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Repressive epigenetic mechanisms, such as the H3K27me3 histone modification, were predicted to affect muscle gene expression and its mineral content in Nelore cattle

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ABSTRACT

Epigenetic repression has been linked to the regulation of different cell states. In this study, we focus on the influence of this repression, mainly by H3K27me3, over gene expression in muscle cells, which may affect mineral content, a phenotype that is relevant to muscle function and beef quality. Based on the inverse relationship between H3K27me3 and gene expression (*i.e.*, epigenetic repression) and on contrasting sample groups, we computationally predicted regulatory genes that affect muscle mineral content. To this end, we applied the TRIAGE predictive method followed by a rank product analysis. This methodology can predict regulatory genes that might be affected by repressive epigenetic regulation related to mineral concentration. Annotation of orthologous genes, between human and bovine, enabled our investigation of gene expression in the *Longissimus thoracis* muscle of *Bos indicus* cattle. The animals under study had a contrasting mineral content in their muscle cells. We identified candidate regulatory genes influenced by repressive epigenetic mechanisms, linking histone modification to mineral content in beef samples. The discovered candidate genes take part in multiple biological pathways, *i.e.,* impulse transmission, cell signalling, immunological, and developmental pathways. Some of these genes were previously associated with mineral content or regulatory mechanisms. Our findings indicate that epigenetic repression can partially explain the gene expression profiles observed in muscle samples with contrasting mineral content through the candidate regulators here identified.

1. Introduction

Adequate mineral content and concentration in mammalian muscle cells is essential to tissue function. Muscle mineral content affects the maintenance of the acid-base homeostasis, osmotic pressure, membrane electric potential, bone and skeletal tissue function, and the transmission of nerve impulses [1]. In addition, mineral content in bovine muscle affects meat quality by impacting sensorial, nutritional, and toxicological factors [2]. Minerals are often components of metalloenzymes and act as enzymatic cofactors, involved in protection against oxidative

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stress, and being relevant to muscle metabolism, hormone synthesis, and protein bonds [3]. Previous work identified genes and genomic regions of relevance to the mineral content of muscle cells in Nelore cattle [4–8]. However, the mechanisms regulating the expression of these genes are not fully understood.

Studies that seek to understand regulatory mechanisms need to consider epigenetics. Amongst epigenetic regulators are the histone modifications (HM), which can repress or activate gene expression, depending on the type of the modification [9]. One of these HM is the Histone-3-Lysine-27-trimethylation (H3K27me3), which usually causes gene inactivation through heterochromatin formation [10,11]. The presence of silencer mechanisms such as H3K27me3 in humans and other eukaryotes are known [10,12]. Often these types of regulatory mechanisms are termed epigenetic repression.

In Holstein cattle, the localization pattern of the H3K27me3 HM along the genome was mapped using lymphocytes [13]. The authors identified that the presence of this HM is enriched in regions of approximately 2 Kb upstream of the transcription start site that is being regulated and that the modification tends to repress genes, especially acting in the promoter regions. In the same cattle breed, genes were differentially affected by H3K27me3 in spermatozoids from bulls that had high or low fertility [14]. The effect of H3K27me3 over gene expression was associated with DNA methylation and replication, as well as nucleosome and chromatin assembling. Kern et al. made publicly available data from genome-wide identification of H3K27me3 in eight tissues from Hereford cattle [15]. Prowse-Wilkins et al. made available the same type of results for six tissues from three lactating Holsteins dairy cows [16]. Briefly, recent literature is accumulating bovine specific data that can be probed to try and understand the regulatory impact of H3K27me3, assumed as an important HM in various tissues. The data accumulated so far emerged from studying *Bos taurus* cattle and may or may not be of relevance to *Bos indicus* cattle.

In this study, we focus on the prediction of gene regulators linked to mineral content in muscle of *Bos indicus* cattle that are putatively repressed by the influence of H3K27me3 or other epigenetic repressive mechanisms. For this, we used Nelore cattle samples that had contrasting mineral content in their muscle tissue. The animals studied were Nelore, the most common beef cattle breed in Brazil. To study patterns of epigenetic repression, we used a prediction methodology called TRIAGE [17]. TRIAGE is an *in-silico* analysis of the impact of the H3K27me3 HM over gene expression, based on the general repressive tendency of H3K27me3 that was previously observed in humans. TRIAGE can identify repressive epigenetic regulation but cannot identify the mechanism linked to this regulation. It is expected that the repression is due to the action of H3K27me3, but it can be done to other repressive epigenetic mechanisms such as methylation or even different histone modifications such as H3K9me3. As H3K27me3 still allows genes to be activated in different cell states [10], *e.g.*, differences in mineral content, we hypothesize that there is a relationship between repressive epigenetic mechanisms and mineral content observed in bovine muscle. To test that, we are proposing the usage of the TRIAGE method with a Rank Product Analysis, enabling sample group comparison. Here, we propose to unveil evidence of this possible relationship, therefore broadening our knowledge of the regulatory processes that affect muscle mineral content, with consequences to tissue function and beef quality.

2. Results

Histone modification H3K27me3 was predicted to regulate muscle gene expression and affect the mineral content observed in beef samples. The identified genes, proposed as being regulators linked to mineral content and repressed by H3K27me3 or other epigenetic repressive mechanisms, had significantly different expressed profiles that could be linked to contrasting mineral content in the *Longissimus thoracis* muscle. Our findings point to an epigenetic regulation for mineral content in the muscle of Nelore cattle.

To perform the TRIAGE [17] combined with Rank Product analyses, we used expression data from an already published RNA-Seq experiment performed with muscle samples selected from Nelore cattle that had been measured for the mass fraction of ten minerals [5,7]. The ten minerals measured were calcium (Ca), copper (Cu), potassium (K), magnesium (Mg), sodium (Na), phosphorus (P), sulphur (S), selenium (Se), iron (Fe) and zinc (Zn). For each mineral, we had Genetic Estimated Breeding Value (GEBV) for the mass fraction so that we could form contrasting groups focusing on the genetic part of the variation, with high GEBV or low GEBV. Mineral content values, GEBV averages and standard deviation errors of each group were published elsewhere [5,7]. These high and low GEBV groups were used to compare H3K27me3 profiles for each mineral through a combination of the TRIAGE methodology [17] and rank product analysis [18]. The TRIAGE methodology [17] calculates the tendency of a gene in a sample to be repressed through its expression value and a specific repressive tendency score (a gene's tendency to be epigenetically repressed). The rank product analysis [18] is a non-parametric method originally proposed to identify differentially ranked genes based on the estimation of false prediction through the comparison of ranks. For more details on these approaches, see the methods section. Fig. 1 shows the steps of our methodology. These methods led to the identification of candidate regulators linked to mineral concentration, *i.e.* genes with a discordant rank, based on discordance scores, that are supposed to be repressed in different forms by epigenetic repression mechanisms when comparing contrasting sample groups for mineral content. The discordance score is a product of the repressive tendency score of a gene and its expression value. Lists of candidate regulators were identified for each mineral, and these results indicate a functional relationship between H3K27me3 or other repressive epigenetic mechanisms and mineral content in Nelore muscle.

2.1. Discordant ranks and discordant scores uncover genes regulated by repressive epigenetic mechanisms in muscle samples with contrasting mineral content

We considered the expression of 14,546 ortholog genes, using the reference *Bos taurus* genome and the reference human genome. These orthologs were considered for further analysis after filtering the RNA-Seq data obtained from the muscle of 113 Nelore steers. We defined discordantly regulated genes (DRGs) for mineral concentration as the candidate regulators for each mineral concentration that are also putatively being affected by a repressive epigenetic mechanism. They have a high tendency to be repressed in several tissues but presented different expression between contrasting groups. They are genes differentially ranked by TRIAGE discordance scores for each contrasting group, considering an estimated percentage of false-positive predictions (pfp) < 0.01 in the rank product analyses. DRGs for a mineral in the high vs low comparison are genes that have a high tendency to be repressed by the presence of the histone modification H3K27me3 and/or other repressive epigenetic mechanisms in the majority of human tissues but present more transcription abundance in the high mineral content group in comparison with the low mineral content group. In the case of the DRGs for the low vs high comparison, the interpretation is the same but with the DRGs presenting more transcription abundance in the low mineral content group in comparison with the high mineral content group. DRGs for both comparisons (high *vs* low and low *vs* high) are genes that tend to be inactivated by H3K27me3 in many cell states but were predicted not to be repressed, although with different expression values, in both extreme conditions of mineral content. The number of DRGs related to the content of each mineral measured, considering both comparisons (high *vs* low and low *vs* high), ranged from 156 (Na) to 218 (K), see Table 1. The results of the rank product analysis for each mineral contrasting group are available in Supplementary Tables S1–S20. Due to the novelty of these analyses, we applied an stringent significance cutoff and only genes with pfp <0.01 were considered significant and used for further analyses.

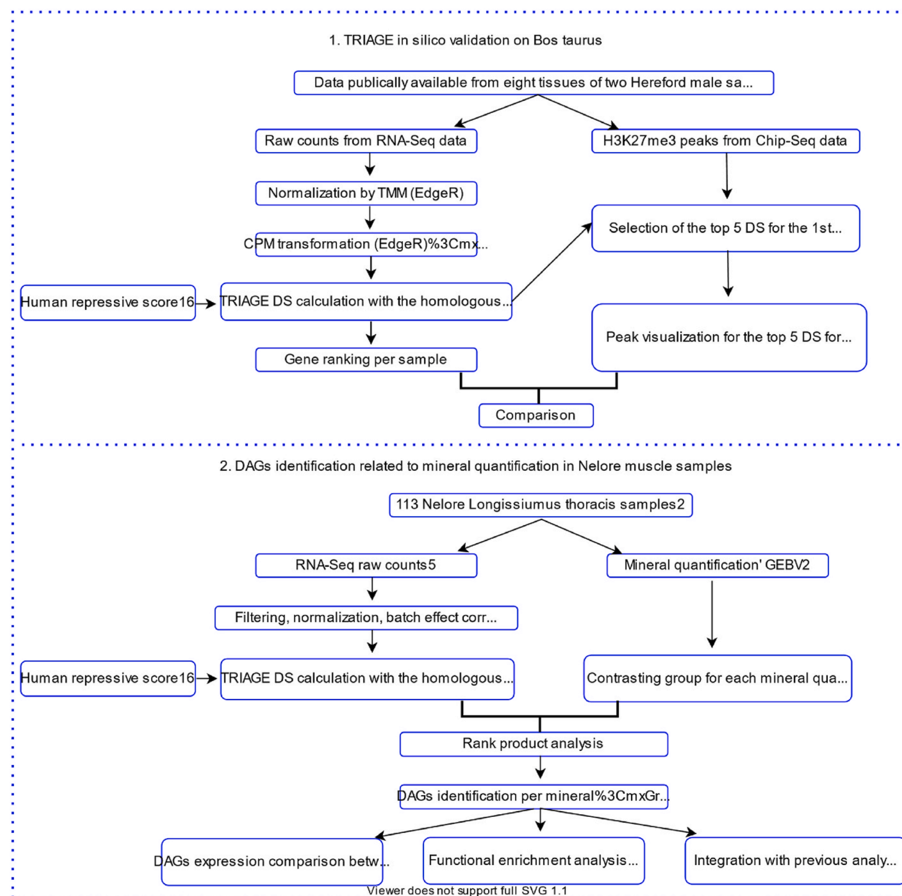

Fig. 1. Steps of the methodology.

Table 1
Number of the discordantly regulated genes (DRGs) between the contrasting groups for each mineral estimated percentage of false-positive predictions 0.01 (pfp).

	High *vs* Low[a]	Low *vs* High[b]	Both[c]	Total
Ca	84	125	0	209
Cu	81	127	1	207
Mg	103	115	5	213
K	106	116	4	218
Na	82	79	5	156
P	100	108	3	205
S	53	112	3	162
Se	102	80	2	180
Zn	96	108	1	203
Fe	65	95	2	158

[a] DRGs in the high *vs* low comparison.
[b] DRGs in the low *vs* high comparison.
[c] Genes that are discordantly activated in both comparisons between contrasting groups.

The *SIM2, LMX1B, COMP, ZIC4, HOXC10, RBFOX3, CDH22, SIM1, CRTAC1, ALX4, PAX5, PAX3, TBX3, NR2E1, GRM4, EN2, LBX1, TMEM132E, PAX9* and *DMRT1* genes were DRGs for at least one comparison for all minerals in this study. These genes have a higher likelihood to be scaping epigenetic repression (*i.e.*, being activated) differently by a mechanism that involves variation in mineral content, turning them into candidate regulators for mineral content in muscle of Nelore cattle. The genes *SIM2, COMP, HOXC10, HOXC4, LMX1B, RBFOX3, CRTAC1, ZIC4, CDH22* and *SIM1*, had a high position in both ranks from each contrasting group (marked in bold in Supplementary Tables S2–S21 and Table 2). Table 2 shows a list of DRGs for each mineral comparison that are also differentially expressed genes (DEGs) [5,7], genes with expression correlated to the specific mineral mass fraction and genes presenting a regulatory impact over the same specific mineral mass fraction (RIF) [8] and the other attributes for these genes, does showing that they were already linked to mineral content in our population. Supplementary Fig. 2 shows the expression differences of the genes in Table 2 between the contrasting groups for the minerals' analysis in which they were DRGs.

Supplementary Table S22 shows the DRGs in common for more than one group comparison. Summarizing the candidate regulators that emerged from the comparisons of contrasting groups for all 10 minerals analized might help to uncover new broadly candidate regulators of mineral content.

We compared the expression values for the DRGs between groups, considering the results for both comparisons together, confirming the expression differences of the DRGs identified for all minerals (*i.e.*, high vs low and low vs high). The expression profiles were used to verify if samples from contrasting groups were clustered, which they did, except for Ca, Na and Se. For these three minerals, one sample in each was out of the expected group, see Supplementary Fig. 1 for expression heatmaps and clustering results. We detailed the Ca analyses results, differences in expression and functional annotation, emphasizing what can be achieved when using TRIAGE and rank product analysis together (Fig. 2A).

2.2. Genes as regulators linked to epigenetic repression, modulated by mineral content, contribute to a variety of biological pathways

The DRGs identified in this study formed a list of candidate regulators linked to mineral content, modulated by epigenetic repression. To study these candidate regulators, we used the STRING app plugin [19] in the Cytoscape software to identify biological pathways enriched within

Table 2
List of discordantly regulated genes (DRGs) for each mineral comparison that are also differentially expressed genes (DEGs) [5,7], genes with expression correlated to the specific mineral mass fraction and genes presenting a regulatory impact over the same specific mineral mass fraction (RIF) [8] and the other attributes for these genes.

Mineral group[a]	DEG	Position[b]	Other attributes	Correlated	Position[b]	Other attributes	RIF	Position[b]	Other attributes
Ca high	*TMEM233*	61							
	SCN3B	67	*trans* eQTL						
Ca low	*COMP*	2		*VDR*	104	TF			
	MAFB	9	TF						
	C1QB	45							
	ITGA11	66							
	MKX	70	TF						
	PDGFRA	74	*trans* eQTL						
	KY	99	*trans* eQTL						
	IGF2	123	*trans* eQTL						
Cu high									
Cu low	*COMP*	1		*BHLHE22*	91	TF			
	EBF1	25	TF						
	COL12A1	44	*cis* eQTL						
	MKX	47	TF						
	RET	63	*cis* e *trans* eQTL						
	C1QB	68							
	TNMD	70							
	C1QC	75							
	CILP2	92							
	PRRX2	107	TF e *trans* eQTL						
K high	***COMP***	42		*ZIC3*	38	TF e *trans* eQTL			
	SCN3B	99	*trans* eQTL						
K low	*CRTAC1*	1		*VDR*	108	TF			
	COMP	35							
	SLIT3	53							
	PRRX2	68	TF e *trans* eQTL						
	ACTC1	89	*trans* eQTL						
	CRABP2	112							
Mg high	***COMP***	28		*ZIC3*	35	TF e *trans* eQTL			
Mg low	*CRTAC1*	1							
	IGF2	12	*trans* eQTL						
	COMP	30							
	SLIT3	62							
	PRRX2	90	TF e *trans* eQTL						
Na high				*ZIC3*	67	TF e *trans* eQTL			
Na low									
P high	***COMP***	80							
P low	*CRTAC1*	1							
	COMP	26							
	PRRX2	58	TF e *trans* eQTL						
	ACTC1	86	TF e *trans* eQTL						
S high	***COMP***	52							
S low	*HPCAL4*	1		*BCL11B*	47	TF			
	COMP	24		*VDR*	72	TF			
				IKZF3	96	TF			
Se high	*DLK1*	32							
	NR4A2	60	TF						
Se low	*COMP*	7		*LGR6*	13				
				SRRM4	35				
Zn high				*ZIC3*	45	TF			
Zn low				*GRM4*	5	*trans*_eQTL			
				TNR	47		*TNR*	47	
Fe high									
Fe low	*HPCAL4*	12		*HPCAL4*	12				
				TSPEAR	27	*cis* e *trans* eQTL			

[a] Contrasting groups regarding mineral mass fraction.
[b] Rank position of the DRGs by the rank product analysis results according to the pfp ranking.

our DRGs lists of candidate regulators for each mineral. STRING was also used to identify the known protein-protein interactions (PPIs) for the proteins codified by the DRGs. The presence of a protein-protein interaction among DRGs taking part in the same pathways or related pathways can imply an inference of this pathway regulation related to mineral content. We identified 19 enriched metabolic pathways, concordant across the analyses for nine minerals, all except Cu. Fig. 2B shows the enriched pathways for the Ca analysis. Supplementary Fig. 3 shows the concordant enriched pathways for all minerals minus the Cu, because there are no significant enriched pathways for the Cu analysis.

The neuronal system metabolic pathway was enriched in the analysis pertaining seven minerals (*i.e.,* Ca, K, Mg, P, S, Zn and Fe). The DRGs in this pathway were not the same for each different mineral, but most of them encode potassium voltage-gated channels and some encode synapse accessory proteins. The second pathway, enriched for K, Mg, Se and Fe, was the presynaptic depolarization and calcium channel opening, with DRGs encoding a combination of two, three or four voltage-dependent R-type calcium channel subunits. Both pathways were related to neural impulse transmission. Supplementary Table S1 lists the candidate regulators and their pathways from this enrichment analysis.

The MAPK signalling pathway, signalling pathways regulating pluripotency of stem cells, and transcriptional mis-regulation in cancer,

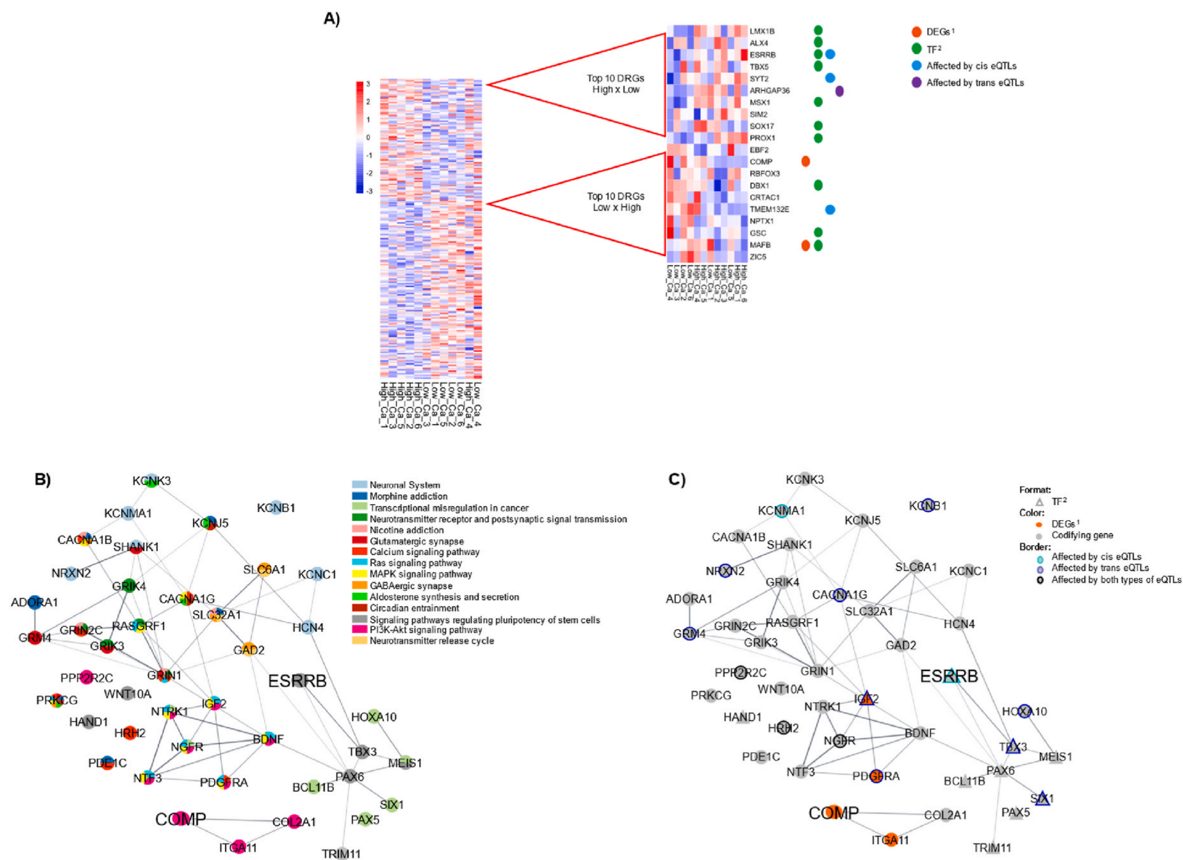

Fig. 2. Expression, metabolic pathways, and attributes representation for the Calcium results. A) Heatmap with the expression of all 209 discordantly regulated genes (DRGs) for the Ca analysis, considering both comparisons together with a more detailed representation of the top 10 DRGs for Ca analysis High x Low and Low x High and its attributes. B) Protein-protein interaction networks connecting the DRGs from Ca analyses were enriched for Ca signalling pathways. Just the genes being part of enriched pathways are shown and the pathways are marked. C) Protein-protein interaction networks predicted by the protein codified by the DRGs for Ca being part of enriched networks. Just the genes being part of enriched pathways are shown and the attributes regarding previously published work are marked. The line thickness between two proteins indicates the strength of data support. [1]Differentially expressed genes between sample groups contrasting for Ca content [7], [2]Transcription factors.

were enriched for three minerals each (Ca, Mg and S; Ca, Mg and Na and Ca, Mg and S, respectively). The DRGs emerging from the analyses for each mineral varied, but they have similarities in terms of their function. They encode genes for Voltage-dependent R-type Ca channel subunits and neurotrophic related proteins in the MAPK signalling pathway. Additionally, they encode Wnt proteins and proteins involved in the pluripotency of stem cell regulation. For transcriptional mis-regulation in cancer, they encode homeobox and paired-box proteins and receptors.

Neurotransmitter receptors and postsynaptic signal transmission were pathways enriched for two minerals, *i.e.,* Ca and Se. Within these pathways, the genes *GRIK4* and *GRIK3* genes encoded Glutamate ionotropic receptors kainite type subunits and were DRGs for both Ca and Se. For Ca alone three genes are of interest: the *GRIN1* and *GRIN2C* DRGs that encode Glutamate ionotropic receptors N-methyl D-aspartate type subunits, and the *RASGRF1* DRG that encodes a Ras protein. In contrast, the DRGs for Se encode GABA and acetylcholine receptors, as well as a potassium channel protein.

The PI3K-Akt signalling pathway was also enriched for two minerals, Ca and Mg, presenting seven DRGs listed for both minerals: *NTRK1, COMP, PDGFRA, IGF2, COL2A1, NGFR* and *PPP2R2C*. These genes encode growth factor receptors, a neurotrophic tyrosine kinase receptor, phosphatase, collagen and matrix proteins.

In addition to the myriad of pathways described above, another 12 pathways were enriched for specific minerals (*i.e.*, not overlapping with other minerals), but also mainly related to receptors, synapses, and signalling. For K, the STRING analysis raised a link with immunology, because the complement cascade pathway was enriched. For Se, the list of DRGs were enriched for genes linked to the development biology pathway.

2.3. Differential expression analyses partially agree with the discordant regulation analyses, and recapitulate regulatory mechanisms previously linked to muscle mineral content

To further characterize the candidate regulators identified above, we compared the lists of DRGs with previous published analyses of RNA-seq data that had identified genes of relevance to muscle mineral content in cattle, with the same samples [5,7]. We identified 24 DRGs that were previously described as differentially expressed genes (DEGs) for mineral content [5,7]. Eleven DRGs were also genes with expression values correlated to the mass fraction of specific minerals [8]. For example, one DRG seems to regulate the Zn mass fraction [8]. For all minerals, except Zn, we identified DEGs that were DRGs for the same mineral. Among them, *COMP* is a DEG that is also a DRG for seven out of the ten studied minerals. Some DRGs are known transcription factors (TFs) [20] connected to *cis* and *trans* expression Quantitative Trait Loci (QTLs) in Nelore muscle [21] (Table 2). However, no DRG was related to allele specific expression QTLs [22]. These annotations for the discovered DRG help to bring gene expression and regulatory context to our findings.

The regulatory context and the enriched pathways were visualized by constructing protein-protein interaction networks (Supplementary Fig. 3). To provide supporting evidence to our visualizations, the DRGs'

networks used known protein-protein interactions and well-established pathway knowledge. An overview of the complete analyses for Ca content, from heatmaps to annotated networks, are provided in Fig. 2. The overlap between our current analyses and the previously published work on differential gene expression enabled further annotation of the DRG network associated with Ca content (Fig. 2C). Annotation of DRGs as being TF or part of *cis* and *trans* expression QTLs contributes to our understanding of how epigenetic mechanisms interplay with other regulatory mechanisms to impact gene expression and mineral content (see Supplementary Tables S2–S22 for detailed annotation of all DRGs discovered).

3. Discussion

Epigenetics has long been an "elephant in the room" for the field of animal breeding and genetics. Epigenetic mechanisms contribute to gene expression, working together with gene-environment interactions to result in a phenotype [23]. Animal genomes still lack in epigenetic annotation, indicating a gap in knowledge where our study can be placed. Broadly speaking, functional annotation of genomes, including epigenetic studies, is relevant to improve livestock systems, as proposed by the FAANG project [24].

Studies on the H3K27me3 histone modification need to consider three points: (i) the recruitment of sequence-specific chromatin modifiers, (ii) the ability of H3K27me3 to be a template for the polycomb repressive complex-2 (PRC2) to bind and modify other neighbouring nucleosomes and, (iii) polycomb group proteins recruitment via polycomb group response elements for polycomb group domain formation [25]. This is a complex histone modification mechanism. Therefore, understanding how the H3K27me3 mark works and what causes its presence or absence across the genome is an onerous task. The TRIAGE method was created to facilitate this task, and it can be applied to any cell type or species where H3K27me3 is a conserved epigenetic mechanism for the regulation of cell identity [17]. Even though it focuses on the H3K27me3 HM, authors of the TRIAGE suggests that it can detect regulators being affected by other repressive epigenetic mechanisms, since different repressive mechanisms can be in place in different cells or different organisms.

In this study, we applied the TRIAGE method to identify gene regulators linked to mineral content in Nelore cattle muscle samples, uncovering epigenetic mechanisms related to gene expression profiles and mineral content. Our results led to the discovery of regulatory genes linked to mineral content, with predicted divergences in their regulation by epigenetic repressive mechanisms, mainly by the H3K27me3 HM. These results, together with previous knowledge, create a working hypothesis for epigenetic modulation of mineral content in muscle cells.

Gene silencing by H3K27me3 is mainly linked to genes associated with cell fate in embryonic development and cell differentiation [10]. A limited number of studies relate H3K27me3 with traits other than developmental stages or diseases. In *Arabidopsis thaliana*, it is known that H3K27me3 histone modification contributes to the regulation of TFs that are themselves key regulators of iron uptake, as a response to Fe deficiency. Hypothetically, H3K27me3 regulates the induction of TFs target genes under Fe deficiency to facilitate iron acquisition while preventing iron overload [26]. This study in a plant model indicates that a relationship between mineral content and H3K27me3 is possible. In another example, during cell inflammation in mice, bacterial products and cytokines induce the expression of Jmjd3 - a protein that binds target genes of the polycomb group and removes H3K27me3 to activate genes involved in macrophage transdifferentiation [27]. Both examples showcase the dynamic aspects of H3K27me3 effects over gene expression, which can be modified in response to a specific stimulus, be that Fe content or the presence of specific proteins.

The link between H3K27me3 or other epigenetic repressive mechanisms and mineral content is a working hypothesis, emerging from our results. Our results do not allow the understanding on how the regulatory processes involved in the gene expression regulation prediction by repressive epigenetic mechanisms linked to mineral content works. The top DRGs candidate regulators linked to Ca content align with previous results from differential expression analyses of groups contrasting for Ca content. These DRGs had been annotated as regulators (*i.e.*, TFs), regulated (affected by expression QTLs) or differentially expressed themselves in groups contrasting for Ca content. In short, our application of the TRIAGE method with the Rank Product Analysis identified regulators linked to mineral content in muscle tissue.

In our work, the DRGs linked to Fe content were enriched in the neuronal system, the presynaptic depolarization pathway, and calcium channel opening. One specific DRG for Fe content was the *HPCAL4* gene, which was found as a differentially expressed gene for Fe content [5]. *HPCAL4* gene encodes a protein from the superfamily of visinin-like Ca binding, which can impair Fe absorption. *HPCAL4* was highly expressed in muscle samples of animals predicted to have low Fe content, as per their genetic estimated breeding values [5]. Furthermore, *HPCAL4* was negatively correlated to Fe content in this same population [8], which corroborates its Ca binding function as a mechanism reducing Fe absorption.

We hypothesize that the 20 DRGs for all minerals are the best candidate regulators emerging from our analyses, due to the corroborating evidence about their epigenetic regulation being related to mineral content. These DRGs encode TFs and proteins linked to development and expression regulation. These are genes that are expected to be regulators linked to mineral concentration and that are regulated by H3K27me3 or other repressive epigenetic mechanisms [28]. Tissue regeneration shares aspects with embryonic development [29]. Among the DRGs, *HOX* [30], *PAX* [31] (specially *PAX3*) and other developmental genes mediate aspects of differentiation and injury response in mammalian adult musculature. These functional mechanisms in muscle might overlap with embryonic development mechanisms and explain why the DRGs in our study were enriched for embryonic development. Embryonic development is a primary known target of H3K27me3 regulation.

The enriched pathways are mostly related to neural impulse transmission through mineral channels, synaptic related proteins, receptors, and neurotropic related proteins. As separate analyses, Ca, Mg, S and Se have enriched pathways related to development and regulatory proteins. In a sense, our enrichment analysis, confirms a link between epigenetic repression, development and regulatory proteins, in adult tissues, that is vastly discussed in the literature [29]. Of note, the complement cascade pathway was enriched for K. This pathway can act on inflammation, a process that can be suppressed by K [32].

The *UTX* gives a known link between H3K27me3 HM and neural impulse transmission. This gene encodes an enzyme that demethylates the H3K27me3, which starts the activation of many genes. The deletion of the *UTX* gene in mice hippocampus leads to reduced potency and amplitude of postsynaptic current, aberrant dendrite development and defective synapse formation [33].

Some of our DRGs were already linked to a mineral content in previous studies with the same Nelore population [5–8]. We believe that our DRGs are gene regulators and are discordantly activated between contrasting mineral content in Nelore muscle samples. We also believe that this discordant activation is due to epigenetic mechanisms. The H3K27me3 is a possible mechanism regulating the DRGs, especially those known to be regulated by this HM between human tissues. Other DRGs were previously identified as TFs, affected by *cis* and *trans* eQTLs or as ASE genes in our population. This suggests that this HM can, to some degree, underlie these eQTLs.

Although it is not possible to claim that all DRGs are regulated by epigenetic mechanisms, our results propose a new perspective to incorporate a layer of epigenetic information regarding gene regulation in Nelore muscle in association to mineral content variation. More direct studies of HM are warranted to confirm our ideas and current results.

4. Conclusion

In summary, muscle gene expression linked to mineral content in Nelore cattle can be partially regulated by epigenetic mechanisms. To provide supporting evidence to the emerging hypothesis, we focused on DRGs that overlapped with pathway enrichment analysis and with previous analyses of differential gene expression linked to mineral content in the same population. Our DRGs are new candidate regulators that deserve further investigation so that they can be placed in the mechanisms that explain H3K27me3 or other epigenetic repression regulating gene expression. This work is also proposing a new application to the TRIAGE method: a framework to link epigenetics to phenotypes with the use of Rank Product Analysis, unveiling gene regulators and the possible role of histone modification in mineral content phenotypes.

4.1. Methods

All methods were performed in accordance with the relevant guidelines and regulations. This study was carried out in compliance with the ARRIVE guidelines. The experimental procedures were approved by the Ethical Committee of Embrapa Pecuária Sudeste (São Carlos, São Paulo, Brazil, CEUA 01/2013).

4.2. Nelore sample population

Our sample population was formed by 113 Nelore steers. These animals were part of a bigger population composed of 373 Nelore steers from half-sibling families born in two different farms and two breeding seasons (2008 and 2009). At approximately 21 months old, the animals were transferred to Embrapa Pecuária Sudeste (São Carlos, São Paulo, Brazil). After 28 days of adaptation, they were maintained in a feedlot with *ad libitum* access to food and water. During this period, the animals received a diet that do not affect the mineral content of the muscle. The diet was formed by 40% of dry matter containing corn silage, crude protein, ground corn, soybean meal, cottonseed, soybean hull, limestone, mineral mixture, urea and monensin (Rumensin®). At the slaughter, with the animals at around 25 months old, samples from *Longissimus thoracis* were dissected between the 11th and 13th ribs, frozen in liquid nitrogen, and conserved at −80 °C until further analysis. Further experimental details were published elsewhere [2].

4.3. Phenotype obtention

From the L. *thoracic* samples, we used here the quantification of Ca, Cu, K, Mg, Na, P, S, Se, Zn and Fe already described elsewhere [2]. Briefly, the muscle samples were lyophilized, digested, and the minerals' mass fractions were quantified. Selenium was determined by inductively coupled plasma mass spectrometry (ICP-MS 820-MS, Varian, Mulgrave, Australia). The other minerals' quantification was obtained by inductively coupled plasma optical emission spectrometry (ICP OES, Vista Pro-CCD with a radial view, Varian, Mulgrave, Australia).

The Genetic estimated breeding values (GEBV) for each mineral mass fraction were calculated elsewhere [2] to all 373 Nelore steers forming the original population, using a Bayesian model implemented in the GenSel software [34]. The linear model considered birthplace, feedlot location and breeding season as attributes in the contemporary groups and age at slaughter as a linear covariate.

4.4. RNA-seq: sequencing, quality control and gene expression data

Total RNA was extracted from 113 Nelore samples using Trizol® (Life Technologies, Carlsbad, CA) and the RNA integrity was verified in a Bioanalyzer 2100® equipment (Agilent, Santa Clara, CA, USA) [35].

We followed RNA-Seq procedures for our samples as previously described [5]. The RNA-sequencing libraries were made with the TruSeq® sample preparation kit and the sequencing was performed with a pair-ending protocol in an Illumina HiSeq 2500® sequencer (Illumina Inc., San Diego, CA, USA). The libraries and sequencing were performed at the Genomics Center at ESALQ, Piracicaba, São Paulo, Brazil.

As a quality control, the reads with less than 65 base pairs and Phred score lower than 24 were excluded from further analysis. The reads that passed this filter were aligned to the bovine reference genome version ARS-UCD 1.2 using the STAR software v.2.5.4 with the default parameters [36]. The same software was used to obtain the gene expressions in counts.

We removed genes not expressed in at least 20% of the samples (22 samples). The batch effect occurrence was tested through a principal component analysis implemented with the NOISeq software v.2.16.0 [37]. We tested for the batch effects of the birthplace, breeding season, age at the slaughter, slaughter group and a combination of sequencing flowcell and lanes. The batch effect test pointed to a significant effect for the combination of sequencing flowcell and lanes. We normalized the expression data with the VST function from DESeq2 software [38] and corrected the batch effect with the ARSyNseq function of the NOISeq software v.2.16.0 [37], as described in our previous work [8].

4.5. Repressive tendency score extrapolation to Bos taurus

We applied the same repressive tendency score extracted from human data to orthologous genes in *Bos taurus* as the original paper suggested [17]. We obtained the list of 16,340 orthologs using the online software BioMart [39] from the Ensembl site, based on the bovine reference genome ARS-UCD 1.2.

The repressive tendency score indicates if a gene is differentially exposed to inactivation due to the presence of the H3K27me3. We extrapolated this score using the same published repressive tendency score [17] for humans in the bovine genes presenting orthology one to one for human genes.

4.6. DS calculation and gene ranking

Based on the repressive tendency score attributed to each expressed gene with orthology one to one between *Bos taurus* and humans, and the gene expression from Nelore muscle sample from the RNA-Seq data, we calculated the discordant score for each gene in each sample as previously described [17]:

$$DS_i = \ln(Y_i + 1).RTS_i$$

Where the discordant score (DS_i) is given for each gene i through the product of its logarithmic (ln) expression (Y_i) and its repressive tendency score (RTS_i). This score indicates the degree of expressional discordance given gene-specific epigenetic repressive tendency. A high discordance score suggests that the gene is highly expressed despite its expected high repressive tendency in diverse tissue types due to the presence of H3K27me3, and therefore can be a regulator in that specific sample. All genes for each sample were separately ranked in accord with the discordant score. We implemented the prediction only in contrasting sample groups for the GEBV for 10 different mineral mass fractions to compare the predictions between mineral extremes.

4.7. Sample groups contrasting for mineral quantification

The contrasting sample groups for the GEBV from mineral mass fraction formed by six samples with high GEBV and six samples with low GEBV for each mineral were the ones used before in a study that identified differentially expressed genes in Nelore cattle muscle related to the mass fraction of all minerals in this study, except Fe. We chose the top 12 contrasting samples for Fe based on the GEBV for Fe mass fraction calculated elsewhere [5].

4.8. Rank product analysis

After determining the discordance scores for all genes across all samples forming the contrasting groups, we performed rank product analysis between low and high GEBV for each mineral. We aimed to identify discordantly ranked genes (DRGs) related to the phenotypes, the candidate regulators linked to each mineral content. We used the R package RankProd to compare the gene rankings between contrasting groups based on the discordance scores for each gene and sample inside the contrasting groups [18]. Genes presenting pfp <0.01 after rank comparison between contrasting groups were considered DRGs. This restrictive threshold was chosen because of the novelty of this analysis and the fact that it is an extrapolation from human data.

DRGs are candidate regulators. Also, they are genes in which the expression varies more concerning the prediction made by TRIAGE between contrasting groups for mineral quantification. These gene sets have a high tendency to be repressed by the presence of the histone modification H3K27me3 or other repressive epigenetic mechanisms in the majority of the tissues, but present different transcription abundance between sample groups in this case. The final results are lists of candidate regulators, DRGs for each mineral in comparisons of high mineral content versus low mineral (high *vs* low) content and low mineral content versus high mineral content (low *vs* high). Both comparisons gave us candidate regulators.

For a gene to be high ranked by the TRIAGE method and then present discordances between groups by the rank product analysis, it needs to have its expression differing among groups give a fix Repressive Tendency Score, which defines each gene's association to H3K27me3 broad domains in humans.

4.9. DRGs' expression

We used the pheatmap R package (https://CRAN.R-project.org/package=pheatmap) to access the expression differences of the DRGs identified for both comparisons of each mineral together. This analysis was performed to identify if the expression patterns of the DRGs are different between contrasting groups of the same mineral.

4.10. Functional enrichment analysis

The STRING app v.1.6.0 [19] for the Cytoscape software v.3.9.1 was used to analyze functional enrichment of known metabolic pathways (*i.e.,* KEGG and Reactome pathways) and known protein-protein interactions (PPI) for the DRGs for each mineral. The STRING app uses the known PPI for your species of interest and shows you the ones formed by your genes of interest, in this case, the separate DRG for each mineral analysis. The app also informs other annotation analyses, such as enriched pathways and biological functions. The search was performed with the *Bos taurus* database, with the option of load enrichment data, considering just pathways with p-values <0.05 and with the threshold parameters (confidence cutoff $=0.4$ and maximum additional interactions $=0$).

4.11. Integration with previous analysis

The DRG lists were compared to gene sets previously published with the same muscle Nelore samples regarding mineral mass fraction. These data encompass expressed TFs [20], differentially expressed genes for mineral mass fraction [5,7], genes correlated to mineral content [8], genes with regulatory impact over mineral mass fraction [8], genes containing SNPs presenting allelic specific expression [40], genes being affected by aseQTLs [22] in the muscle and genes containing or being affected by eQTLs [21] identified in muscle.

Author contribution

J.A., W.J.S., M.B., M.R.S.F., C.F.G., A.R.A.N. and L.C.A.R. designed the experiments and analysis. J.A. and W.J.S. performed the analysis. G.B.M performed the GEBV analyses. A.Z. was responsible for the data organization and maintenance. J.A., W.J.S., M.B., M.R.S.F., W.J.S.D., A.O.L., M.I.P.R., T.F.C., J.J.B., L.L.C. and L.C.A.R. interpreted the results and J.A., W.J.S. and L.C.A.R. drafted the manuscript. All authors revised and approved the final manuscript.

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Declaration of competing interest

The authors claim no conflict of interests.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrep.2023.101420.

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