**Integrated regulatory network reveals novel candidate regulators in the development of negative energy balance in cattle**

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**Supplementary Material S6** *An overview of the integrated regulatory modules. Each module is discussed separately below.*

**Module zero**

Module zero is composed of 43 genes and four assigned regulators (bta-mir-19a, FOXM1, KLF7, and SOX5) (Fig 5). All of the gene targets of these regulators were enriched for cell cycle-related categories, revealing a coherent module. This result is consistent with the previous study that found cell-cycle enrichment for gene targets of miR-17-5p in NEB([Fatima *et al.*, 2014](#_ENREF_5)). Moreover, the functions of these regulators are reported in NEB related processes in previous studies. The miR-19a is known to regulate gene expression in adipose tissue in response to high-fat diet in cattle ([Romao *et al.*, 2012](#_ENREF_21)). It is reported that the overexpression of FOXM1 is associated with the development of liver cancer cells in humans ([Calvisi *et al.*, 2009](#_ENREF_3)). KLF7 is a candidate gene for obesity and type two diabetes ([Zobel *et al.*, 2009](#_ENREF_32)), which is known to regulates differentiation and proliferation of precursor adipose cells. The results suggested that the module and its regulators may be important in NEB. This possibility became even stronger when looking at the functions of the different genes present in this module, as we found some genes which have a clear function in the liver metabolic processes. For example, ECT2 plays an important role in liver regeneration in mice ([Sakata *et al.*, 2000](#_ENREF_22)). Also, TOP2A is known as a regulator of regrowth in liver tissue ([Dovey *et al.*, 2009](#_ENREF_4)). Interestingly, this module contained three known targets being regulated by FOXM1 (CCNB1 gene) ([Li *et al.*, 2011](#_ENREF_12)) and bta-mir-19a (FAM83D and CIT genes) ([Pedernera *et al.*, 2010](#_ENREF_20)) in human.

**Module 8**

Module 8 is a rather large module and contained 52 genes. Based on the previous studies, the function of some of these genes (such as P2RX4 and RGS16 ([Huang *et al.*, 2006](#_ENREF_7))) are related to lipid metabolism and liver metabolic processes. The module was predicted to be regulated by three TFs (TFE3, XBP1 and GRHL1) and two miRNAs (bta-let-7d and bta-mir-2346) that were associated with NEB related process based on previous studies (Fig 5). GO terms related to regulation of brown fat cell differentiation and regulation of heat generation were significantly enriched for gene targets of bta-let-7d. This result is consistent with the literature as it has been demonstrated that the let-7 family members are related to the development and abnormalities in liver, glucose and insulin metabolism ([Ma *et al.*, 2013](#_ENREF_15)). This finding indicated that the potential role of this regulator in NEB. The other regulator in the module, TFE3, is a well-known regulator of insulin signaling pathway in liver ([Shimano, 2007](#_ENREF_24)). The third regulator is XBP1, which reported as responsible for regulating lipogenesis in liver cells ([Lee *et al.*, 2008](#_ENREF_11)).

**Module 18**

Module 18 is a small module composed of seven genes and two assigned miRNAs (bta-mir-1277 and bta-mir-193b) (Fig 5). It is reported that miR-193b, regulates proliferation, migration and invasion of cancer cells in the liver ([Xu *et al.*, 2010](#_ENREF_28)). NEB can lead to mobilization of non-esterified fatty acids (NEFAs) from adipose tissue and transported into the liver and oxidized for energy production. Incomplete oxidation of NEFAs in the liver, resulted in increased production of ketone bodies such as BHB (β-hydroxybutyrate) and lead to oxidative stress development ([McCabe *et al.*, 2012](#_ENREF_16)). Surprisingly, gene targets of miR-193b were significantly enriched for cellular response to oxidative stress and NEB related process. We interpreted these findings as early evidence that the module and their regulators, especially bta-mir-193b, can be related to NEB.

**Module 88**

Our results showed that six miRNAs (bta-mir-1468, bta-mir-132, bta-mir-10b, bta-mir-182, bta-mir-26b, bta-mir-30d) and two TFs (XBP1, ZNF219) regulated 63 genes of the module 88 (Fig 5). Based on the previous reports, the roles of these regulators are associated with liver metabolic processes. It has been shown that miR-10b can explain the pathogenesis of nonalcoholic fatty liver disease ([Zheng *et al.*, 2010](#_ENREF_31)). Also, significantly enriched GO terms of gene targets of this miRNA were linked to short-chain fatty acid biosynthesis process. Of the putative targets of miR-10b, SERPINE1 is involved in liver metabolic processes ([Asselah *et al.*, 2008](#_ENREF_1)). It has been shown that overexpression of miR-132 lead to oxidative stress in alcoholic liver abnormalities in the mouse model ([Bala *et al.*, 2009](#_ENREF_2)). The role of mir-30d in tumor invasion and metastasis in cancer liver cells is demonstrated ([Yao *et al.*, 2010](#_ENREF_30)). Also, surveys such as that conducted by ([Yao *et al.*, 2010](#_ENREF_30)) showed that mir-182 is associated with insulin resistance. Recent evidence suggested that the expression of miR-26b as an obesity-related regulator can be regulated by free fatty acids and glucose ([Xu *et al.*, 2014](#_ENREF_29)). Researchers showed that XBP1 is responsible for regulating lipogenesis in liver cells ([Lee *et al.*, 2008](#_ENREF_11)). Interestingly, the validity of this module was further confirmed by the fact that the module contained one and two known targets of bta-mir-26b (PDLIM1) and bta-mir-30d (SERPINE1 and HABP4) in human, respectively ([Pedernera *et al.*, 2010](#_ENREF_20)). From which, the function of SERPINE1 was related to obesity and response to restriction of nutritional energy ([Lopez-Legarrea *et al.*, 2013](#_ENREF_13)). These results validated the potential biological relevance of the module with the NEB.

**Module 110**

Module 110 contained (36 genes) genes involved in different NEB related biological processes. Two TFs (CREB3L4 and SMAD4), and three miRNAs (bta-mir-2346, bta-mir-487b and bta-mir-543) were assigned to this module (Fig 5). CREB3L4 is known to be as a negative regulator of adipogenesis. Therefore, this TF can be considered as a useful therapeutic target in the fight against obesity and metabolic syndrome ([Kim *et al.*, 2014](#_ENREF_10)). It has been reported that SMAD4 is a tumor suppressor and lack of the protein expression is observed in human liver metastasis ([Losi *et al.*, 2007](#_ENREF_14)). bta-mir-487b was previously shown to be expressed in cancerous liver cell ([Murakami *et al.*, 2013](#_ENREF_17)). It is worth noting that target genes of all these regulators were significantly enriched in different GO terms. For example, some of the GO terms such as MAPK signaling pathway (regulation of steroid production) ([Singh *et al.*, 1999](#_ENREF_25)), Wnt signaling pathway (increases lipid accumulation in liver cells) ([Kaur *et al.*, 2011](#_ENREF_9)), white fat cell differentiation and unsaturated fatty acid biosynthetic process are associated with NEB related processes. Moreover, it is reported that synthesis suppression of long chain polyunsaturated fatty acids (such as arachidonic acid and eicosapentaenoic acid) can be occurred in SNEB cows and lead to the poor fertility ([McCabe *et al.*, 2012](#_ENREF_16)). The fact that these processes are significantly enriched in the same module suggests a strong relationship between them. Also, the module contained one known target of miRNA-543 (SIRT1) in human. A previous study has been revealed that the inhibition of miR-543 lead to increased mRNA and protein expression of SIRT1 and decreases insulin resistance ([Hu *et al.*, 2015](#_ENREF_6)). From these findings and the functions of these genes, we can conclude that most of the module 110 genes and regulators are likely involved in NEB.

**Module 143**

This module contained 15 genes. One TF (ZNF239) and two miRNAs (bta-mir-24-2 and bta-mir-326) were assigned to this module (Fig 5). mir-24-2 is known to be involved in metastasis and liver cirrhosis ([Huang *et al.*, 2008](#_ENREF_8)). Also, gene targets of this regulator was significantly enriched in liver metabolic related GO terms such as negative regulation of lipid metabolic process, regulation of steroid metabolic process and regulation of ketone biosynthetic process. One of these gene targets is DKK3, which is reported as a regulator of Wnt signaling pathway ([Nakamura *et al.*, 2007](#_ENREF_18)). The other regulator of this module, mir-326, is known to be induced in the process of fat cell differentiation ([Tang *et al.*, 2009](#_ENREF_26)).

**Module 145**

Five regulators, including three miRNAs (bta-mir-143, bta-mir-23a, bta-mir-127) and two TFs (TFEB and ZNF219) were assigned to module 145 (Fig 5). Through a literature review, we found that most of these regulators were related to liver metabolic processes. It has been revealed that mir-143 is associated with bovine intramuscular fat proliferation and differentiation ([Li *et al.*, 2011](#_ENREF_12)). miR-127 facilitates liver cell proliferation during liver regeneration in mice ([Pan *et al.*, 2012](#_ENREF_19)). miR-23a is known to inhibit glucose production in liver cells ([Wang *et al.*, 2012](#_ENREF_27)). TFEB is known as a regulator of genes involved in several stages of lipid metabolism ([Settembre *et al.*, 2013](#_ENREF_23)). These observations support the idea that this module might be related to NEB.

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