Transcriptome profiling reveals stress responsive gene networks in cattle muscles

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The conditions to which cattle are subjected before slaughter (social isolation, transportation, deprivation of food and water) are sources of emotional and physical stress that may affect muscle physiology and qualities of meat from these animals. Using a bovine microarray, we examined the muscle transcriptomes in the *Longissimus thoracis* muscle (LT) and the *Semitendinosus* muscle (ST) in cows exposed to stress (n=16) vs cows handled with limited stress (n=16).Changes in transcript abundances (62 differentially expressed genes (DEG) in LT, 32 DEG in ST, including 8 common transcription factors (TF)) illustrated a transcriptomic response to stress. Promoter analysis of the DEG in stressed cows showed that 25 cis transcriptional modules were over-represented of which 9 were detected in common across muscles. Molecular interaction networks were built from the DEG targeted by the most represented cis modules and enabled identifying common regulators and common targets contributing to the response to stress. They provided elements showing that the transcriptional response to stress is likely to i) be controlled by regulators of energy metabolism, factors involved in the response to hypoxia, and inflammatory cytokines; ii) initiate metabolic processes, angiogenesis, response to corticosteroids, immune system processes, and activation/quiescence of satellite cells.The results of this study demonstrate a core transcriptomic response to stress across muscles including changes in expression for TF. These factors could relay the physiological adaptive response of cattle muscles to cope with emotional and physical stress. The study provides elements to understand further the consequences of these molecular processes for meat quality and find strategies to attenuate them.

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Abstract

The conditions to which cattle are subjected before slaughter (social isolation, transportation, deprivation of food and water) are sources of emotional and physical stress that may affect muscle physiology and qualities of meat from these animals. Using a bovine microarray, we examined the muscle transcriptomes in the *Longissimus thoracis* muscle (LT) and the *Semitendinosus* muscle (ST) in cows exposed to stress (n=16) vs cows handled with limited stress (n=16).Changes in transcript abundances (62 differentially expressed genes (DEG) in LT, 32 DEG in ST, including 8 common transcription factors (TF)) illustrated a transcriptomic response to stress. Promoter analysis of the DEG in stressed cows showed that 25 cis transcriptional modules were over-represented of which 9 were detected in common across muscles. Molecular interaction networks were built from the DEG targeted by the most represented cis modules and enabled identifying common regulators and common targets contributing to the response to stress. They provided elements showing that the transcriptional response to stress is likely to i) be controlled by regulators of energy metabolism, factors involved in the response to hypoxia, and inflammatory cytokines; ii) initiate metabolic processes, angiogenesis, response to corticosteroids, immune system processes, and activation/quiescence of satellite cells.The results of this study demonstrate a core transcriptomic response to stress across muscles including changes in expression for TF. These factors could relay the physiological adaptive response of cattle muscles to cope with emotional and physical stress. The study provides elements to understand further the

consequences of these molecular processes for meat quality and find strategies to attenuate them.

Introduction

In livestock species, psychological/emotional factors (including unfamiliar environment or social regrouping), physical factors (including noise or vibrations associated), climatic factors (including temperature and humidity associated with transfer to the slaughterhouse), and deprivation of food and water are sources of emotional and physical stress. Exposition of animals to stress has several adverse impacts including metabolic and health troubles, and degraded welfare. Most of the above conditions are often met prior to or during slaughter with detrimental effect for nutritional and organoleptic meat quality as reported in pigs and poultry (Debut et al. 2005; Debut et al. 2003; Monin & Sellier 1985). There is also compelling evidence to demonstrate that pre-slaughter stress has non-desirable effect on meat quality traits (e.g. low tenderness) in both beef and lamb (Ferguson & Warner 2008; Muchenje et al. 2009; Terlouw 2015; Terlouw et al. 2008; Terlouw et al. 2021). The impact of stress on meat quality has been explained by changes in physiological and metabolic properties of the muscle which is converted to meat *post-mortem*: higher depletion of glycogen before slaughter, less production of lactic acid (a by-product of *post-mortem* glycolysis), and thus insufficient pH decline. Muscles with borderline pH (5.9-6.1) end up being very tough (Marsh et al. 1987) leading to a defect known as dark cutting beef or dark, firm, and dry (DFD) meat.

Changes may be related to variations in protein activities, as well as in protein levels driven by changes in gene expression. Some studies have reported alterations in the muscle proteome of farmed pigs (Morzel et al. 2004), chicken (Hazard et al. 2011; Zanetti et al. 2013), and fishes (Silva et al. 2012). However, there is less data on the transcriptional response to stress in the muscle of meat producing animals despite few studies in pigs (Davoli et al. 2009) and in steers following surgery (Zhao et al. 2012). Herein, we have examined the transcriptomic response in two different skeletal muscles of cows submitted to pre-slaughter stress conditions. We used these responses to infer stress-induced changes in biological and physiological function of these muscles, and discussed the biological functions affected by exposure to stress of psychological and physical origins and their potential impact on meat quality.

Materials & Methods

Animals and samples

The experiment was conducted with Normand cull cows (n=32) of 48–60 months of age housed in the experimental farm of the INRAE research centre (UE Herbipôle - Low mountain ruminant farming systems facility; doi.org/10.15454/1.5572318050509348E12) as described by Gobert et al. (2009) and Delosière et al. (2020). The animals were bought from different private farms in the West of France. Cows should be of pure breed, non-pregnant and non-lactating with a medium fatness score. One technician of the INRAE experimental farm “Herbipôle” visited different private farms to choose and buy the cows the most adapted to the experimental design.

Thirty-two Normand cull cows (mean live weight 642 kg) received a straw (30%) and concentrate (70%) based diet supplemented with lipids (40 g oil/kg diet DM) obtained from extruded linseeds (60%) and rapeseeds (40%) during 101 ± 3 days. For one group of the cows, this diet was the control diet. For another group, the diet was supplemented with vitamin E (155 IU/kg of diet DM) and plant extracts rich in polyphenols (7 g/kg diet DM, respectively; EP diet). The plant extracts were prepared from rosemary (Rosemarinus officinalis), grape (Vinis vitifera), citrus (Citrus paradisi) and marigold (Calendula officinalis) by Phytosynthèse (Riom, France) (INRA patent #P170-B-23.495 FR). The cows received a morning and evening meal representing a daily quantity of 1.8 kg of concentrate and 0.8 kg of hay. Water was provided ad libitum. The cows received an isoenergetic and isonitrogenous ration for a mean daily body weight gain of 1.6 kg for a 101 ± 3 d finishing period. Animals were housed in groups of 4 in 6×6m pens with straw bedding, according to a balanced design relative to feeding treatments. Pens were equipped with electronic feeding gates and individually offered their appropriate allowance of concentrates and straw per day. This allowance, adjusted periodically, supplied the required amount of nutrients to achieve a target growth rate of 1150 g/d. In our study, the finishing period of 101 days was slightly longer than the French standards for cattle (around 70 d.) and was chosen in order to achieve good production conditions and to facilitate experimental organization.

Cows were finished and slaughtered under conditions of limited stress (n = 16) or physical and psychological stress (n = 16) groups included the same numbers of animals for each diet. For limited stress conditions, the cows were transported accompanied by a non-experimental conspecific to avoid social isolation stress, and were handled calmly. For stress conditions, each cow was individually transported in a lorry (social isolation) towards unfamiliar farm. The cow was unloaded at the entrance of a labyrinth built partly outdoors and indoors and taken thrice through it by 2 purposely noisy experimenters (shouting and hitting metal structures with a stick) over a period of 30 min. It was then immediately transported for 15 min to the experimental slaughterhouse. Thus, the stress applied to the cows was a combination of psychological stress (novelty, social isolation, presence of active humans, noise) and physical activity (walking). The principal investigator and the staff of the experimental farm were aware of the group allocation. All cows were slaughtered without any electrical stimulation in a commercial way in compliance with INRAE ethical guidelines for animal welfare in the experimental slaughterhouse of INRAE 105controlled and approved by the French Veterinary Services as any other commercial slaughterhouse. Cows were slaughtered over a period of 8 weeks with one slaughter day per week. Each slaughter day, two experimental cows from a same pen were slaughtered in the same conditions. Slaughter took place between 07h30 and 11h10 am. After unloading, the cows were immediately stunned by captive bolt gun and exsanguinated, as in the French commercial slaughterhouses. Carcasses were stored in a chilling room (4 °C) approximately 45 min following exsanguination. Carcasses were sold for human consumption as in any controlled commercial slaughterhouse. Stress status was evaluated through the plasma and urinary cortisol and heart rate as described in a previous publication (Bourguet et al. 2010). Muscle samples from the *Semitendinosus* (ST) m. (a himbmuscle involved in locomotion) and the *Longissimus thoracis* (LT) m. (a support muscle for the body) were excised within 15 minutes after slaughter. The muscle samples were immediately frozen in liquid nitrogen and stored at -80°C until RNA extraction.

As indicated in previous publications (Bourguet et al. 2010; Delosière et al. 2020), experimental procedures and animal holding facilities respected French animal protection legislation, including licensing of experimenters. They were controlled and approved by the French Veterinary Services (agreement B63 345 17). The animal experimental design was describe in and registered in the research unit quality management database.

Muscle Transcriptome analysis

Transcriptome analyses were carried out with Agilent gene expression microarrays designed with 10,064 probes (including 1614 control probes) for 4,210 bovine genes including more than 3,000 specific muscular genes. The microarray was designed for monitoring transcriptional changes for genes involved in muscle growth (including energy and protein metabolism), carcass composition, fat metabolism and beef quality (including marbling). The microarray was first described in (Hocquette et al. 2012a) and subsequently used in (Costa et al. 2018).

Total RNA was extracted using TrizolTM as described by the manufacturer. Extraction was followed by a purification using RNeasy Mini kit (QIAgenTM).Total RNA was quantified with a Nanodrop ND.1000 spectrophotometer (Thermo Scientific, World Headquarters Location, Waltham, USA). RNA integrity was evaluated with the 2100 bioanalyzer (Agilent Technologies, Massy, France) and the RNA 6000 Lab Chip kit. The total RNA was amplified and labeled with Cyanine 3 using Agilent's Low RNA Input Linear Amplification Kit, PLUS, One-Color (Agilent Technologies) following the detailed protocol described by Agilent. Briefly, 500 ng of total RNA was reverse transcribed to double-strand cDNA using a poly dT-T7 promoter primer. cDNA products were then used as templates for in vitro transcription to generate fluorescent cRNA. Labeled cRNA-s were finally purified using QIAGEN's RNeasy mini spin columns and eluted in 30 µl of nuclease-free water. After amplification and labeling, cRNA quantity and cyanine incorporation were determined using a Nanodrop ND.1000 spectrophotometer (Thermo Scientific). For each hybridization, 600 ng of Cyanine 3 labeled cRNA were fragmented and hybridised at 65°C for 17 hours to an Agilent 8 x 15 K custom Oligo Microarray. After washing, microarrays were scanned using an Agilent DNA G2505B scanner. The Feature Extraction 10.1 software (Agilent Technologies) was used to extract fluorescent hybridization signals.

The data were pre-processed by Feature Extraction 10.1 for all samples and probes. The probes not meeting the quality criteria (saturation and uniformity of spots, intensity above background noise...) were filtered out. Each array was normalized by dividing the raw values of its probe intensities by the median of the Control probes of the array. Each probe intensity was then normalized by dividing its raw value by the median of the corresponding probes from all arrays. After filtration out of the probes with missing values, a log2 transformation was applied to data.

Transcriptomic data were submitted to Gene Expression Omnibus (GEO) under accession number GSE119912. Differential analyses were conducted via linear modelling with the diet supplementation* period * stress interaction factor to explain the probes' levels. An empirical Bayes method was used to moderate the standard errors of the estimated log-fold changes using the R/Limma package (http://bioinf.wehi.edu.au/limma/) as described in Smyth et al. (2003) with a Benjamini and Hochberg multiple testing correction (Benjamini & Hochberg 1995). The genes for which at least 80% of the probes were differential at the adjusted p-value 10%

and consistent were retained and considered differentially expressed genes (DEG). All probe ratios were found consistent for each DEG meaning that for one gene all probe ratios were lower than 1 or greater than 1.

Gene Ontology Enrichment

Functional enrichment according to Gene Ontology Biological Process (GO BP) and wikipathway was performed by submitting lists of accession numbers (for DEG) or gene names (for common regulators and targets of the DEG) to the ProteINSIDEv2 workflow (Kaspric et al. 2015, https://umrh-bioinfo.clermont.inrae.fr/ProteINSIDE_2/) which enables to analyse lists of protein or gene identifiers from ruminant species and gathers biological information provided by functional annotations, putative secretion of proteins and proteins interactions. This workflow queries the g:Profiler database based of the most complete information available for Bos Taurus. The list of all the probes present on the array was used as background list for enrichment analysis of the DEG lists. GO enrichment test was declared significant for P value Benjamini-Hochberg FDR < 0.05 or 0.08. The results expressed as –log10 (p-value) to visually plot them on graphs.

Identification of Cis-Transcriptional modules

Promoter sequences were extracted using the program Gene2Promoter (Genomatix software suite 3.4.1, www.genomatix.de) using default settings, 500 bp upstream and 100 bp downstream of transcription start site. We selected the bovine promoters with at least one relevant transcript and preferentially a high quality level (experimentally verified 5'transcript or with 5'end confirmed by PromoterInspector prediction), and for whose number of conserved orthologous promoters was at least 50% of loci. This was performed for DEG and for the totality of the genes of the microarray. As the co-regulation of mammalian genes usually depends on combination of TFs rather than individual TF alone, cis-acting-regulatory elements are often organized into frameworks of motifs called cis-transcriptional modules. The selected promoters were submitted to the 'ModelInspector' task of GEMS Launcher (version 4.1, Genomatix software, www.genomatix.de) to search for cis transcriptional modules. For this purpose, the promoter sequences of the genes were scanned for matches to the Promoter Module 5.4 Library (Vertebrate Module section). A Fisher's exact test was then used to enlighten over-represented cis-transcriptional modules in the DEG set compared to the total genes set of the microarray.

Construction of interaction networks

Network analysis was performed with Pathway Studio software version 12.0.1.9 using the Elsevier's Resnet Mammal DataBase (Ariadne Genomics, Rockville, Md., USA). Gene interaction networks were built with the DEG targeted by the most represented cis transcriptional modules for each muscle (targeting at least 5 and 4 DEG for LT and ST respectively), and with the DEG targeted by the 9 over-represented cis transcriptional modules common to both muscles, generating 2 muscle specific networks and 1 common network of stress response. For each set of genes, to reconstruct the network, Pathway Studio search known relations between them and add regulators and the expression targets common to them.

Filters were applied to identify only key expression regulators and targets of each network. To be added in the network, targets genes must be linked to minimum 3 bibliographic references and have at least 6 known relations in the Pathway Studio Database. For regulators, also 3 bibliographic references are needed, and 2 known relations, except for regulators of the 9 common modules for which a cut-off of 5 relations has been chosen.

Lastly, Venn diagrams were used to identify the major regulator genes and major targets genes common to the DEG targeted by cis-transcriptional modules specific to LT, ST and common to both muscles. Sub-networks between DEG and their major regulator genes and between DEG and their major targeted genes were extracted.

Validation of differential expressions

A RT-qPCR assay was performed on the LT samples of 10 animals/group for 4 genes (ATF3, CEBPD, SMAD7 and FOS) with the StepOne Plus™ Real-Time PCR System, using the Power SYBR1 Green master mix (both Applied Biosystems, Foster City, CA, USA). The *GeNorm* algorithm (Vandesompele et al. 2002) was used to determine the optimal number of reference genes required for effective normalization of *qPCR data.* Four housekeeping genes were selected: UXT, MRPL39, CLN3 and TOP2B. Primer sequences (Supplemental Table S1) were designed with an annealing temperature of 60 °C using Primer3 software. qPCR were runned using the StepOnePlus thermocycler (Applied Biosystems). PCR efficiency was tested for each primer pair by a 10-fold dilution series of purified cDNA. Each reaction was subjected to melting curve analysis to ensure the specificity and integrity of the PCR product. Student *t*-test was used to test the significance of the difference between limited stress and stress groups.

Quantitative trait loci (QTL) analysis

Query of genetic information from the lists of the DEG and the common regulators and targets of the DEG was performed with the QTL module included in ProteINSIDEv2 in order to retrieve information on the location of the genes encoding proteins of interest within published QTL for tenderness. This module interrogates a publicly available QTL library in Animal QTL database that contains cattle QTL and the published data associated.

Results

Transcriptomic profiles

We recorded changes in gene expression profiles in the m. *Longissimus thoracis* (LT) and the m. *Semitendinosus* (ST). Individual data is available at GEO repository under accession number GSE119912. No effect was detected for the EP diet, nor for the stress*EP diet or the stress*diet in both muscles, while an effect of the stress was detected ($P<0.1$). In the stressed cows compared to cows handled with limited stress, microarray analysis revealed changes in abundance for 67 transcripts in the LT (including 43 up and 24 down-regulated; $p<0.1$, Supplemental Table S2) corresponding to 62 DEG with unique Gene Names (Figure 1). In the ST, changes were detected for 36 transcripts (including 33 up- and 3-down-regulated; $P<0.1$, Supplemental Table S2) corresponding to 32 DEG with unique Gene Names (Figure 1). Among differential transcripts, 27 are common to both muscles, corresponding to 24 unique Gene

Names (Figure 1). They included 8 known transcription factors (TF): SMAD7, ETS2, MYOG, ATF3, HES6, CEBPD, HEYL, and FOS (Supplemental Table S2). In addition, muscle specific DEG were detected according to the stress status (38 in the LT, and 8 in the ST; Figure 1). They included four TF (MYOD1, MYF6, CEBPB, and HES1) and one transcription co-factor (MED23) in the LT, and a transcriptional activator (ZNF750) in the ST. The differential abundance of four TF transcripts (ATF3, CEBPD, SMAD7 and FOS) was checked by qPCR experiments in the LT and confirmed the observed changes as illustrated in Table 1.

Lists of DEG according to the stress status were submitted to biological information mining through Gene Ontology (GO) term enrichment compared to the background list of the microarray (Supplemental Data S1). In the LT, 9 GO terms Biological process (GO BP) and 1 wikipathway were enriched ($P<0.08$). In the ST, 26 GO BP and 1 wikipathway were enriched ($P<0.08$). As illustrated in Figure 2, 9 GO terms were found in common between the LT and the ST: regulation of gene expression (23 genes in LT, 13 in ST), transcription by RNA polymerase II (16 genes in LT, 12 in ST), regulation of transcription by RNA polymerase II (16 genes in LT, 12 in ST), regulation of biosynthetic process (21 genes in LT, 14 in ST), regulation of cellular biosynthetic process (20 genes in LT, 13 in ST), regulation of macromolecule biosynthetic process (20 genes in LT, 13 in ST), regulation of cellular macromolecule biosynthetic process (20 genes in LT, 13 in ST), skeletal muscle cell differentiation (4 genes in LT, 3 in ST), muscle organ development (8 genes in LT, 5 in ST) and the wikipathway Hypertrophy Model (the same 3 genes in both muscles). For each considered GO BP, the list of genes included both common and muscle- specific DEG.

Cis-Transcriptional modules

A promoter analysis was performed with Gene2promoter of the Genomatix Software Suite in order to seek common TF binding sites in the promoter regions of genes – called cis transcriptional modules- that may account for co-regulation amongst differential transcripts. For 52 of the DEG in the LT, 168 promoters were retrieved from the Genomatix Promoter Database of which 111 were selected according to their relevance for further analysis (Supplemental Table S3). ModelInspector enabled to retrieve 288 different cis-transcriptional modules (on 1378 locations). For 28 of the DEG in the ST, 84 promoters were retrieved. Of these, 57 promoters were further analysed with ModelInspector, 201 cis transcriptional modules were found (on 675 match positions). The same analysis was performed for all the genes represented on the microarray. As illustrated in Supplemental Table S3, 24 cis transcriptional modules were detected as over-represented on DEG compared to the genes represented on the micro-array ($P<0.1$) in the LT and 25 in the ST. Nine of the over-represented modules were common to both muscles. The cis transcriptional modules and the DEG targeted by these modules in each muscle as identified by ModelInspector are listed in Table 2. Cis transcriptional modules with binding sites for TF of the ETS family and SP1 family had a high occurrence in the promoters of the DEG in both muscles.

Interaction networks and identification of regulators and mains targets of DEG

Lastly, with Pathway Studio 2, we constructed interactions networks between the DEG targeted by the over represented cis-transcriptional modules for each muscle, and between the DEG

targeted by the 9 over represented cis transcriptional modules common to both muscles We thus generated muscle specific networks and one core network of stress response. Then thanks to the Pathway Studio 2 database, we looked for the main regulators and the main targets of the 3 networks (Supplemental Data S2, sheets 1-6). We next computed the intersection between the lists obtained from these datasets to identify the key common regulators and targets (Supplemental Data S2, sheets 7-8). Ten main regulators of the stress responsive genes including AKT1, EGF, HIF1A, IFNG, IL1B, INS, MAPK1, MAPK14, TGFB1, and TNF were identified. GO mining showed that they were highly related ($P<0.001$) to regulation of oxidoreductase activity and specifically regulation of monooxygenase activity, inflammatory response, immune system, carbohydrate metabolism (transport and metabolic process) (Supplemental Data S2, sheet 7). A list of 14 main targets of the DEG included BCL2, BGLAP, CDKN1A, COL3A1, ERBB2, FN1, ICAM1, IL6, MMP2, PPARG, SELE, SLC2A4, TLR4, and VEGFA was established. GO mining showed that they were highly related ($P<0.001$) to response to cytokine, response to oxygen levels, response to glucocorticoids, response to stress, immune system, angiogenesis, and carbohydrate homeostasis (Supplemental Data S2, sheet 8). The networks between the DEG and the common regulators and between the DEG and common targets are presented in Figure 3. The list of DEG (LT specific, ST specific and common DEG), the main regulators and the main targets of the DEG are summed-up in Table 3. Twenty-four of the corresponding genes were located in QTL linked to meat quality: shear force (CDIPT, CEBPD, DNAJB4, GPAM, RAB3IL1, MAPK1, and TLR4), muscle compression (ADRB2), tenderness score (ADRB2, CDIPT, RAB3IL1, and IFNG), muscle pH (DLL4, ERBB2), juiciness (ATP1B1, DFFB, RAB3IL1, SELE), and marbling (CEBPD, DLL4, ERBB2, GADD45, ICAM1, IL1B, IL6, LEAP2, MYF6, PDK4, PMP22, SMAD7, TNF).

Discussion

Transcriptional response to stress. Understanding how pre-slaughter stress impacts muscle physiology would provide elements for the management of beef quality especially tenderness. In this study, we have examined the muscle transcriptional profiles of cows exposed to a combination of pre-slaughter emotional and physical stress compared to control cows handled with limited stress. We made the hypothesis that this approach may be useful in investigating the molecular mechanisms of the stress response and their potential impact on meat quality. We recorded changes in the abundance of several gene transcripts in two muscles of cows exposed to stress. We found evidences of a common transcriptional response across muscles albeit their different metabolic type and activity even though some muscle specific DEG were detected. Obviously, there was a core stress-response across muscles as shown by common DEG and common GO (mainly related to regulation of gene expression, and muscle development) and pathway (cardiac/skeletal muscle hypertrophy) between muscles. The highest number of DEG was detected in the LT. This may be related to the more oxidative metabolism of LT (Hocquette et al. 2012b) that makes it more prone to changes in oxidative status and therefore to susceptibility to cope with stress. Muscle gene expression in response to stress likely depends also on gender (Oster et al. 2014), nature and intensity or duration of the stress, and breed which may explain some differences in results between our study and a previous one on Angus animals (Zhao et al. 2012). Nevertheless, regulation of genes involved in carbohydrate, lipid, and protein metabolism is likely to occur in many cases as observed in

this study, as well as in studies in cattle (Buckham Sporer et al. 2007; Zhao et al. 2012) and in pigs (Davoli et al. 2009).

Newly synthetized transcription factors and their related biological pathways. While the short-term response to stress may be primarily driven by changes in protein phosphorylation (e.g. reversible phosphorylation, (Mato et al. 2019)), as well as enzyme activity or protein abundance, our study provided convincing evidence that the response to stress includes a transcriptional component as previously reported in two studies (Davoli et al. 2009; Zhao et al. 2012). Indeed, functional annotation of the lists of DEG showed an enrichment in GO terms related to the regulation of gene expression and transcription. It is well accepted that the primary response to stress involves the activation of pre-existing TF by phosphorylation (Sabban & Kvetňanský 2001). Our data indicate that newly synthetized TF may also relay the stress response as illustrated by 11 DEG encoding TF. Eight of them were common between the muscles, of which some were detected as nodes in the molecular networks associated with the response to stress. The majority of differential TF were upregulated except two muscle regulatory factors (MYOG and MYOD1) and a transcription repressor (HES1). MYOG and MYOD1 are basic helix-loop-helix family TF essential for myogenesis including during regenerative process (Zammit 2017). HES1 is a Notch downstream target (Borggrefe & Oswald 2009). It is also a master regulator of glucocorticoid receptor dependant gene expression. It is silenced by the primary stress hormones glucocorticoids (Revollo et al. 2013). Down-regulation of HES1 was not surprising since the stressed cows showed higher plasma and urinary cortisol levels (Bourguet et al. 2010). Of the up-regulated TF, 4 were basic leucine zippers (bZip) TF: FOS, ATF3, CEBPB, and CEBPD. ATF3 - a member of the mammalian cAMP responsive element-binding proteins (CREB) family- is induced by various stresses. ATF3 is a sensor for a wide range of conditions and modulates the immune response, atherogenesis, cell cycle, apoptosis, and glucose homeostasis (Jadhav & Zhang 2017). ATF3 has been considered an adaptive response gene with a dual mode of action to activate (as a homodimer) or repress (as a heterodimer) target gene expression. It was proposed that ATF3 functions as a "hub" of the cellular adaptive-response network that helps cells to adapt to disturbances of homeostasis (Hai et al. 2010). ATF3 was also found differentially expressed following acute stress induced by surgery in Angus beefs (Zhao et al. 2012). The bZip proteins CEBPB and CEBPD are members of the C/EBP family, which participate in a number of biological responses including energy metabolism, cell proliferation and differentiation, or immune response (Ramji & Foka 2002). Their binding sites are found in the regulatory regions of a large number of acute phase proteins. A dual role was proposed for the C/EBP proteins as mediators of both inflammatory responses and effects of glucocorticoids (Nerlov 2007; Roos & Nord 2011). CEBPD expression is induced by inflammatory effectors and hypoxia, and promotes pro-inflammatory signalling and adaptation to hypoxia (Balamurugan & Sterneck 2013). CEBPB has also been recently identified as a novel regulator of satellite cell homeostasis that promotes differentiation at the expense of self-renewal (Lala-Tabbert et al. 2016).

Overrepresented binding sites of TF in the promoters of DEG and related biological pathways. Several cis transcriptional modules were located in promoters of the DEG. Common modules to both muscles were detected mainly in promoters of common DEG while muscle specific cis transcriptional modules were in promoters of muscle specific DEG albeit some specific cis transcriptional modules were in the promoters of common DEG. FOS was targeted

by 4 common modules in both muscles plus 1 specific module in the ST. ATF3 was targeted by 1 common module in both muscles, and by 3 specific modules (1 in the LT and 2 in the ST). Examination of cis transcriptional modules of DEG from both muscles revealed that binding sites for the transcription factor SP1 and for members of the ETS family are often included in those modules. SP1 is ubiquitously expressed and in addition to functioning as a 'housekeeping' TF may be a key mediator of gene expression induced by insulin and other hormones (Solomon et al. 2008). ETS1 is a highly conserved TF throughout evolution that controls cytokines and chemokines, and angiogenesis (Russell & Garrett-Sinha 2010). ETS binding sites were found in the promoter of common differential TF as well as in the promoter of 11 of the 15 LT specific DEG. Similar results were found in the muscles of rats exposed to stress with the same modules identified (our unpublished data).

Other biological pathways related to the response to stress. Our study provided additional evidences that the response to stress interplays with immune response, inflammatory response, and chemotaxis, as well as production of Interleukins (IL-16 in LT, IL-1 B and IL-6 as main regulators and targets of the DEG, and IL-10 and IL-13 in the list of common targets of the DEG). This is consistent to previous studies examining the response to stress in livestock animals: A transcriptional shift of pathways of acquired and innate immunity was reported in the peripheral blood of psychosocially stressed pigs (Oster et al. 2014). Amplified inflammatory activity was also detected in blood neutrophil expression in young bulls following truck transportation for 9 hours (Buckham Sporer et al. 2007) and in the LT muscle of Angus beef exposed to acute stress induced by surgery (Zhao et al. 2012). Moreover, a conserved transcriptional response to chronic social stress involving increased expression of proinflammatory genes (including IL-6, IL-8) has been reported in blood leukocytes (Powell et al. 2013) in mice and humans. In our study, changes in chemokines and cytokines expression in muscle were most probably part of adaptive mechanisms contributing to the stress response (Figure 3). IL-16 is a lymphocyte chemoattractant factor also classified as an "alarmin" (Rider et al. 2017). IL-6 and IL-8 are also regarded as myokines released from muscle in response to contractions (Brandt & Pedersen 2010). Muscle-derived IL-6 may mediate some of the anti-inflammatory and insulin-sensitizing effects of physical exercise (Covarrubias & Horng 2014).

Another striking result of our study is the up-regulation of transcripts related to the carbohydrate metabolic pathway e.g. transcripts encoding PDK4 (an inactivator of Pyruvate dehydrogenase complex; targeted by 1 cis transcriptional module in the LT and 3 modules in the ST), PFKFB3 (a glycolysis regulator; targeted by 1 cis transcriptional module in the LT and 2 cis transcriptional modules in the ST) and SLC25A25 (a mitochondrial ATP transporter; targeted by 2 cis transcriptional modules in the LT). This illustrates a switch in energy metabolism in the muscles of animal exposed to exercise and psychological stress, towards anaerobic metabolism to support ATP production for muscle contraction. PDK4 plays a pivotal role in controlling metabolic flexibility (Zhang et al. 2014) showing increased expression in response to moderate intensity exercise.

Analysis of molecular networks highlighted also the contribution of the response to oxygen levels / hypoxia in the response to stress albeit different transcripts and different contractile and metabolic muscle types. Consistently, the Hypoxia Inducible Factor (HIF1A) TF was identified as a main common regulator of the DEG. This could be a signature of oxygen imbalance or of the physical activity imposed to the cows. Thus, it may not be surprising that expression of

PFKB3 a downstream target of HIF was up-regulated. Hypoxia was also demonstrated to cross-talk with the Notch signalling pathways which regulates the satellite cells quiescence and self-renewal (Liu et al. 2012). Since quiescent satellite cells have a low metabolic rate, fewer mitochondria and an anaerobic metabolism, this is likely part of the adaptive signature of muscle to stress. Thus, the combined signatures of hypoxia, Notch signaling pathway (Fukada et al. 2007), down regulation of MYOD1 (Kopan et al. 1994), and up-regulation of IRFD1 (an inducer of the regenerative myogenesis) further designate quiescent satellite cells as stress targets in our study, and most probably as physical activity targets.

Putative effects on meat quality. Lastly, the transcriptomic muscle response to pre-slaughter stress may have impact on meat quality through energy metabolism and hypoxia. Indeed, the anaerobic glycolysis is highly relevant to beef quality since it is involved in *post-mortem* protein degradation and hence beef tenderization during meat ageing (reviewed by Maltin et al. (2003)). This process is regulated by the decline in muscle pH due to a conversion of glycogen into lactate following the lack of oxygen after slaughtering. Stress was shown to highly affect meat tenderness by increasing *post-mortem* ultimate pH (Purchas 1990) due a depletion of glycogen stores by stress prior slaughtering, which is leads to dark-cutting meats. Reliable indicators of the occurrence of ultimate high pH and pre-slaughter stress were identified in the sarcoplasmic proteome of muscle (Fuente-Garcia et al. 2019; Sentandreu et al. 2021). They were mainly involved in metabolic, chaperone/stress-related, muscle contractility/fiber organization, and transport activities. In our study, several genes encoded by the DEG and the common regulators or targets of the DEG were located in bovine QTL associated with meat quality criteria known to be impacted by stress: muscle pH (DLL4, ERBB2), shear force (CDIPT, CEBPD, DNAJB4, GPAM, RAB3IL1, MAPK1, and TLR4), tenderness score (ADRB2 CDIPT RAB3IL1, and IFNG), and compression ((ADRB2)). However the relationships between transcript level and meat quality parameters remain to be studied.

Conclusions

Exposition to emotional and physical stress prior to slaughter induced a transcriptional response in two muscles of cows. Our data provide evidence of a coordinated response across muscles of the stressed animals thanks to the identification of common target genes and associated functions, cis transcriptional modules, and regulators and downstream targets. The response included an interplay between metabolic changes (glycolytic), hypoxia, inflammatory process, and renewal/quiescence of satellite cells, likely due to elevated cortisol. However, the relative contribution of mechanisms related to stress and to physical activity induced by walking the labyrinth remains to be elucidated.

From an animal production perspective, the gene networks activated by stress will help to understand the molecular mechanisms of meat conversion and beef quality defects caused by pre-slaughter stressful conditions suffered by cattle. Target stress-responsive gene network could be modulated by management factors (on farm nutrition, antioxidant supplementation…) in order to reduce the adverse impact of stress.

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Table 1(on next page)

Validation of some differentially expressed genes following pre-slaughter stress in the *Longissimus thoracis* muscle

The abundance of some DEG detected by microarray analysis was quantified by qRT PCR in the *Longissimus thoracis* muscle of stress cows *vs* cows handled with limited stress (2n=20). Variation of reference genes used for normalization was computed with the GeNorm software package. Student *t*-test was used to test the significance of the difference between the two conditions.

Table 1:

	Fold change (qPCR)	P-value	Fold change (Microarray)
ATF3	2.1	**0.006**	2.6
CEBPD	4.	**0.001**	3.6
FOS	1.4	0.143	2.5
SMAD7	1.8	**0.006**	1.7

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Table 2(on next page)

Over-represented transcriptional modules in the promoter of the stress-responsive genes in the muscles of cows.

The transcriptional modules were searched with the module inspector function of Genomatix, their occurrence was examined in the promoters of genes of the experimental datasets and the number of target genes was determined in each dataset.

1 **Table 2**

2

Muscle	Module	pValue	Occurrence of module	Number of target genes	Gene ID
LT	ETSF_ETSF_01[a]	0.076	16	15	IL16 SERPINE1 HES1 HSPBAP1 CDIPT XYLT2 TUBB3 THBS1 MED23 GPAM PIGM *CEBPD HEYL HES6 MYOD1*
	SP1F_CAAT_02	0.040	10	9	NME6 SDC4 PDPR *HES6* PFKFB3 CDIPT PMP22 THBS1 IFRD1
	CAAT_AP1F_01	0.035	8	8	SLC25A25 SERPINE1 NME6 IMP3 HSPBAP1 SLC2A3 THBS1 *ATF3*
	SP1F_EBOX_SP1F_01	0.024	8	7	DFFB GLUL PDK4 IMP3 PMP22 XYLT2 *CEBPD*
	CAAT_SP1F_01	0.088	5	5	SERPINE1 ATP1B1 GLUL GEM *HES6*
	GATA_GATA_GATA_01	0.037	5	3	NME6 SLC16A6 MED23
	YY1F_SRFF_02	0.016	3	3	SLC2A3 *ATF3 FOS*
	SORY_SORY_EGRF_01	0.061	3	3	MUSK ATP1B1 RAB3IL1
	NFKB_NFKB_01	0.064	3	3	SLC25A25 GLUL GEM
	HNF1_GATA_01	0.098	3	3	MED23 PLD1 ATP1B1
	KLFS_NR2F_KLFS_01[a]	0.024	3	2	SERPINE1 TUBB3
	STAF_SP1F_01	0.026	2	2	GLUL *HEYL*
	RXRF_EBOX_01	0.043	2	2	PDPR RAB3IL1
	AP1F_SMAD_01	0.055	2	2	IL16 THBS1
	ETSF_AP1F_04	0.067	2	2	ACOT11 HSPBAP1
	CEBP_MYBL_03	0.076	2	2	ACOT11 HSPBAP1
	AARF_CEBP_01	0.091	2	2	ABRA NME6
	BRNF_RXRF_02	0.066	4	1	DLL4
	NFKB_ETSF_01	0.007	2	1	DLL4
	SRFF_AP1F_01	0.047	1	1	*FOS*
	ETSF_SP1F_SMAD_01	0.062	1	1	*HEYL*
	YY1F_SRFF_01	0.076	1	1	*FOS*
	PAX8_NKXH_01	0.076	1	1	PMP22
	ETSF_SRFF_01	0.091	1	1	*FOS*
ST	SP1F_SP1F_06	0.002	30	14	PGF GADD45A SLC16A6 ADAMTS9 CYP1A1 SLC2A8 SDC4 PMP22 TUBB3 IFRD1 HYAL2 *ATF3 HES6 HEYL*
	NFKB_SP1F_03	0.002	12	8	SLC2A8 SDC4 MYLK4 PGF LRP4 PMP22 *HEYL CEBPD*
	SP1F_ETSF_04	0.087	8	8	ABRA SDC4 PGF LCAT PMP22 CYP1A1 *SMAD7 HES6*
	SMAD_E2FF_01	0.088	12	7	SLC2A8 SDC4 IFRD1 PDK4 *CEBPD HES6 FOS*
	SP1F_YY1F_01	0.044	10	7	PGF *A*BRA GEM SDC4 SLC2A8 *ATF3 HES6*
	SP1F_CAAT_02	0.039	6	5	SDC4 PFKFB3 IFRD1 PMP22 *HES6*
	SP1F_EBOX_SP1F_01	0.085	4	4	MYLK4 PMP22 PDK4 *CEBPD*
	RUSH_EGRF_01	0.049	3	3	SDC4 GADD45A SPOCK2
	IRFF_NFAT_01	0.084	3	3	MYLK4 ADAMTS9 IFRD1
	GATA_GATA_GATA_01	0.013	4	2	SLC16A6 ADAMTS9

Muscle	Module	pValue	Occurrence of module	Number of target genes	Gene ID
	MYOD_MYOD_03	0.066	3	2	SPOCK2 *HES6*
	AP1F_ETSF_04	0.013	2	2	IFRD1 HYAL2
	YY1F_SRFF_02	0.028	2	2	*ATF3 FOS*
	ZFHX_ZFHX_NKXH_01	0.037	2	2	GADD45A ADAMTS9
	SMAD_HIFF_01	0.032	2	1	PFKFB3
	SP1F_MZF1_01	0.035	2	1	PMP22
	ETSF_SP1F_SMAD_01	0.016	1	1	*HEYL*
	SRFF_AP1F_01	0.024	1	1	*FOS*
	YY1F_SRFF_01	0.039	1	1	*FOS*
	PAX8_NKXH_01	0.039	1	1	PMP22
	ETSF_SRFF_01	0.047	1	1	FOS
	MEF2_MYOD_01	0.054	1	1	SLC16A6
	KLFS_CREB_KLFS_01	0.070	1	1	SLC2A8
	CAAT_SREB_01	0.077	1	1	IFRD1
	GATA_HNF1_02	0.077	1	1	PDK4

LT: *Longissimus thoracis* muscle; ST: *Semitendinosus* muscle

[a] these modules were also found in rats exposed to stress (our unpublished data)

Modules in bold were in common between muscles

Genes capitalized in italics are TF

Table 3(on next page)

Components of the molecular response initiated by pre-slaughter stress in two muscles of cows as revealed by transcriptomic signatures.

The Differential expressed genes (DEG), the main regulators and the main targets of the 3 datasets of the DEG as identified by Pathway Studio are listed. Query of genetic information was performed with the ProteQTL module included in ProteINSIDE in order to retrieve information on the location of the genes encoding proteins of interest within published Quantitative trait loci (QTL) for tenderness. This module interrogates a publicly available QTL library in Animal QTL database that contains cattle QTL and the published data associated.

Table 3

Type of response	Gene Name	Transcription regulator	Location in a bovine QTL
LT specific DEG	**ACOT11**		
	ADRB2		Tenderness score, Muscle compression
	ARL6IP2		
	ATP1B1		Juiciness
	CDIPT		Tenderness score, Shear force
	CEBPB	TF	
	CXCR6		
	DFFB		Juiciness
	DLL4		Muscle pH, Marbling score
	DNAJB4		Shear force
	GLUL		
	GPAM		Shear force
	HES1	TM	
	HSPB1*		
	IDS		
	IL16		
	IMP3		
	ITGAE		
	LEAP2		Marbling score
	MED23	TM	
	MUSK		
	MYF6		Marbling score
	MYLC2		
	MYOD1	TF	
	NME6		
	NOL6		
	PDPR		
	PIGM		
	PITPNM2		
	PLD1		
	RAB3IL1		Tenderness score, Shear force, Juiciness, Marbling score
	SERPINE1		
	SLC25A25		
	SLC2A3		

	THBS1		
	TREM1		
	XYLT2		
	YWHAZ		
ST-specific DEG	**ADAMTS9**		
	ATL2		
	CYP1A1		
	HYAL2		
	MYLK4		
	PPP2B		
	SLC2A8		
	ZNF750	TM	
Common DEG			
	ABRA		
	ATF3	TF	
	CEBPD	TF	Shear force, Marbling score
	ETS2	TF	
	FOS	TF	
	GADD45A		Marbling score
	GEM		
	HES6	TF	
	HEYL	TF	
	IFRD1		
	LCAT		
	LRP4		
	MYOG	TF	
	PDK4		Marbling score
	PFKFB3		
	PGF		
	PMP22		Marbling score
	RGS2		
	SDC4		
	SLC16A6		
	SMAD7	TF	Marbling score
	SORBS1		
	SPOCK2		
	TUBB6		
Common main regulators			
	AKT1		
	EGF		
	HIF1A	TF	

	IFNG		Tenderness score
	IL1B		Marbling score
	INS		
	MAPK1		Shear force
	MAPK14		
	TGFB1		
	TNF		Marbling score
Common main targets			
	BCL2		
	BGLAP		
	CDKN1A		
	COL3A1		
	ERBB2		Muscle pH, Marbling score
	FN1		
	ICAM1		Marbling score
	IL6		Marbling score
	MMP2		
	PPARG	TF	
	SELE		Juiciness
	SLC2A4		
	TLR4		Shear force
	VEGFA		

* proposed as a protein biomarker for high ultimate pH (pHu) meat in Sentandreu et al, 2021.

Figure 1

Venn diagram visualizing the intersection of the lists of the Gene Names of the differentially expressed genes (DEG) in response to pre-slaughter stress in the *Longisssimus thoracis* (LT) muscle and in the *Semitendinosus* (ST) muscle.

A subset of 24 common DEG was assigned to a set of core stress responsive genes. The two subsets of DEG only in the LT (n=38) or in the ST (n=8) were considered as components of the muscle-specific response to stress. Transcriptional regulators are underlined: Transcription factor (unbroken line), transcriptional modulator (dotted line).

Figure 1

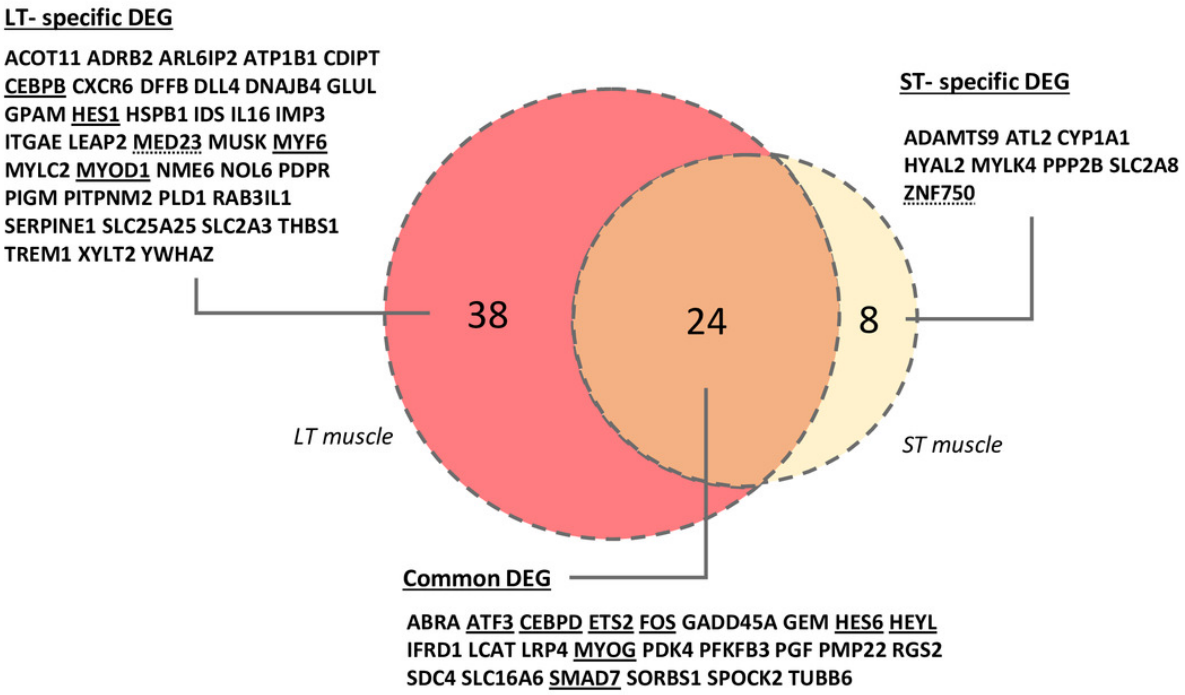

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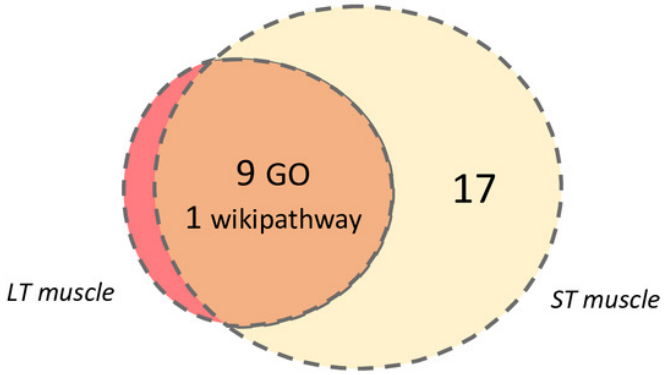
Figure 2

Common GO terms across muscles for the differentially expressed genes (DEG) in response to pre-slaughter stress.

Lists of DEG were submitted to functional annotation compared to the microarray background (data available in Additional file 3). The intersection of the lists of GO terms and wikipathway was computed at http://bioinformatics.psb.ugent.be/webtools/Venn/ . Gene Names capitalized in bold are common DEG between muscles. Gene Names capitalized in italics are muscle-specific DEG. LT: *Longisssimus thoracis* muscle ; ST: *Semitendinosus* muscle

Figure 2



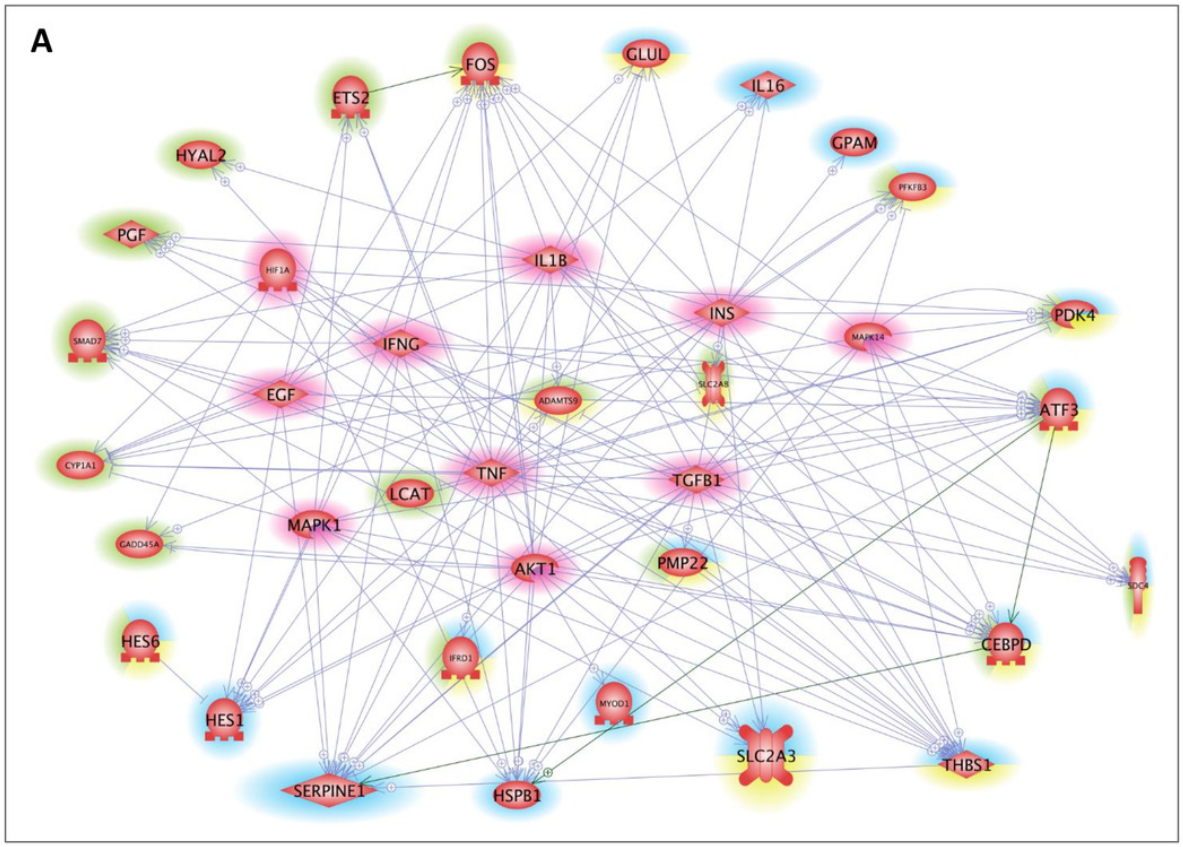
Common GO BP terms	LT muscle	ST muscle
Muscle organ development	**ATF3** *DLL4* **FOS HEYL** *MYF6 MYOD1* **MYOG SMAD7**	**ATF3 FOS HEYL MYOG SMAD7**
Skeletal muscle cell differentiation	**ATF3 FOS HEYL** *MYOD1*	**ATF3 FOS HEYL**
Transcription by RNA polymerase II	**ABRA** *ADRB2* **ATF3** *CEBPB DLL4* **ETS2 FOS GADD45A** *HES1* **HES6 HEYL IFRD1** *MED23 MYOD1* **MYOG SMAD7**	**ABRA ATF3 ETS2 FOS GADD45A HES6 HEYL** *HYAL2* **IFRD1 MYOG SMAD7** *ZNF750*
Regulation of transcription by RNA polymerase II	**ABRA** *ADRB2* **ATF3** *CEBPB DLL4* **ETS2 FOS GADD45A HES1 HES6 HEYL IFRD1** *MED23 MYOD1* **MYOG SMAD7**	**ABRA ATF3 ETS2 FOS GADD45A HES6 HEYL** *HYAL2* **IFRD1 MYOG SMAD7** *ZNF750*
Regulation of gene expression	**ABRA** *ADRB2* **ATF3** *ATP1B1 CEBPB* **CEBPD** *DLL4* **ETS2 FOS GADD45A** *GPAM HES1* **HES6 HEYL** *HSPB1* **IFRD1** *MED23 MYF6 MYOD1* **MYOG** *PLD1* **SMAD7** *THBS1*	**ABRA ATF3 CEBPD ETS2 FOS GADD45A HES6 HEYL** *HYAL2* **IFRD1 MYOG SMAD7** *ZNF750*
Regulation of biosynthetic process	**ABRA** *ADRB2* **ATF3** *CEBPB* **CEBPD** *DLL4* **ETS2 FOS GADD45A** *HES1* **HES6 HEYL IFRD1** *MED23 MYF6 MYOD1* **MYOG PDK4** *PLD1* **SMAD7** *THBS1*	**ABRA ATF3 CEBPD ETS2 FOS GADD45A HES6 HEYL** *HYAL2* **IFRD1 MYOG PDK4 SMAD7** *ZNF750*
Regulation of macromolecule biosynthetic process	**ABRA** *ADRB2* **ATF3** *CEBPB* **CEBPD** *DLL4* **ETS2 FOS GADD45A** *HES1* **HES6 HEYL IFRD1** *MED23 MYF6 MYOD1* **MYOG** *PLD1* **SMAD7** *THBS1*	**ABRA ATF3 CEBPD ETS2 FOS GADD45A HES6 HEYL** *HYAL2* **IFRD1 MYOG SMAD7** *ZNF750*
Regulation of cellular biosynthetic process	**ABRA** *ADRB2* **ATF3** *CEBPB* **CEBPD** *DLL4* **ETS2 FOS GADD45A** *HES1* **HES6 HEYL IFRD1** *MED23 MYF6 MYOD1* **MYOG PDK4** *PLD1* **SMAD7** *THBS1*	**ABRA ATF3 CEBPD ETS2 FOS GADD45A HES6 HEYL** *HYAL2* **IFRD1 MYOG PDK4 SMAD7** *ZNF750*
Regulation of cellular macromolecule biosynthetic process	**ABRA** *ADRB2* **ATF3** *CEBPB* **CEBPD** *DLL4* **ETS2 FOS GADD45A** *HES1* **HES6 HEYL IFRD1** *MED23 MYF6 MYOD1* **MYOG** *PLD1* **SMAD7** *THBS1*	**ABRA ATF3 CEBPD ETS2 FOS GADD45A HES6 HEYL** *HYAL2* **IFRD1 MYOG SMAD7** *ZNF750*
Wikipathway : Hypertrophy Model	**ATF3 IFRD1 MYOG**	**ATF3 IFRD1 MYOG**

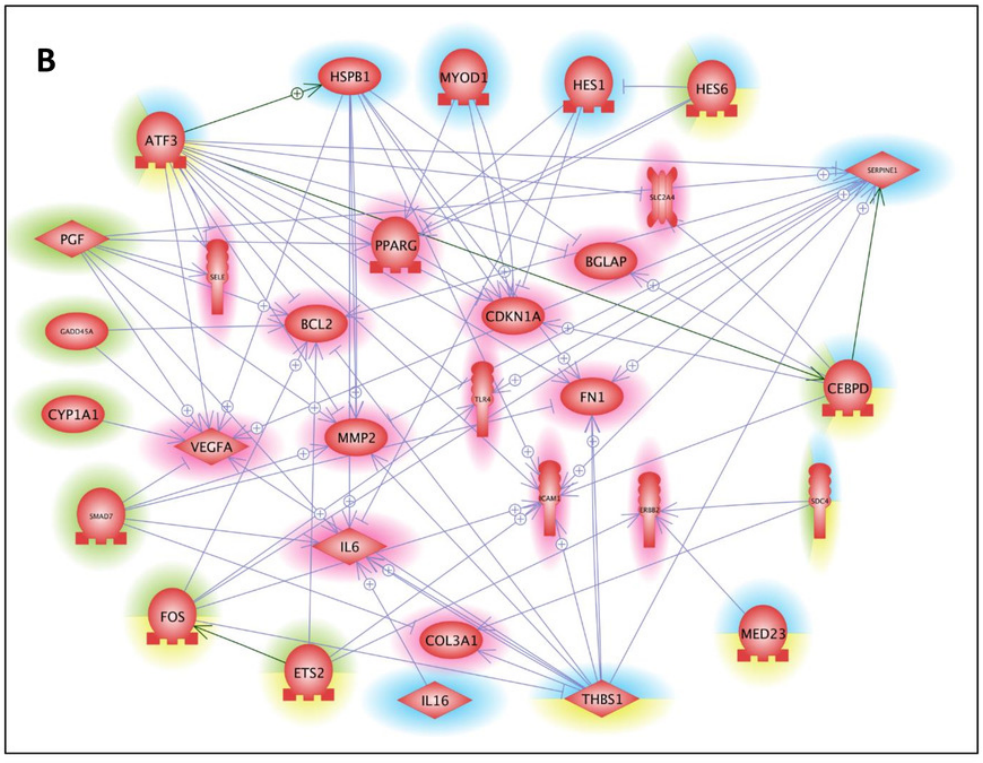
Figure 3

Common regulators and common targets between the DEG targeted by cis-modules in the LT and ST muscle

A/ Common regulators between the DEG targeted by the most represented cis modules for the LT (highlighted in blue) and the ST (highlighted in green), and the DEG targeted by the cis-modules common to both muscles (highlighted in yellow). The list of 10 potential regulators of stress responsive genes included: AKT1, EGF, HIF1A, IFNG, IL1B, INS, MAPK1, MAPK14, TGFB1, and TNF. B/ Common targets between the DEG targeted by the most represented cis modules for the LT (highlighted in blue) and the ST (highlighted in green), and the DEG targeted by the cis modules common to both muscles (highlighted in yellow). The list of 14 potential targets of the DEG included: BCL2, BGLAP, CDKN1A, COL3A1, ERBB2, FN1, ICAM1, IL6, MMP2, PPARG, SELE, SLC2A4, TLR4, and VEGFA

Figure 3





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