expression analyses and significance of PTc scores

Differential expression analyses were carried out with the *edgeR* package [37] by considering the exonic fraction of mRNAs, as well as miRNA expression profiles from RNA-Seq and small RNA-Seq datasets, respectively, and in the two experimental systems under study. Expression filtered raw counts for exonic reads were normalized with the trimmed mean of M-values normalization (TMM) method [38] and the statistical significance of mean expression differences was tested with a quasi-likelihood F-test [37]. Correction for multiple hypothesis testing was implemented with the Benjamini-Hochberg false discovery rate approach [39]. Messenger RNAs were considered as differentially expressed (DE) when the absolute value of the fold-change (FC) was higher than 2 ($|FC| > 2$) and $q$-value $< 0.05$. For miRNAs, $|FC| > 1.5$ and $q$-value $< 0.05$ were used instead. This more flexible threshold was motivated by the fact that miRNAs are commonly lowly expressed and show more stable and subtle expression changes compared to mRNAs [7,40]. The statistical significance of the post-transcriptional (PTc) scores was evaluated by incorporating the intronic quantification as an interaction effect for exonic abundances [10]. The fasting pigs (*AL-T0*) from Duroc skeletal muscle, as well as *obese* pigs from Duroc-Göttingen adipocyte expression profiles were considered to be the baseline controls, i.e., any given upregulation in ΔEx or ΔInt values represents and overexpression in fed (*AL-T2*) Duroc gilts and *lean* Duroc-Göttingen minipigs with respect to their fasting (*AL-T0*) and *obese* counterparts.

## 2.6. miRNA target prediction

Putative interactions between the seed regions of expressed miRNAs (small RNA-seq datasets) and the 3'-UTRs of expressed protein-coding mRNA genes (RNA-seq datasets) were predicted on the basis of sequence identity using the Sscrofa11.1 reference assembly. The annotated 3'-UTRs longer than 30 nts from porcine mRNAs were retrieved from the Sscrofa11.1 v.103 available at BioMart (http://www.ensembl.org/biomart) and miRBase [41] databases. Redundant seeds from mature porcine microRNAs were removed. The seedVicious v1.1 tool [42] was used to infer miRNA-mRNA interactions. MiRNA-mRNA 8mer, 7mer-m8 and 7mer-A1 interactions were considered as the most relevant among the full set of canonical and non-canonical interactions [2,43,44].

Based on the study of Grimson et al. [43], the *in silico*-predicted miRNA-mRNA interactions matching any of the following criteria were removed: (i) Binding sites located in 3'-UTRs at less than 15 nts close to the end of the open reading frame (and the stop codon) or less than 15 nts close to the terminal poly(A) tail (E criterion), (ii) binding sites located in the middle of the 3'-UTR in a range comprising 45-55% of the central region of the non-coding sequence (M criterion), and (iii) binding sites lack AU-rich elements in their immediate upstream and downstream flanking regions comprising 30 nts each (AU criterion).

Covariation patterns between miRNAs and their predicted mRNA targets were assessed by computing Spearman's correlation coefficients ($\rho$) with the TMM normalized and $\log_2$ transformed expression profiles of the exonic fractions of mRNA and miRNA genes. To determine the contribution of miRNAs to post-transcriptional regulation in the two experimental systems under study, only miRNA-mRNA predicted pairs comprising DE upregulated miRNAs (FC > 1.5; $q$-value < 0.05) and mRNA genes with relevant PTc

scores (see post-transcriptional signal prioritization section) were taken into consideration.

### 2.7. miRNA target enrichment analyses

We sought to determine if the overall number of mRNA genes with high post-transcriptional signals (the ones with the top 5% negative PTc scores and reduced ΔEx values > 2-folds) were significantly enriched to be targeted by at least one upregulated miRNA (FC > 1.5; *q*-value < 0.05), compared with the whole set of expressed mRNAs genes with available 3'-UTRs from both datasets. Enrichment analyses were carried out using the Fisher's exact test implemented in the *fisher.test* R function. Significance level was set at nominal *P*-value < 0.05.

We also tested whether these genes were enriched for binding sites of the top 5% most highly expressed miRNA genes, excluding significantly upregulated miRNAs, as well as for binding sites of significantly downregulated miRNAs (FC < -1.5; *q*-value < 0.05). Given the relatively low statistical significance of DE miRNAs observed in the UNIK Duroc-Göttingen minipigs (l*ean* vs *obese*), we considered that miRNAs were significantly upregulated in this particular data set when FC >1.5 and *P*-value < 0.01.

As a control randomized test for enrichment analyses between miRNAs and mRNAs with high post-transcriptional downregulatory signals, we implemented a bootstrap corrected iteration to generate 100 random sets of 10 expressed mature miRNA genes without seed redundancy, which were used as input for miRNA target prediction with the sets of mRNA genes with the high post-transcriptional signals (top 5% PTc scores and at least 2-folds ΔEx reduction). The distribution of odds ratios obtained after iterating over each random set of miRNAs (N = 100) were then compared with odds ratios obtained with the set of significantly upregulated miRNAs.

The $P$-value for the significance of the deviation of observed odds ratios against the bootstrapped odds ratios distribution was defined as, $P - value = 1 - \frac{r+1}{k+1}$, where $r$ is the number of permuted odds ratios with values equal or higher than the observed odds ratio for enrichment analyses with the set of upregulated miRNAs, and $k$ is the number of defined permutations (N = 100).

### 2.8. Gene covariation network and covariation enrichment score

We computed pairwise correlation coefficients among the whole set of DE mRNA genes in the *AL-T0* vs *AL-T2* ($q$-value < 0.05, N = 454) and *lean* vs *obese* ($q$-value < 0.05, N = 299). These correlations were compared with those corresponding to the set of genes with relevant post-transcriptional signals and putatively targeted by DE upregulated miRNAs. Normalized exonic and intronic estimates in the $\log_2$ scale obtained from EISA analyses were used to compute Spearman's correlation coefficients ($\rho$) for each potential pair of DE mRNA genes plus those with post-transcriptional signals but without significant DE. Self-correlation pairs were excluded. Significant correlations were identified with the Partial Correlation with Information Theory (PCIT) network inference algorithm [45] implemented in the *pcit* R package [46]. Non-significant covarying pairs were set to zero, while a value of 1 was assigned to the significant ones with both positive or negative coefficients $|\rho| > 0.6$.

The potential contribution of miRNAs to shape the observed covariation patterns was assessed by calculating a covariation enrichment score (CES) following Tarbier et al. 2020 [47]. If multiple genes are downregulated by any upregulated miRNAs in a coordinated manner, we would expect to observe a reduced abundance in their mature spliced mRNA forms but not in the corresponding primary transcripts, i.e., we would detect a covariation only for the exonic fractions (but not for the intronic ones). In other

words, the intronic fraction, eventually spliced and degraded in the nucleus, should not reflect any posterior post-transcriptional regulatory effects in the cytoplasm, so little or null covariation might be expected. Significant differences among the set of exonic, intronic and control CES values were tested with a non-parametric approach using a Mann-Whitney U non-parametric test [48]. Further details can be found in **Supplementary Methods**.

### 2.9. Estimating the expression levels of miRNAs and several of their predicted mRNA targets by qPCR

The same total RNA extracted from adipocytes and used for sequencing was subsequently employed for cDNA synthesis and qPCR verification. Five mRNAs (*LEP*, *OSBLP10*, *PRSS23*, *RNF157* and *SERPINE2*) among the top 5% negative PTc scores and showing at least 2-folds reduction in their ΔEx values were selected for qPCR profiling. Two reference genes (*TBP* and *ACTB*, as defined by Nygard et al. 2007 [49]) were used for normalization. Accordingly, three of the most DE miRNAs were selected for qPCR profiling (ssc-miR-92b-3p, ssc-miR-148a-3p and ssc-miR-214-3p), plus two highly expressed non-DE miRNAs for normalization (ssc-let-7a and ssc-miR-23a-3p) from the *lean* vs *obese* small RNA-Seq dataset. Further details about qPCR experimental procedures are available in **Supplementary Methods**. All primers for mRNA and miRNA expression profiling are available at **Table S2**. Raw Cq values for each assay are available at **Table S3**.

## 3. Results

### 3.1. The analysis of post-transcriptional regulation in muscle samples from fasting and fed Duroc gilts

*Identification of genes predicted to be post-transcriptionally regulated by miRNAs*

After the processing, mapping and quantification of mRNA and miRNA expression levels in GM skeletal muscle samples from Duroc gilts, an average of 45.2 million reads per sample (~93%) were successfully mapped to 31,908 genes annotated in the Sscrofa11.1 v.103 assembly (including protein coding and non-coding genes). Besides, an average of 2.2 million reads per sample (~42%) mapped to 370 annotated porcine miRNA genes.

A total of 30,322 (based on exonic reads) and 22,769 (based on intronic reads) genes were successfully quantified after splitting the reference Sscrofa11.1 v.103 assembly between exonic and intronic features. The exonic fraction displayed an average of 1,923.94 estimated counts per gene, whereas the intronic fraction showed an average of 83.02 counts per gene. In other words, exonic counts were ~23 fold more abundant than those corresponding to intronic regions.

Differential expression analyses based on exonic fractions identified 454 DE genes. Among those, only genes with |FC| > 2 were retained, making a total of 52 upregulated and 80 downregulated genes in the *AL-T0* vs *AL-T2* comparison (**Table S4, Fig. S2A**). Besides, differential expression analyses on small RNA-seq data revealed 16 DE miRNAs, of which 8 were upregulated in *AL-T2* pigs, representing 6 unique miRNA seeds (**Table S5**). These non-redundant seeds of significantly upregulated miRNAs in fed animals (ssc-miR-148a-3p, ssc-miR-7-5p, ssc-miR-30-3p, ssc-miR-151-3p, ssc-miR-374a-3p and ssc-miR-421-5p) were selected as potential post-transcriptional regulators of GM muscle mRNA expression in response to nutrient supply. EISA results made possible to detect 133 genes with significant effects (**Table S6**), of which three had > 2-

folds reduced ΔEx fractions and two of them had significantly negative PTc scores (**Table 1**).

To detect mRNA genes putatively downregulated by miRNAs at the post-transcriptional level, mRNA genes displaying the top 5% negative PTc scores with at least 2-folds ΔEx reduction were selected as putative miRNA-targets (**Fig. S2B**). With this approach, 26 mRNA genes were selected (**Table 1**). One of them (ENSSSCG00000049158) did not have a properly annotated 3´-UTR so it was excluded from further analyses. Among this set of genes with high post-transcriptional signals, 18 appeared as significantly downregulated (FC < -2; *q*-value < 0.05) in the differential expression analysis considering their exonic fractions (**Table 1** and **Table S4**).

**Table 1:** mRNA genes with the top 5% post-transcriptional (PTc) scores and at least 2-folds exonic fraction (ΔEx) reduction (equivalent to -1 in the $\log_2$ scale) from *gluteus medius* skeletal muscle expression profiles of fasting (*AL-T0*, N = 11) and fed (*AL-T2*, N = 12) Duroc gilts.

| ID | Gene | $\log_2$FC | ΔEx | $PT_c$ | *P*-value | *q*-value | DE | miRNA target |
|---|---|---|---|---|---|---|---|---|
| ENSSSCG00000032094 | *DKK2* | -2.010 | -1.431 | -4.738 | 1.654E-05 | 3.830E-03 | • | x |
| ENSSSCG00000015334 | *PDK4* | -2.108 | -5.250 | -4.698 | 4.693E-03 | 1.330E-01 | x | x |
| ENSSSCG00000015037 | *IL18* | -1.655 | -1.191 | -3.682 | 4.787E-03 | 1.340E-01 | • | x |
| ENSSSCG00000005385 | *NR4A3* | -1.337 | -3.082 | -3.646 | 4.038E-02 | 4.098E-01 | x | x |
| ENSSSCG00000003766 | *DNAJB4* | -1.391 | -1.008 | -3.348 | 8.358E-03 | 1.905E-01 | | x |
| ENSSSCG00000015969 | *CHRNA1* | -1.561 | -1.339 | -3.341 | 2.606E-03 | 9.406E-02 | x | x |
| ENSSSCG00000039419 | *SLCO4A1* | -1.055 | -2.279 | -3.180 | 2.820E-02 | 3.544E-01 | x | x |
| ENSSSCG00000049158 | | -1.107 | -1.096 | -3.164 | 3.182E-02 | 3.735E-01 | | x |
| ENSSSCG00000004347 | *FBXL4* | -1.298 | -1.126 | -3.133 | 1.422E-03 | 6.520E-02 | x | x |
| ENSSSCG00000004979 | *MYO9A* | -1.239 | -1.003 | -3.043 | 7.296E-03 | 1.731E-01 | | x |
| ENSSSCG00000013351 | *NAV2* | -1.163 | -1.196 | -2.863 | 2.605E-04 | 2.301E-02 | x | x |
| ENSSSCG00000032741 | *TBC1D9* | -0.913 | -1.061 | -2.736 | 1.534E-02 | 2.583E-01 | • | x |
| ENSSSCG00000031728 | *ABRA* | -1.238 | -1.393 | -2.704 | 1.295E-03 | 6.116E-02 | x | x |
| ENSSSCG00000006331 | *PBX1* | -0.891 | -1.039 | -2.480 | 1.135E-02 | 2.177E-01 | x | x |
| ENSSSCG00000035037 | *SIK1* | -1.357 | -1.289 | -2.475 | 3.999E-03 | 1.212E-01 | x | x |
| ENSSSCG00000038374 | *CIART* | -1.027 | -1.321 | -2.052 | 1.543E-02 | 2.587E-01 | x | |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| ENSSSCG00000023806 | *LRRN1* | -0.776 | -1.013 | -1.983 | 1.580E-01 | 7.074E-01 | | x |
| ENSSSCG00000009157 | *TET2* | -0.381 | -1.123 | -1.792 | 4.880E-01 | 9.582E-01 | | x |
| ENSSSCG00000011133 | *PFKFB3* | -0.022 | -2.256 | -1.785 | 9.712E-01 | 9.987E-01 | x | x |
| ENSSSCG00000002283 | *FUT8* | -0.578 | -1.286 | -1.784 | 9.887E-02 | 6.059E-01 | x | x |
| ENSSSCG00000023133 | *OSBPL6* | -0.432 | -1.088 | -1.772 | 3.835E-01 | 9.108E-01 | x | |
| ENSSSCG00000017986 | *NDEL1* | -0.767 | -1.644 | -1.759 | 1.006E-02 | 2.081E-01 | x | x |
| ENSSSCG00000031321 | *NR4A1* | -0.630 | -1.328 | -1.720 | 6.298E-02 | 5.006E-01 | x | |
| ENSSSCG00000035101 | *KLF5* | -0.519 | -1.487 | -1.708 | 2.942E-01 | 8.488E-01 | x | x |
| ENSSSCG00000004332 | *BACH2* | -0.714 | -2.105 | -1.705 | 9.089E-02 | 5.861E-01 | x | x |
| ENSSSCG00000017983 | *PER1* | -0.773 | -1.073 | -1.627 | 3.000E-02 | 3.662E-01 | x | |

[a]$Log_2FC$: estimated $log_2$ fold change for mean exonic fractions from *gluteus medius* expression profiles of fasted *AL-T0* and fed *AL-T2* Duroc gilts; [b]ΔEx: exonic fraction increment ($Ex_2 – Ex_1$) when comparing exon abundances in *AL-T0* ($Ex_1$) vs *AL-T2* ($Ex_2$) Duroc gilts; [c]PTc: post-transcriptional signal (ΔEx – ΔInt) in z-score scale; [d]q-value: q-value calculated with the false discovery rate (FDR) approach [39]. The "x" symbols represent differentially expressed (DE) genes (FC < -2; *q*-value < 0.05) according to their exonic fractions, as well as those targeted by at least one of the upregulated miRNAs excluding redundant seeds (N = 6, **Table S5**). The "•" symbol represents suggestive differential expression (*P*-value < 0.01, **Table S4**).

*Context-based pruning of predicted miRNA-mRNA interactions removes spurious unreliable target events*

As a first step to determine if genes with highly negative PTc scores and showing a marked reduction in exonic fractions were repressed by upregulated DE miRNAs (**Table S5**), we investigated the accuracy and reliability of *in silico* predictions regarding miRNA binding sites in their 3'-UTRs (**Table S7**). We evaluated the presence of enriched binding sites over a random background of expressed genes with no context-based removal of predicted binding sites, applying each one of the three selection criteria reported in Methods independently, as well as by combining them pairwise or altogether. As depicted in **Fig. S3A** and **S3B**, introducing additional context-based filtering criteria for removing spurious unreliable binding site predictions resulted in an overall increased enrichment of miRNA targeted genes within the top 1% (**Fig. S3A**) and 5% (**Fig. S3B**) negative PTc

scores and displaying > 2-folds ΔEx reduction. This significantly increased enrichment was more evident when using the AU criterion, as shown in **Fig. S3A**. However, we also detected a slight increment when adding the other two context-based removal criteria (M and E). These findings were less evident when taking into consideration the list of the top 5% genes (**Fig. S3B**). Nevertheless, an increased enrichment for targeted mRNAs by DE upregulated miRNAs was detectable for all combined filtering criteria, especially for 7mer-A1 binding sites, and probably at the expense of the scarcer and more efficient 8mer binding sites. Based on these results, we decided to apply the three joint criteria (AU, M and E) for enrichment analyses between the set of mRNA genes with high post-transcriptional signals and putatively targeted by miRNAs.

*Genes with relevant post-transcriptional signals are enriched for putative miRNA binding sites in their 3'-UTRs*

Target prediction and context-based pruning of miRNA-mRNA interactions for mRNA genes displaying the top 5% negative PTc scores and at least 2-folds reductions in the ΔEx exonic fraction (N = 25 after excluding ENSSSCG00000049158; **Fig. 1A**) made possible to detect 11 8mer, 21 7mer-m8 and 22 7mer-A1 miRNA binding sites (**Table S7**) corresponding to the 6 non-redundant seeds of DE miRNAs upregulated in *AL-T2* gilts. This set of 25 mRNA genes showing putative post-transcriptional repression by miRNAs showed a significant enrichment in 8mer, 7mer-m8 and 7mer-A1 sites (**Fig. 1B**), and this was especially relevant when combining these three types of binding sites. More importantly, 21 out of these 25 genes were predicted as putative targets of miRNAs upregulated in the GM muscle samples from *AL-T2* fed gilts (**Table 1** and **Table S5**).

We also evaluated the enrichment of the mRNA genes with the top 5% negative PTc scores and at least 2-folds ΔEx reduction (N = 25, **Table 1**) to be targeted by the following

sets of miRNAs: (i) Non-redundant miRNAs downregulated in *AL-T2* fed gilts (ssc-miR-1285, ssc-miR-758, ssc-miR-339, sc-miR-22-3p, ssc-miR-296-5p, ssc-miR-129a-3p, ssc-miR-181c and ssc-miR-19b, **Table S5**), (ii) the top 5% most expressed miRNAs, excluding those being upregulated (ssc-miR-1, ssc-miR-133a-3p, ssc-miR-26a, ssc-miR-10b, ssc-miR-378, ssc-miR-99a-5p, ssc-miR-27b-3p, ssc-miR-30d, ssc-miR-486 and ssc-let-7f-5p), and (iii) for an iteration (N = 100) of random sets of 10 expressed miRNAs, irrespective of their DE and abundance status, as a control test. None of these additional analyses recovered a significant enrichment for any type of the three considered miRNA target subtypes (**Fig. 1B**).

The mRNA with the highest and significant PTc score was the Dickkopf WNT Signaling Pathway Inhibitor 2 (*DKK2*), and thus, this was the gene with the highest putative miRNA-driven downregulation and no additional transcriptional influence (**Table 1**). Moreover, the DKK2 locus was the only gene harboring two miRNA 8mer binding sites (**Table S7**), although it was not among the most highly DE mRNA genes according to differential expression analyses (**Table S4**). As depicted in **Table 1**, only suggestive significant differences ($P$-value < 0.01) in mean expression were found for *DKK2* transcripts. Besides, among the set of mRNA genes displaying the top 5% PTc scores and at least 2-folds ΔEx reduction (**Table 1**), 5 were not DE, and 3 of them only showed suggestive DE, while the rest of DE mRNAs displayed a wide range of significance (**Table S4**). The divergence between EISA and differential expression results is also shown in **Fig. 1C**, where not all the mRNA genes highlighted by EISA (**Fig. 1A**) were DE downregulated loci.

The miRNAs with the highest number of significant miRNA-mRNA interactions (in terms of correlations between their expression levels) were ssc-miR-30a-3p and ssc-miR-421-5p, which showed 9 and 8 significant miRNA-mRNA interactions, followed by ssc-

miR-148-3p with 4 significant interactions with mRNA genes showing significant post-transcriptional signals (**Table S7**).

*Genes showing post-transcriptional regulatory signals predominantly covary at the exonic level*

To further elucidate whether genes displaying the top 5% PTc scores are strong candidates to be regulated by miRNAs according to *in silico* predictions (N = 21), we evaluated the covariation patterns among them and with the whole set of 454 mRNA genes DE between *AL-T0* and *AL-T2* gilts.

By calculating CES values (see Methods) for the 21 genes putatively targeted by DE upregulated miRNAs, we obtained an estimation of the fold change in their observed covariation with respect to other 435 DE mRNAs. CEs values were measured for both their exonic and intronic fractions. Our analyses revealed that 19 out of these 21 genes showed an increased covariation of approximately 2-folds in their exonic fractions when compared to their intronic fractions (**Table S8**, **Fig. 1D**). When we iteratively analyzed the observed fold change in covariation for random sets of genes (N = 1,000), they displayed CES ≈ 1, indicative of no covariation (**Fig. 1D**). The observed CES distributions of exonic, intronic and control sets were significantly different (*P*-value = 3.663E-06) after running non-parametric tests (**Fig. 1D**), thus supporting that the genes displaying the top 5% PTc scores are probably repressed by DE upregulated miRNAs.

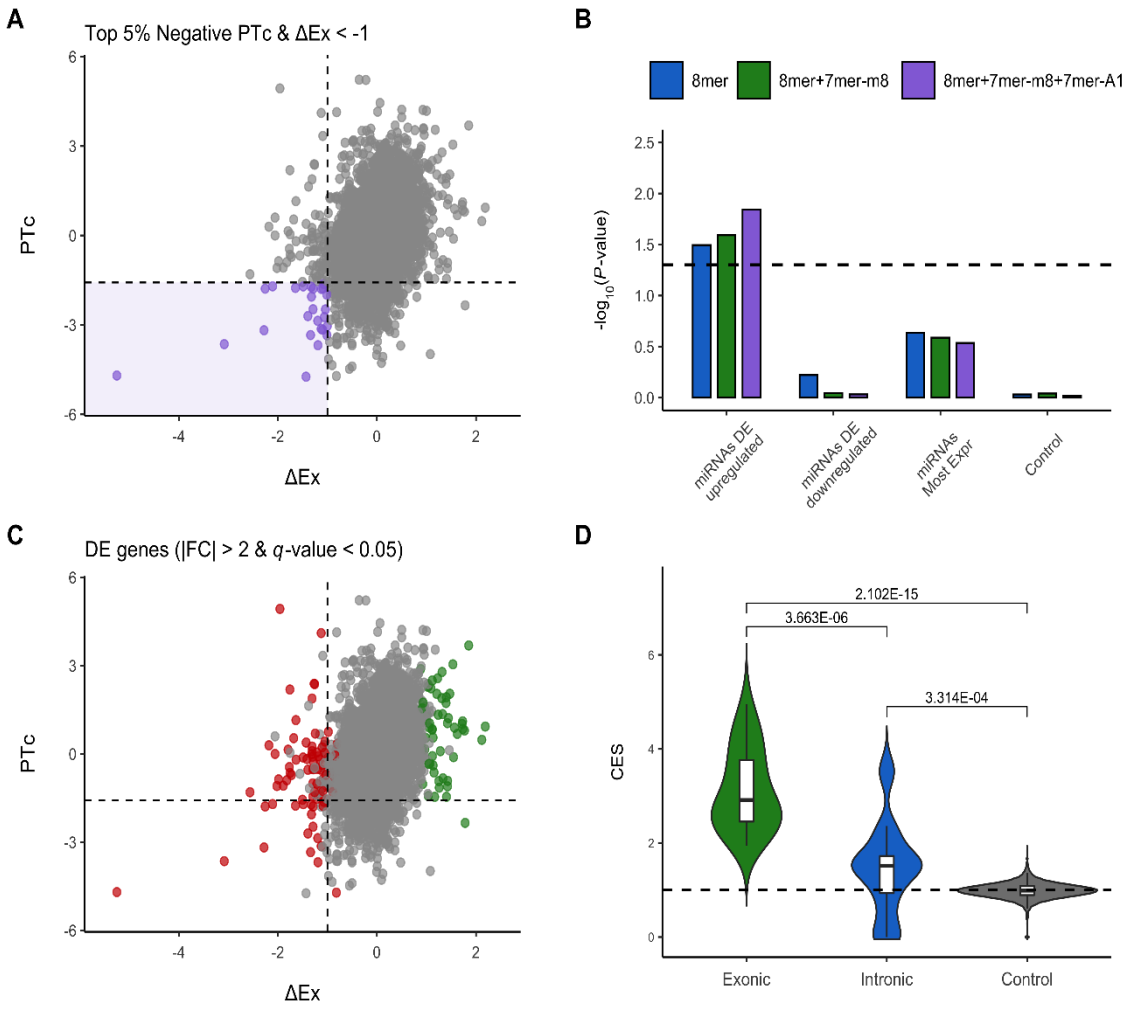


**Figure 1:** (**A**) Scatterplot depicting mRNA genes with the top 5% negative PTc scores and at least 2-folds ΔEx reduction according to exonic (ΔEx) and PTc (ΔEx – ΔInt) values (in purple) and putatively targeted by DE upregulated miRNAs (FC > 1.5; *q*-value < 0.05) from *gluteus medius* skeletal muscle expression profiles of fasted (*AL-T0*, N = 11) and fed (*AL-T2*, N = 12) Duroc gilts. (**B**) Enrichment analyses of the number of mRNA genes with the top 5% negative PTc scores and at least 2-folds ΔEx reduction putatively targeted by DE upregulated miRNAs (FC > 1.5; *q*-value < 0.05), DE downregulated miRNAs (FC < -1.5; *q*-value < 0.05) and the top 5% most highly expressed miRNAs, excluding DE upregulated miRNAs. (**C**) Scatterplot depicting DE upregulated (in green) and downregulated (in red) mRNA genes (|FC| > 2; *q*-value < 0.05) according to exonic (ΔEx)

and PTc (ΔEx – ΔInt) values. (**D**) Covariation enrichment scores (CES) for the exonic and intronic fractions of mRNA genes with the top 5% negative PTc scores and at least 2-folds ΔEx reduction, putatively targeted by upregulated miRNAs from *gluteus medius* skeletal muscle expression profiles of fasted (*AL-T0*, N = 11) and fed (*AL-T2*, N = 12) Duroc gilts. The control set of CES values were generated by permuted (N = 1,000) random sets of exonic and intronic profiles of genes with the same size as those used before (N = 21). Significant differences were assessed using a Mann-Whitney U non-parametric test [48].

### 3.2. Studying post-transcriptional signals in fat metabolism using an independent Duroc-Göttingen minipig population

After pre-processing and filtering of sequenced reads from adipocytes samples, we were able to retrieve ~98.1 and ~0.87 million mRNA and small RNA reads per sample, and ~96.5% and ~73.4% of these reads mapped to annotated porcine mRNA and miRNA genes, respectively. Differential expression analyses revealed a total of 299 DE mRNAs, of which 52 were downregulated and 95 were upregulated, respectively (**Table S9**). Regarding miRNAs, only one gene (ssc-miR-92b-3p) was significantly upregulated in *lean* pigs, while 7 additional miRNAs showed suggestive differential expression (*P*-value < 0.01), of which 4 were downregulated, and one (ssc-miR-92a) had the same seed sequence as ssc-miR-92b-3p (**Table S10**).

After running EISA on the mRNA expression profiles for exonic and intronic fractions, a total of 44 downregulated mRNAs in *lean* pigs displayed the top 5% PTc scores with reduced ΔEx > 2-folds (**Table S11, Fig. 2A**). One of them (ENSSSCG00000016928) did not have a properly annotated 3´-UTR so it was excluded from further analyses. The

whole set of mRNA genes from EISA results is available at **Table S12**. Among this set of genes with high post-transcriptional signals, 13 appeared as significantly downregulated (FC < -2; *q*-value < 0.05) and 9 had suggestive repression (*P*-value < 0.01) in the differential expression analysis considering their exonic fractions (**Table S9** and **S11**). In this dataset, the sestrin 3 (*SESN3*) locus showed the second highest negative PTc score, but was the only one among the rest of highlighted genes where a significant post-transcriptional signal was observed (**Table S11**), i.e., equivalent to the *DKK2* gene in Duroc pigs, this was the only locus showing post-transcriptional regulation with no additional co-occurring transcriptional signal. Such strong downregulation was also evidenced in differential expression analyses, where it was the most significantly DE gene (**Table S9**).

From the set of downregulated genes, 25 out of the 43 mRNA genes analyzed for miRNA binding sites were classified as putative targets of the set of miRNAs upregulated in *lean* pigs (ssc-miR-92b-3p, ssc-miR-148a-3p, ssc-miR-204 and ssc-miR-214-3p; **Table S10**). Target prediction and context-based pruning of miRNA-mRNA interactions for these 43 mRNA genes made it possible to detect 8 8mer, 21 7mer-m8 and 24 7mer-A1 miRNA binding sites (**Table S13**) corresponding to the non-redundant seeds of selected upregulated miRNAs (N = 4) in *lean* minipigs (**Table S10**). The *SESN3* gene showed the highest number of predicted putative miRNA target sites in its 3´-UTR (**Table S13**).

Enrichment analyses for the set of putative miRNA target genes with the top 5% negative PTc scores and at least 2-folds ΔEx reduction (N = 25, **Table S11**) revealed no significant enrichment for the three types of miRNA target sites considered, although a slight increase when considering 8mer + 7mer-m8 binding sites and all three types together could be observed (**Fig. 2B**). Among this set of 25 genes, 7 appeared as significantly downregulated (FC < -2; *q*-value < 0.05) and 6 had suggestive repression (*P*-value < 0.01)

in the differential expression analysis considering their exonic fractions (**Table S9** and **S11**, **Fig. 2C**).

In agreement with results obtained for the first studied skeletal muscle expression dataset, the exonic fraction of the mRNA genes putatively targeted by upregulated miRNAs in lean pigs showed approximately 2-folds significantly increased covariation (*P*-value = 2.703E-02) with regard to their intronic fraction (**Fig. 2D**). Besides, 18 out of these 25 mRNA genes showed an overall increased covariation in their exonic fractions compared with their intronic fractions, expressed as the increment in their CES values (ΔCES = exonic CES – intronic CES, **Table S14**).

Because adipose tissue samples were available, qPCR analyses were performed to assess whether mRNAs among the top 5% negative PTc scores and with at least 2-folds ΔEx reduction together with upregulated miRNAs displayed patterns of expression consistent with those obtained in the RNA-seq and small RNA-seq experiments. To this end, we selected five mRNAs (*LEP*, *OSBPL10*, *PRSS23*, *RNF157* and *SERPINE2*) and 3 miRNAs (ssc-miR-148a-30, ssc-miR-214-3p and ssc-miR-92b-3p) for qPCR verification. All the analyzed mRNA genes showed a reduced expression in *lean* pigs compared with their *obese* counterparts (**Fig. 2E**) and the *LEP* gene was the most significantly downregulated gene ($\log_2$FC = -1.953; *P*-value = 1.120E-03). This result was in agreement with the strong downregulation observed in differential expression analyses based on RNA-Seq data (($\log_2$FC = -1.957; *q*-value = 3.443E-03, **Table S9**). With regard to miRNAs, the opposite pattern of expression was observed, with all the three profiled miRNA genes being upregulated in *lean* pigs. Moreover, as reported in **Table S10**, ssc-miR-92b-3p was the miRNA with the most significant upregulation as evidenced in qPCR analyses (*P*-value = 3.57E-02, **Fig. 2F**).

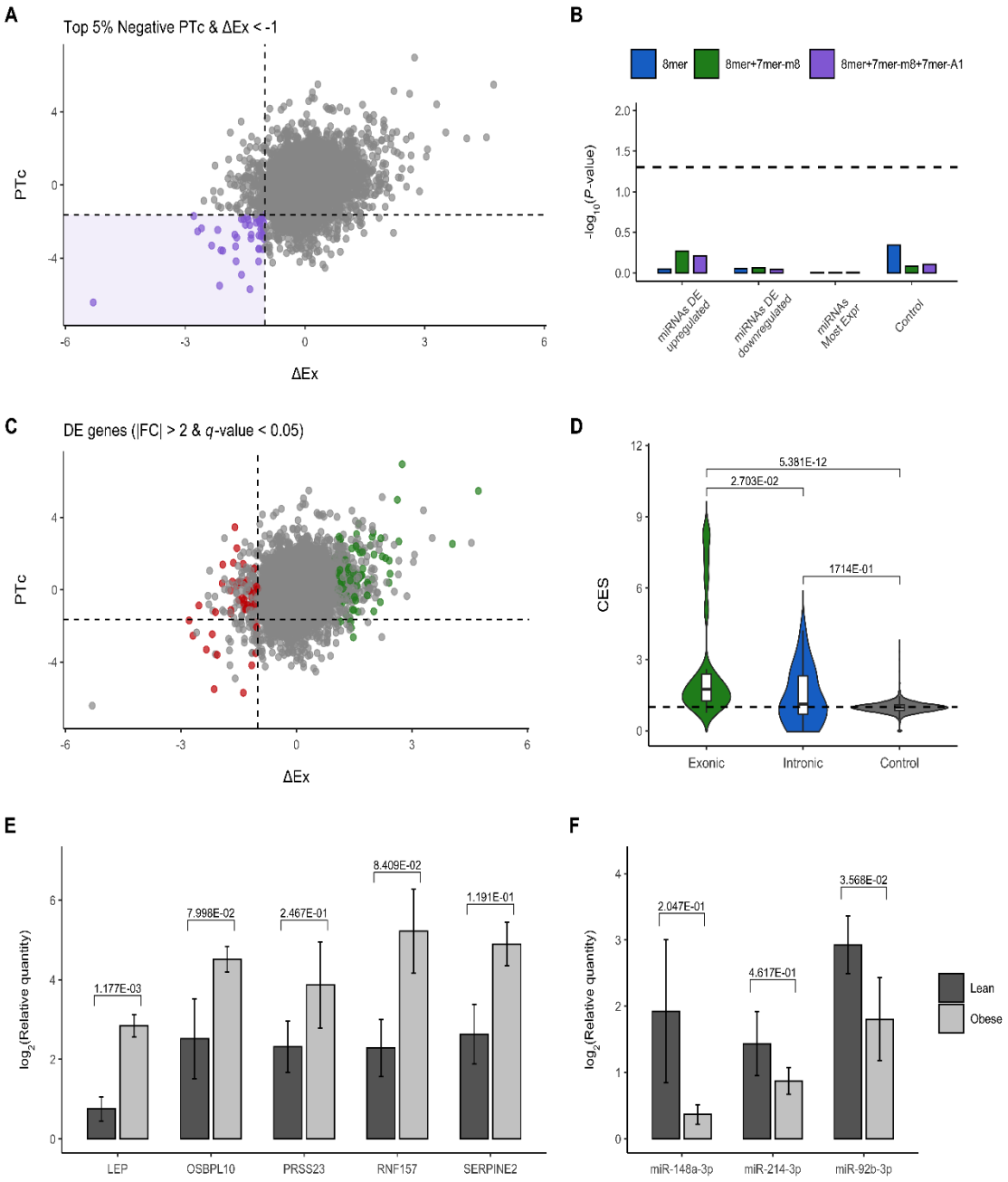


**Figure 2:** (**A**) Scatterplot depicting mRNA genes with the top 5% negative PTc scores and at least 2-folds ΔEx reduction according to exonic (ΔEx) and PTc (ΔEx – ΔInt) values (in purple) and putatively targeted by upregulated miRNAs (FC > 1.5; *P*-value < 0.01) from adipocyte expression profiles of *lean* (N = 5) and *obese* (N = 5) Duroc-Göttingen minipigs according to their body mass index (BMI). (**B**) Enrichment analyses of the number of mRNA genes with the top 5% negative PTc scores and at least 2-folds ΔEx

reduction putatively targeted by DE upregulated miRNAs (FC > 1.5; *q*-value < 0.05), DE downregulated miRNAs (FC < -1.5; *q*-value < 0.05) and the top 5% most highly expressed miRNAs, excluding DE upregulated miRNAs. (**C**) Scatterplot depicting DE upregulated (in green) and downregulated (in red) mRNA genes (|FC| > 2; *q*-value < 0.05) according to exonic (ΔEx) and PTc (ΔEx – ΔInt) values. (**D**) Covariation enrichment scores (CES) for the exonic and intronic fractions of mRNA genes with the top 5% negative PTc scores and at least 2-folds ΔEx reduction, putatively targeted by upregulated miRNAs from adipocyte expression profiles of *lean* (N = 5) and *obese* (N = 5) Duroc-Göttingen minipigs. The control set of CES values were generated by permuted (N = 1,000) random sets of exonic and intronic profiles of genes with the same size as those used before (N = 25). Significant differences were assessed using a Mann-Whitney U non-parametric test [48]. (**E**) Barplots depicting qPCR $\log_2$ transformed relative quantities (Rq) for *LEP*, *OSBPL10*, *PRSS23*, *RNF157* and *SERPINE2* mRNA transcripts measured in adipocytes from the retroperitoneal fat of *lean* (N = 5) and *obese* (N = 5) Duroc-Göttingen minipigs. (**F**) Barplots depicting qPCR $\log_2$ transformed relative quantities (Rq) for ssc-miR-148a-3p, ssc-miR-214-3p and ssc-miR-92b-3p miRNA transcripts measured in the isolated adipocytes from the retroperitoneal fat of *lean* (N = 5) and *obese* (N = 5) Duroc-Göttingen minipigs.

# 4. Discussion

In this study, we undertook the inference of functional miRNA-mRNA interactions using EISA for post-transcriptional signals prioritization, combined with target prediction of upregulated miRNAs, as well as enrichment and covariation analyses.

We observed that the majority of mRNA genes with highly negative PTc scores, i.e., predominantly downregulated at their exonic fractions, also had a coordinated downregulatory effect of their intronic fractions, taken as a proxy of transcriptional repression. This was evidenced by the overall low significance of post-transcriptional signals within the mRNA genes with the top 5% negative PTc scores and reduced ΔEx in both analyzed experimental conditions. Only two genes (*DKK2* and *NAV2*) in *AL-T0* vs *AL-T2* and one gene (*SESN3*) in the *lean* vs *obese* contrasts showed significant PTc scores, revealing that, overall, a coordinated downregulatory effect at both transcriptional and post-transcriptional levels was present, which is in agreement with previous studies using EISA [14,15]. However, it is worth noting that we did not consider the significance of PTc scores as a relevant criterion for prioritizing putative post-transcriptionally downregulated genes, as these will appear as significant when the post-transcriptional activity is the only mechanism modulating the target gene expression profile. Only co-occurring yet opposite transcriptional and post-transcriptional events or single post-transcriptional signals would arise as significant, excluding genes with both coordinated downregulation at the transcriptional and post-transcriptional level.

We decided to use the intronic fraction of expressed mRNAs as a proxy of their transcriptional activity, a source of yet unspliced mRNA transcripts leading to the accumulation of intronic sequences prior to their debranching and degradation by exonucleases. This allows the use of RNA-seq datasets to apply EISA without the need of further experimental procedures, and it can also be applied to investigate transcriptional regulatory signals [15]. Previous reports have also explored the use of specific techniques to capture nascent mRNA transcripts before they are spliced [50–52], and these have been used to account for the transcriptional activity in a similar approach to EISA [53]. Although more advanced methodologies measuring nascent transcription of mRNAs

might provide a better resolution for future experimental designs [54], EISA would still be useful to explore the large amount of available RNA-seq data where additional experiments are no longer possible.

Since the efficacy of miRNA-based regulation on mRNA targets depends on the context of the target site within the 3'-UTR [43], we have described the usefulness of introducing context-based filtering criteria for removing spurious *in silico*-predicted target sites for miRNAs. Using enrichment analyses, we were able to link the downregulated mRNAs at their exonic fractions to upregulated miRNAs that were putatively targeting them and triggering their observed decay in differential expression analyses. The influence of other non-DE highly expressed miRNAs or downregulated miRNAs was discarded by the lack of predicted targeted mRNA genes with high post-transcriptional downregulatory signals for such miRNAs. Overall, the increase in enrichment significance shown for targeted mRNAs with post-transcriptional signals by upregulated miRNAs revealed the ability of context-based filtering criteria to discriminate and remove weak or false positive target sites located within unfavored regions of the 3'-UTR. However, highly efficient target sites such as those of type 8mer, although scarcer than 7mer-m8 sites, might still be functional even at unfavored positions [43,55,56]. This may partially explain the relative lack of 8mer sites found in the top post-transcriptionally regulated mRNA genes in both experimental setups.

Although miRNAs were the major post-transcriptional regulators that we considered in this study, it is important to remark that other additional post-transcriptional modifications and interactions might be responsible for the observed downregulation of mRNAs. For instance, long non-coding RNAs [57], circular RNAs [58,59], RNA methylation [60] or RNA binding proteins [61–63] can all act as post-transcriptional regulators of targeted mRNAs. We further hypothesized that genes showing relevant post-

transcriptional downregulatory effects might be regulated by the same set of significantly upregulated miRNAs, which could induce shared covariation in their expression profiles at the exonic level. On the contrary, their intronic fractions would be mainly unaffected, as introns would have been excised prior to any given miRNA-driven downregulation, if occurring. In this way, an increased gene covariation might be detectable within the sets of commonly targeted mRNA genes with relevant post-transcriptional signals at the exon but not at the intron level, as opposed to covariation events of these set of genes with the rest of DE genes. Our results revealed an increased degree of covariation between genes with high post-transcriptional signals at their exonic fractions, highlighting a putative coordinated downregulation by the set of significantly upregulated miRNAs.

From the analysis of top mRNA genes showing the strongest post-transcriptional downregulatory effects in fasted vs fed gilts, several biological functions putatively regulated by miRNAs were revealed. The *DKK2* gene was the one showing the highest negative PTc score, and its post-transcriptional regulatory signal was also significant, indicating that no additional coordinated transcriptional downregulation was found for this particular gene. Moreover, this gene also showed the strongest covariation difference in its exonic fraction compared with its intronic fraction. This consistent post-transcriptional regulatory effect might be explained by the presence of two miRNA target sites of type 8mer in its 3´-UTR for ssc-miR-421-5p and ssc-miR-30a-3p, two highly DE and upregulated miRNAs. Besides, ssc-miR-30e-3p, a miRNA sharing its seed and regulatory effect with ssc-miR-30a-3p, was also upregulated in fed (*AL-T2*) gilts, which would reinforce the repression of their targeted mRNA transcripts. The *DKK2* gene is a member of the dickkopf family that inhibits the Wnt signaling pathway through its interaction with the LDL-receptor related protein 6 (LRP6). Its repression has been associated with reduced blood-glucose levels and improved glucose uptake [64], as well

as with improved adipogenesis [65] and inhibition of aerobic glycolysis [66]. These results agreed with the increased glucose usage and triggered adipogenesis in muscle tissue after nutrient supply. Other additional relevant post-transcriptionally downregulated mRNA genes found by EISA were: (i) pyruvate dehydrogenase kinase 4 (*PDK4*), a mitochondrial enzyme that inhibits pyruvate to acetyl-CoA conversion and hinders glucose utilization promoting fatty acids oxidation in energy-deprived cells under fasting conditions [67,68]; (ii) interleukin 18 (*IL18*), involved in controlling energy homeostasis in the muscle by inducing AMP-activated protein kinase (AMPK) [69], a master metabolic regulator that is suppressed upon nutrient influx in cells [70]; (iii) nuclear receptor subfamily 4 group A member 3 (*NR4A3*), which activates both glycolytic and glycogenic factors [71], as well as β-oxidation in muscle cells [72]; (iv) acetylcholine receptor subunit α (*CHRNA1*) of muscle cells, that is linked to the inhibition of nicotine-dependent *STAT3* upregulation [73] that results in protection against insulin resistance in muscle [74]; (v) PBX homeobox 1 (*PBX1*), a regulator of adipocyte differentiation [75]; (vi) Tet methylcytosine dioxygenase 2 (*TET2*), linked to glucose-dependent AMPK phosphorylation [76]; and (vii) BTB domain and CNC homolog (*BACH2*), associated with mTOR complex 2 (mTORC2) glucose-dependent activation [77,78] and the repression of forkhead box protein O1 (*FOXO1*) [79] and *PDK4* in a coordinated manner [7,80]. Overall, the highlighted downregulated genes in the muscle of gilts after nutrient supply pointed towards a common regulatory function of miRNAs in modulating glucose uptake and energy homeostasis pathways of the skeletal myocytes.

Alternative post-transcriptional regulatory factors other than miRNAs might explain the presence of non-miRNA targets within the top post-transcriptional signals related to regulatory mechanisms not directly involved in energy homeostasis or glucose usage. For instance, three circadian clock-related mRNA genes that showed high post-transcriptional

signals were the circadian associated repressor of transcription (*CIART*), period 1 (*PER1*) and salt inducible kinase 1 (*SIK1*), yet the first two were not detected as targets of DE miRNAs. As previously reported for this experimental design [22], the presence of several genes showing abundance differences might reflect a tight feedback interplay among them, where their expression and accumulation are coordinately regulated.

Regarding EISA results in RNA-seq profiles of adipocytes from *lean* vs *obese* Duroc-Göttingen minipigs, several of the mRNA genes that showed high post-transcriptional repression are involved in the regulation of lipid metabolism and energy homeostasis. The gene showing the highest post-transcriptional signal was the estrogen related receptor γ (*ESRRG*), which modulates oxidative metabolism and mitochondrial function in adipose tissue and imposes downregulation of adipocyte differentiation when repressed [81]. The second locus highlighted by EISA was sestrin 3 (*SESN3*), an activator of mTORC2 and PI3K/AKT signaling pathway [82] that protects against insulin resistance and promotes lipolysis when inhibited [83]. This gene showed the most significant downregulation in *lean* pigs, and gathered multiple putative binding sites for all the four upregulated miRNAs analyzed. Other genes showing significant post-transcriptional regulation were the following: The sterile α motif domain containing 4A (*SAMD4A*) that has been linked to the inhibition of preadipocyte differentiation and leanness phenotype in knockdown experiments [84,85]. The prostaglandin F2- receptor protein (PTGFR), for which overexpression has been associated with hypertension and obesity risk [86], and its repression appears to improve insulin sensitivity and glucose homeostasis [87]. The expression of serpin E1 and E2 (*SERPINE1*, *SERPINE2*) is linked to obesogenic states and diabetic symptoms [88], while their inhibition improved glucose metabolism [89]. The serine protease 23 (PRSS23), which regulates insulin sensitivity and cytokine expression in adipose tissue, and its downregulation confers protective effects against

inflammation and reduced fasting glucose level improving insulin resistance [90]. A high expression of ring finger protein 157 (*RNF157*) has been linked to high fatness profiles and increased autophagy in adipose tissue [91]. Silencing of ORP10 protein, encoded by the *OSBLP10* gene, promotes low-density lipoprotein (LDL) synthesis and inhibits lipogenesis [92]. The serum levels of glycosylphosphatidylinositol phospholipase 1 (GPLD1) are regulated by insulin and glucose metabolism [93] and linked to the development of insulin resistance and metabolic syndrome [94]. Overexpression of neutrophil cytosolic 2 (*NCF2*), the gene showing the highest increase in covariation at the exonic fraction, was described in obese humans [95]. The repression of RAP1 GTPase activating protein (RAP1GAP) promotes RAP1 activity, which protects against obesity and insulin and glucose resistance [96,97]. Finally, leptin production was also decreased in lean pigs. This key adipokine is mainly produced in adipose tissue [98] and regulates appetite, energy expenditure and body weight [99,100]. In summary, similar to what we found for glucose metabolism and energy homeostasis in the first experimental population, we were also able to describe a set of post-transcriptionally downregulated genes in Duroc-Göttingen minipigs tightly related to adipose tissue metabolism regulation.

# 5. Conclusions

In this study we have implemented an exon/intron split analysis of RNA-seq data from skeletal muscle and adipose tissue of pigs in two independent populations. In this way, we were able to identify a set of genes putatively regulated by miRNAs at the post-transcriptional level. Many of these genes were involved in the regulation of energy homeostasis and development of both skeletal muscle and adipose tissues analyzed in the

present study, which were in agreement with metabolic modifications in response to nutrient supply and the fatness profile of pigs. Overall, the use of EISA for post-transcriptional signals prioritization combined with target prediction of upregulated miRNAs, as well as enrichment analyses on the set of selected targeted genes and their coordinated covariation, reinforced the usefulness of this approach to infer functional miRNA-mRNA interactions using exonic and intronic fractions of commonly available RNA-seq datasets.

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**Data availability**

The RNA-seq and small RNA-seq datasets from skeletal muscle tissue used in the current study are available at the Sequence Read Archive (SRA) database with BioProject codes PRJNA386796 and PRJNA595998, respectively. For the adipose tissue samples, RNA-seq and small RNA-seq datasets are available at PRJNA563583 and PRJNA759240.

**Conflict of interest**

The authors declare no conflict of interest.

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# Supplementary Tables

**Table S1:** Phenotype values for selected Duroc-Göttingen minipigs from the F2-UNIK resource population according to their body mass indexes.

**Table S2:** Primers for qPCR validation of selected mRNA and miRNA genes according to EISA results in the F2-UNIK Duroc-Göttingen minipig population comparing *lean* (N = 5) and *obese* (N = 5) individuals.

**Table S3:** Raw Cq values from qPCR analyses after efficiency correction measuring adipocyte expression profiles of selected mRNAs and miRNAs from *lean* (N = 5) and *obese* (N = 5) Duroc-Göttingen minipigs.

**Table S4:** Genes detected by *edgeR* tool as differentially expressed when comparing *gluteus medius* expression profiles of fasted *AL-T0* (N = 11) and fed *AL-T2* (N = 12) Duroc gilts.

**Table S5:** microRNAs detected by *edgeR* tool as differentially expressed when comparing *gluteus medius* expression profiles of fasted *AL-T0* (N = 11) and fed *AL-T2* (N = 12) Duroc gilts.

**Table S6:** EISA results for post-transcriptional signals (PTc) detected in *gluteus medius* skeletal muscle expression profiles of fasted (*AL-T0*, N = 11) and fed (*AL-T2*, N = 12) Duroc gilts.

**Table S7:** Binding sites for differentially upregulated miRNAs found in mRNA genes with the top 5% negative $PT_c$ scores and at least 2-folds reduction in the exonic fraction (ΔEx) of *gluteus medius* skeletal muscle expression profiles from fasting (*AL-T0*, N = 11) and fed (*AL-T2*, N = 12) Duroc gilts.

**Table S8:** Covariation enrichment scores (CES) for the exonic and intronic fractions of mRNA genes with the top 5% negative post-transcriptional signals (PTc) and at least 2-folds reduction in their exonic (ΔEx) fraction, that were putatively targeted by DE upregulated miRNAs from *gluteus medius* skeletal muscle expression profiles *AL-T0 vs AL-T2* Duroc gilts.

**Table S9:** Genes detected by *edgeR* tool as differentially expressed when comparing adipocyte expression profiles from *lean* (N = 5) and *obese* (N = 5) Duroc-Göttingen minipigs according to their body mass index.

**Table S10:** microRNA genes detected by *edgeR* tool as differentially expressed when comparing adipocyte expression profiles from *lean* (N = 5) and *obese* (N = 5) Duroc-Göttingen minipigs according to their body mass index.

**Table S11:** mRNA genes with the top 5% post-transcriptional signals (PTc) and at least 2-fold exonic fraction (ΔEx) reduction (equivalent to -1 in the log2 scale) from adipocyte expression profiles of *lean* (N = 5) and *obese* (N = 5) Duroc-Göttingen minipigs according to their body mass index.

**Table S12:** EISA results for post-transcriptional signals (PTc) detected in adipocyte expression profiles of *lean* (N = 5) and *obese* (N = 5) Duroc-Göttingen minipigs according to their body mass index.

**Table S13:** Binding sites for differentially upregulated miRNAs found in mRNA genes with the top 5% negative PTc scores and at least 2-folds reduction in the exonic fraction (ΔEx) of adipocyte expression profiles from *lean* (N = 5) and *obese* (N = 5) Duroc-Göttingen minipigs according to their body mass index.

**Table S14:** Covariation enrichment scores (CES) for the exonic and intronic fractions of mRNA genes with the top 5% negative post-transcriptional signals (PTc) and at least 2-folds reduction in their exonic (ΔEx) fraction, that were putatively targeted by DE

upregulated miRNAs from adipocyte expression profiles of *lean* vs *obese* Duroc-Göttingen minipigs.

# Supplementary Figures

**Figure S1:** Diagram depicting the consecutive steps implemented for studying miRNA-driven post-transcriptional regulatory signals applying the EISA approach and additional enrichment and covariation analyses.

**Figure S2:** Scatterplots depicting the exonic (ΔEx) and intronic (ΔInt) fractions from *gluteus medius* skeletal muscle expression profiles of fasting (*AL-T0*, N = 11) and fed (*AL-T2*, N = 12) Duroc gilts. (**A**) mRNA genes with the top 5% post-transcriptional (PTc) negative scores and at least 2-folds reduced exonic (ΔEx) fractions (equivalent to -1 in the $\log_2$ scale), suggestive of miRNA-driven post-transcriptional regulation. (**B**) mRNA genes differentially expressed showing upregulation (FC > 2; *q*-value < 0.05, in green) and downregulation (FC < -2, *q*-value < 0.05, in red) in fed (*AL-T2*, N = 12) Duroc gilts with respect to their fasted (*AL-T0*, N = 11) counterparts.

**Figure S3:** Enrichment analyses of the number of genes with the (**A**) top 1% and (**B**) top 5% negative PTc scores and at least 2-fold reduced exonic fractions (ΔEx) putatively targeted by upregulated miRNAs (FC > 1.5; *q*-value < 0.05) from *gluteus medius* skeletal muscle expression profiles of fasting (*AL-T0*, N = 11) and fed (*AL-T2*, N = 12) Duroc gilts. Results show the change in enrichment significance when incorporating context-based pruning of miRNA binding sites of type 8mer, 7mer-m8 and 7mer-A1. R: Raw enrichment analyses without any additional context-based pruning. AU: Enrichment

analyses removing miRNA binding sites without AU-rich flanking sequences (30 nts upstream and downstream). M: Enrichment analyses removing miRNA binding sites located in the middle of the 3'-UTR sequence (45-55%). E: Enrichment analyses removing miRNA binding sites located too close (< 15 nts) to the beginning or the end of the 3'-UTR sequences. The black dashed line represents a *P*-value = 0.05.