HSPs, Ubiquitins and Antioxidants aid in Heat Tolerance in Tharparkar Indicine cattle

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Abstract

Background

Heat stress induced by high environmental temperature and humidity affects livestock production and health. With global warming on the uprise, indigenous cattle known for their heat tolerance are gaining importance than the crossbreds. However, systems biology behind this phenotype in indigenous cattle is less known. In this study using transcriptome analysis, we identified key molecules and pathways that may lead to the heat tolerance phenotype in indigenous cattle (Tharparkar breed)

Results

The number of DEGs in Crossbred were found to be more than in Tharparkar suggesting a greater dysregulation in systems biology in Crossbred. A contrast in gene expression was observed with 18.5 % of upregulated genes in Crossbred (Vrindavani cattle) downregulated in Tharparkar and 17.5% upregulated genes in Tharparkar downregulated in Crossbred. The increased HSPs levels have been found positively correlated with tolerance in many species. Upregulation of *HSF*, *HSP70*, *HSP90*, and activation of eIF2 signaling pathway in Tharparkar and vice-versa in Crossbred delineates how Tharparkar withstands heat stress. Unlike Crossbred, Tharparkar is not only endowed with higher expression of the scavengers (*UBE2G1*, *UBE2S*, and *UBE2H*) of misfolded proteins but also with protectors (*VCP*, *Serp1*, and *CALR*) of naïve unfolded proteins. Further, the apoptotic genes that were dysregulated in both genetic groups indicated a relatively higher probability of apoptosis in Crossbred than in Tharparkar. Also, higher expression of the antioxidants in Tharparkar enables it to cope up with higher levels of free radicals generated as a result of heat stress.

Conclusion

In this study we found relevant molecules/genes dysregulated in Tharparkar in the direction that can counter heat stress. To best of our knowledge this is a comprehensive comparison between Tharparkar and crossbred at a global level using transcriptome analysis.

Background

Cattle being homoeothermic, modulate their internal body temperature with sync to environmental temperature by equilibrating the amount of heat produced within the body and its dissipation to the ambient environment. The stress that arises due to disproportionate thermodynamic behavior between cattle and its surrounding environment is termed as heat stress [1]. Environmental induced hyperthermic stress lowers feed intake, which in turn reduces growth, milk production, reproductive efficiency thereby negatively affecting the economics of livestock keepers [2-4]. It has been associated with reduced fertility through its deleterious impact on oocyte maturation and early embryo development [5]. Increased morbidity and mortality was observed by lowering the immune response in immune-compromised animals under heat stress [6].

India has a wide variety of indigenous cattle breeds distributed throughout its agro-climatic zones. These are known for their natural tolerance to tropical heat [7, 8].To meet the growing demand for milk in India several crossbreeding programs were taken up. Every state had its own crossbreeding policy, which is agro-climatic and breed-specific. Crossbreds were found notwithstanding harsh climate, being susceptible to tropical diseases and require a constant input of good management conditions [8]. The adaptive capacity to heat stress varies between species and genetic groups within species. Among various adaptive mechanisms, physiological adaptability seems to be the primary step in cattle. Sahiwal cows better regulate body temperature in response to heat stress than Karan Fries [8]. It was observed that Ongole cattle rely on the respiration rate to maintain thermal balance, while, Bali cattle rely on rectal temperature [9]. In Brazil, Sindhi and Girolando breeds showed better physiological response to thermal stress than Gir cattle [10]. Increase in respiration rate was reported in Nellore breed when exposed to heat load [11].

Though it is known that Indigenous breeds are heat tolerant than the exotic breeds and Crossbreds, studies explaining the difference between these genetic groups have been done mainly to address the physiological responses vis – a – vis heat stress and very few studies at the genomic level have been taken up [12, 13]. The systems biology behind a phenotype is most commonly studied by transcriptome profiling [14, 15]. RNA-seq is a high throughput approach to measure relative global changes in the transcripts under specific condition(s) [14, 16, 17]. RNA - seq allows for analysis of transcriptome in an unbiased way, with, a tremendous dynamic detection range (>8,000 fold), and low background signals [18]. It has been used as an investigating tool in understanding disease pathogenesis [19, 20] and differential physiological response to various biotic and abiotic factors [21, 22].

In this study, we evaluated the whole genome expression level of the Peripheral blood mononuclear cell (PBMCs) of the two genetic groups- Crossbred (Vrindavani) and Indigenous cattle breed (Tharparkar) to understand their differential response to heat stress.

Results

Physiological Parameters

The overview of the analysis is given in Figure 1. Respiration rate (RR), rectal temperature (RT) and T3 level increased significantly ($p<0.05$) on 7th-day post heat stress in both the genetic groups (n=5) (Figure 2). However, the increase was found significantly higher in Crossbred than in Tharparkar.

Comparison of DEGs of Crossbred and Tharparkar under heat stress

Under heat stress, global expression profiles of Crossbred and Tharparkar were identified with 6042 and 4718 DEGs, respectively (Supplementary Table 1) . Among these, 3481 DEGs were found common between the two genetic groups, and 2561 and 1238 DEGs were uniquely found in Crossbred and Tharparkar, respectively (Figure 3a). Additionally, 3132 and 2924 genes were upregulated and downregulated in Crossbred and, 2367 and 2358 genes were upregulated and downregulated in Tharparkar, respectively (Figure 3b). On comparison of upregulated and downregulated genes, 724 and 1416 genes were found uniquely upregulated and 514 and 1145 genes were found uniquely downregulated in Tharparkar and Crossbred, respectively. The comparison also revealed that 17.5% of upregulated genes (1278) in Tharparkar were downregulated in Crossbred and 18.5% downregulated genes (1344) in Tharparkar were found upregulated in Crossbred. However, the number of common upregulated and downregulated genes in both the genetic groups were 357 (4.9%) and 498 (6.8%), respectively (Figure 3c).

Functional analysis of knowledge-based genes

Heat shock genes have been found dysregulated under heat stress in both breeds. Most of the HSPs -*HSPA4*, *HSPB8*, *HSPA1A* (HSP 70), *HSPA8* (HSP 70), *HSP90AB1* (HSP 90alpha) and *HSP90AA1* (HSP 90beta) and heat shock protein regulating factors- *HSF1* and EEF1A1 have been found to be downregulated/not-differentially expressed in Crossbred but upregulated in Tharparkar. However, CAMK2D that is involved in the regulation of expression of heat shock genes are upregulated in Crossbred and downregulated in Tharparkar. Among the apoptotic genes, *BCL2L11*, *FASLG*, *TICAM2*, *TLR4*, *APC*, *CASP3*, *MAPK8*, *MLKL*, *XIP*, *VIM*, and *HMGB2* were found to be upregulated in Crossbred and downregulated in Tharparkar. The number of upregulated genes involved in antioxidants was found to be more in Tharparkar than in Crossbred. Among these, *GPX3*, *NUDT2*, *CAT*, *CYCS*, *CCS*, *PRDX5*, *PRDX6*, *PRDX1*, *SOD1*, and *CYBB* were found either downregulated/not-differentially expressed in Crossbred and upregulated in Tharparkar. More number of genes involved in Ubiquitination were differentially expressed in

Crossbred than in the Tharparkar. Genes like *UBE2G1, UBE2S, UBE2H, UBA52,* and *UBA1* have been found downregulated/ not-differentially expressed in Crossbred and upregulated in Tharparkar. However, *VCP, RNF40*, and *UBE2L3* have been found downregulated in Crossbred but not-differentially expressed in Tharparkar. Among the genes involved in Unfolded Protein folding response (UPR) - *MBTPS1, CREB3L1, SERP1 GSK3A, EIF2S3, SRPA, CALR,* and *SERP1* have been found downregulated in Crossbred and upregulated in Tharparkar (Figure 4).

PPI network revealed functional importance of HSP70 (*HSPA8* and *HSPA1A)* and ubiquitin (UBB, UBA52), in coordinating genes involved in heat stress

The PPI networks were constructed for the knowledge-based genes that were common between Tharparkar and Crossbred. In the PPI networks, hubs define the functional and structural importance of a network. The genes, which act as hubs in PPI networks were found to be *UBB, UBA52, HSPA8,* and *HSPA1A* (Figure 5). Among the 4, *UBB* was downregulated in both genetic groups and the rest were downregulated in Crossbred and upregulated in Tharparkar.

Canonical pathway analysis by IPA revealed contrast in signaling pathways in Crossbred and Tharparkar

Canonical pathways associated with Crossbred and Tharparkar are represented in Figure 6a and 6b. In Crossbred, Oncostatin M Signaling, Phospholipase C Signaling, EIF2 Signaling, Integrin Signaling, IL-3 Signaling, and CXCR4 Signaling were found to be highly inactivated and PTEN signaling was found to be highly activated. In Tharparkar, EIF2 Signaling, Androgen Signaling, Oncostatin M Signaling, α-Adrenergic Signaling, BMP signaling pathway, and UVC-Induced MAPK Signaling were found to be highly activated and PTEN signaling was found to be inactivated. The canonical pathway Oncostatin M Signaling and eIF2 Signaling were found to have the highest ratio of genes involved vis-a-vis the genes in the database in Crossbred and Tharparkar, respectively.

While carrying out comparative analysis through IPA, Calcium-induced T Lymphocyte Apoptosis, BMP signaling pathway, UVC-Induced MAPK Signaling, Regulation of Cellular Mechanics by Calpain Protease, fMLP Signaling in Neutrophils, Melatonin Signaling, and Leukocyte Extravasation Signaling, were found inactivated in Crossbred and activated in Tharparkar (Figure 7). Genes involved in Oncostatin M Signaling- *GRB2, HRAS, JAK1, JAK3, MAP2K1, MAPK1, OSM, RAP1B, RAP2A, STAT1, STAT5B, TYK2,* and *RRAS* were found

downregulated in Crossbred and upregulated in Tharparkar (Figure 8a, b). While the key genes involved in PTEN Signaling pathway – *FASLG, RAP2A, BIM, CASP3* and *MSP58* were found upregulated in Crossbred and downregulated in Tharparkar as well (Figure 9a, b).

Variation in microRNAs and Transcription factors

In Crossbred, 111 miRNAs were found to be inactivated and 37 activated. In Tharparkar, 205 miRNAs were found to be inactivated and 272 activated. Among them, 52 microRNAs were found common between the two genetic groups. Most of the common miRNAs were found activated in Crossbred and inactivated in Tharparkar (Figure 10). miR-4779, miR-4651, miR-1207-5p, miR-6967-5p and miR-504-3p are the top 5 miRNAs that were activated in Crossbred and inactivated in Tharparkar.

Various Transcription factors were found to regulate the expression of the identified DEGs. Transcription factors, 19 in Tharparkar (11activated and 8 inactivated) and 26 in Crossbred (8activated and 18 inactivated) were identified in IPA that regulate the expression of DEGs. Among them, *PAX5, MTA3, MYC, PROX1* and *SMAD7* in Crossbred and, *HMGA1, MAF, MAX NOTCH22* and *NCOR1* in Tharparkar are top5 upregulated and activated TFs. On comparing the TFs of Tharparkar and Crossbred, it was found that *BHLHE40, HMGA1, HMGB1, IKZF1,* and *TCF7* were found to be common. *BHLHE40, HMGA1,* and *TCF7* were found to be activated in Tharparkar and inactivated in Crossbred and it was vice - versa with *HMGB1* and *IKZF1* (Figure 11)

Real-time validation.

To confirm the dysregulation of genes, qRT-PCR was used to validate the expression under heat stress. The expression of genes (selected based on their role in heat stress) was in concordance with the RNA- Seq results (Figure 12)

Discussion

Heat stress is a natural phenomenon that affects domestic animals in tropical, sub-tropical and often in temperate regions of the world during summer months. Heat and humidity during the

summer months combine to make an uncomfortable environment for dairy cattle. Heat stress negatively impacts a variety of dairy parameters resulting in economic losses [23]. Response to heat stress varies with species and genetic groups within species [5, 24, 25]. In this study, the global transcriptome of genetic groups – Crossbred and Tharparkar cattle under heat stress was evaluated to understand their differential response to heat stress.

Animals (n=5) of both the genetic groups were exposed to a temperature of 42 °C for 7 days. Around 5th- 6th day, short term heat acclimation occurs [26, 27]. This time point was selected to understand the differences in systems biology to heat stress in the two genetic groups. Initially, heat stress indicators - RR, RT, and T3 level were evaluated. RR was found to increase in both genetic groups under heat treatment and the increase in Crossbred was found to be significantly ($P<0.05$) different from that in Tharparkar. A positive correlation exists between RR and heat treatment [28-30]. This increase is an attempt to dissipate excess body heat by vaporizing more moisture in expired air or response to a greater requirement of oxygen by tissues under heat stress. Also, the physiological response to heat stress includes reduced heat production, which is achieved by lowering feed intake and thyroid hormone secretion [31]. T3 level increases under heat stress [32, 33]. A significant increase in T3 level in Crossbred as compared to Tharparkar indicates an effective regulatory mechanism in modulating T3 levels in Tharparkar in response to heat stress. The T3 triggered metabolism may be one of the reasons that increase heat production resulting in increased rectal temperature in Crossbred in comparison to Tharparkar as was found in our study. The significant increase in RR, RT and T3 level in Crossbreed than in Tharparkar, suggests the inability of Crossbred to cope up with high stress in comparison to Tharparkar.

A phenotype is defined by the changes in systems biology. Transcriptome profiling by RNA-seq is the most common methodology to study the changes in systems biology. RNA profiling based on next-generation sequencing enables to measure and compare gene expression patterns [16]. The global transcriptome of Tharparkar and Crossbred indicated differential response to heat stress as evident from the DEGs, that are either distinct to both or have a difference in expression. The number of DEGs in Crossbred were found to be more than in Tharparkar suggesting a greater dysregulation in systems biology in Crossbred. Among the dysregulated genes, the number of upregulated genes were more than the downregulated genes in both genetic groups. However, a contrast in expression was observed with 18.5 % of upregulated genes in

Crossbred downregulated in Tharparkar and 17.5% upregulated genes in Tharparkar downregulated in Crossbred.

While exploring the DEGs at a functional level, it was found that most of heat shock genes were found upregulated in Tharparkar and downregulated in Crossbred. The increased HSPs levels have been found positively correlated with tolerance in many species[34, 35]. HSF1, that positively regulates the transcription of *HSP70* and *HSP90* [36, 37] was found upregulated in Tharparkar and downregulated in Crossbred. Pathway analysis through IPA revealed eIF2 signaling pathway to be highly activated in Tharparkar and inactivated in Crossbred under heat stress. Upregulation of *HSF, HSP70, HSP90*, and activation of eIF2 signaling pathway in Tharparkar and vice-versa in Crossbred delineates how Tharparkar withstands heat stress. In an attempt to ensure that the HSP70 in Tharparkar is maintained at an optimum level, dysregulation of *CAMK2D* and *GSK3A* seems to act as negative feedback. CAMK2D that induces the transcription of HSP70 via HSF1 [38] has been found downregulated in Tharparkar. GSK3A that inhibits the trimerization of HSF1 that is needed for the induction of HSP70 [39] has been found upregulated in Tharparkar. The decreased level of HSP70 in Crossbred makes it inevitable that such negative feedbacks would further reduce its level and *GSK3A* was found downregulated and *CAMK*, upregulated.

Ubiquitination is positively correlated with heat tolerance [40, 41]. Ubiquitin-Proteasome System (UPS) regulates the levels of proteins and acts by removing the misfolded or damaged proteins that may accumulate as a result of exposure to abiotic stress. Malfunctioning of ubiquitin-proteasome system UPS could have negative consequences for protein regulation, including loss of function [42]. In Tharparkar after heat acclimation, HSP70 tends to activate the ubiquitination pathway to minimize the accumulation of the unfolded proteins that can't be refolded by it [43]. This pathway activation is supported by upregulation of E3 ligases - *UBE2G1, UBE2S*, and *UBE2H* that catalyze covalent attachment of E3 to unfolded proteins [44-47] in Tharparkar. USP7 that deubiquitinates target proteins [48, 49] was found upregulated in Crossbred and downregulated in Tharparkar. Further, a group of molecules – *VCP, Serp1*, and *CALR* that ensure the protection of naïve proteins during their transport within the cell [50-52]were found upregulated in Tharparkar and downregulated in Crossbred. Unlike Crossbred, Tharparkar is not only endowed with higher expression of the scavengers of misfolded proteins but also with protectors of naïve unfolded proteins.

Activation of apoptosis pathway is one of the major physiological processes linked with heat stress. Among the apoptotic genes, *BCL2L11, FASLG, MLKL, CASP3, MAPK8,* and VIM have been found upregulated in Crossbred and downregulated in Tharparkar under heat stress. BCL2L11 induces apoptosis by neutralizing key molecules of pro-survival BCL2 sub-family [53, 54], FASLG transduces the apoptotic signal into cells[55, 56], CASP3 activates caspases and executes apoptosis [57], and MAPK8, MLKL, and VIM also induce apoptosis [58, 59]. PTEN signaling pathway that drives apoptosis [60, 61] was found inactivated in Tharparkar and activated in Crossbred. This indicates a relatively higher probability of finding apoptosis in Crossbred than in Tharparkar.

The ability to balance the ROS and antioxidant level, is one of the key factors that would determine the tolerance of an individual to heat stress. The antioxidant triad of GPX, SOD, and CAT that forms the first line of defense against reactive oxygen species [62-64], was found upregulated in Tharparkar and downregulated in Crossbred. Additionally, genes belonging to Peroxiredoxins - *PRDX3, PRDX5* and *PRDX6* that catalyzes the reduction of hydrogen peroxide and organic hydroperoxides [65-69],were also found upregulated in Tharparkar and were either downregulated or not-differentially expressed in Crossbred. Higher expression of the antioxidants in Tharparkar enables it to cope up with higher levels of free radicals generated as a result of heat stress while Crossbred is unable to do so.

Conclusion

A contrast in expression was observed with 18.5 % of upregulated genes in Crossbred downregulated in Tharparkar and 17.5% upregulated genes in Tharparkar downregulated in Crossbred. Transcripts of molecules that stimulate heat shock response, Ubiquitination, unfolded protein response and antioxidant level were found upregulated in Tharparkar and downregulated in Crossbred. EIF2 Signaling that promotes protein translation and PTEN signaling that drives apoptosis were found activated and inactivated in Tharparkar, respectively and vice-versa in Crossbred. We found relevant molecules/genes dysregulated in Tharparkar in the direction that can counter heat stress. A proposed contrasting interplay of molecules in both two groups is shown

in Figure 13. To best of our knowledge this is a comprehensive comparison between Tharparkar and crossbred at a global level using transcriptome analysis.

Methods

Experimental condition and Ethical Statement

The animals used for the study were from the Indian Veterinary Research Institution. The permission to conduct the study was granted by Indian Veterinary Research Institutional Animal Ethics Committee (IVRI-IAEC) under the Committee for Control and Supervision of Experiments on Animals (CPCSEA), India, vide letter no 387/CPSCEA. Prior to experiment, the animals -5 Tharparkar and 5 Crossbred (Vrindavani) cattle, were acclimatized for 15 days outside the Psychometric chamber. Tharparkar is one among the best dairy breeds in the Indian subcontinent, adapted to the Indian states of Punjab and Haryana. Tharparkar cattle are lyre horned type of zebu cattle that have been frequently used for upgrading local low-yielding cattle breeds [70, 71]. Vrindavani is a synthetic Crossbred cattle strain of India with exotic inheritance of Holstein-Friesian, Brown Swiss, Jersey and indigenous inheritance of Hariana cattle [72]. The experiment was conducted during October when the environmental THI was 73.0242. These animals were exposed in Psychometric chamber at 42 °C for six hours for 7 days (THI =78.5489). All the animals were fed with wheat straw and concentrate mixture in 60:40 ratios. Respiration rate (RR) and rectal temperature (RT) of animals from each genetic group were measured on 0 day (Control) before exposure to Psychometric chamber and on 7th day of heat exposure (Treated). Blood samples were collected from the animals at the mentioned time points and serum concentration of Triiodothyronine (T3) was estimated by RIA technique using T_3 ^{125}I (Immunotech) as per the manufacturer's instructions.

RNA sequencing

PBMCs were collected from the blood Samples **.** Total RNA from each of the collected samples (PBMCs) was isolated using the RNeasy Mini kit (Qiagen GmbH, Germany) according to the manufacturer's protocol. The integrity and quantity of isolated RNA were assessed on a Bioanalyzer 2100 (Agilient Technologies, Inc). The library was prepared using NEBNext Ultra RNA Library Prep Kit for Illumina (NewEngland Biolabs Inc.) following the manufacturer's

protocol. Approximately, 100ng of RNA from each sample was used for RNA library preparation. The quality of the libraries was assessed on Bioanalyzer. Libraries were quantified using a Qubit 2.0 Fluorometer (Life technologies) and by qPCR. Library (1.3ml, 1.8pM) was denatured, diluted and loaded onto a flowcell for sequencing. cDNA library preparation and Illumina Sequencing was performed at Sandor Life Sciences Pvt. (Hyderabad, India). Finally, the RNA-seq data were provided in FASTQ format.

Raw data processing

The reads generated were paired end and 150bp in length. Quality control checks on raw sequence data from each sample were performed using FastQC (Babraham Bioinformatics). Processing of the data was performed using prinseq-lite software [73] to remove reads of low quality (mean phred score 25) and short length (< 50) for downstream analysis.

Identification of Differentially Expressed Genes (DEGs)

Bos taurus reference genome (release 94) and its associated gene transfer file (GTF) were downloaded from Ensembl FTP genome browser [74]. The reference genome was prepared and indexed by RNA-Seq by expectation maximization (RSEM) [75] by rsem-prepare-reference command. Further, the clean reads obtained from filtering of raw data were aligned to the indexed reference genome by Bowtie2 [76] to estimate transcript abundance in counts by rsem-calculate-expression command. To compare the gene expression levels among different samples, the aligned reads were used to generate a data matrix by rsem-generate-data-matrix command. The data was submitted to the GEO database with accession number GSE136652. In each genetic group, all the samples of day 0 (Control) were compared with the day 7 (treated) for the calculation of differential gene expression by edgeR [77] package. A P-value threshold of 0.05 was applied to determine transcripts significantly differentially expressed between treated and control samples. The Ensemble IDs were converted to the respective gene ID by g: Convert of g: Profiler [78, 79].

Functional Analysis of DEGs

Under heat stress four major physiological processes are found to be usually associated - Induction of apoptosis [80, 81]; Ubiquitination [82, 83]; elicitation of unfolded protein response (UPR) in cells [84] and ; Imbalance in production of ROS and antioxidants [85, 86]. The genes involved in these processes were retrieved from Reactome database [87]. From this data set the

genes that were differentially expressed in both the genetic groups were extracted to study the contrast in their expression between genetic groups. Their protein-protein interaction network was also studied in both the genetic groups.

Predicted protein-protein interaction of the knowledge-based genes

Protein-protein interaction (PPI) network among the knowledge-based DEGs that were found common to both Tharparkar and Crossbred was retrieved using interactions available in the String database [88]. The degree was calculated using igraph package (https://cran.r-project.org/web/packages/igraph/ index.html). The PPI network was then visualized using Cytoscape software V. 3.7 [89]

Ingenuity Pathway Analysis (IPA) Analysis

QIAGEN's IPA (QIAGEN, Redwood City, USA) [90] was used to analyze the identified DEGs of Crossbred and Tharparkar. The list of DEGs was used to identify the canonical pathways and the most significant biological processes against Ingenuity Pathways Knowledge Base (IKB). Core analysis for each dataset was performed to know activated (Z score > 2) or inactivated (Z score < −2) canonical pathways. Upstream regulators- Transcription factors and microRNAs were also identified.

Validation of reference genes identified

Genes - *BCL2L11, FASLG, CASP3, CAT, SOD1, GSK3A, CALR, HSF1, APC,* and *GPX3* were selected based on their role in heat stress and qRT-PCR was performed on Applied Biosystems 7500 Fast system. *GAPDH* was taken as the internal control. Each of the samples was run in triplicates and relative expression of each gene was calculated using the $2^{-\Delta\Delta CT}$ method with control as the calibrator [91].

Statistical Analysis

Student's *t*-test was done in JMP9 (SAS Institute Inc., Cary, USA) to test the significance of the difference between the control and treated. Differences between groups were considered significant at $P \leq 0.05$.

Declarations

Ethics approval and consent to participate

The permission to conduct the study was granted by Indian Veterinary Research Institutional Animal Ethics Committee (IVRI-IAEC) under the Committee for Control and Supervision of Experiments on Animals (CPCSEA), India, vide letter no 387/CPSCEA.

Consent for publication

Not applicable.

Availability of data and materials

The data was submitted to the GEO database with accession number GSE136652

Competing interests

None of the authors had a conflict of interest to declare

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Authors' contributions

AKT and RG conceived and designed the research. SG, SmS, AS,AV, VV , PK , ShS and GS conducted the wet lab work. RINK, ARS, NH, WAM, MRP, SK , AP and RG analyzed the data. RINK, ARS, MRP, RG , AS and GS helped in manuscript drafting and editing. AKT and RG proofread the manuscript

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Abbreviations

NUDT2	Nudix Hydrolase 2
BIM	Bcl-2-like protein 11
MSP58	Microspherule protein 1
SMAD7	Mothers against decapentaplegic homolog 7
XIP	Late endosomal/lysosomal adaptor and MAPK and MTOR activator 5
HSPA4	Heat shock 70 kDa protein 4
GPX3	Glutathione peroxidase 3
UBE2G1	Ubiquitin-conjugating enzyme E2 G1
VCP	Valosin-containing protein
MBTPS1	Membrane-bound transcription factor site-1 protease
GRB2	Growth factor receptor-bound protein 2
FASLG	Tumor necrosis factor ligand superfamily member 6
PAX5	Paired box protein Pax-5
HMGA1	High mobility group protein A
BHLHE40	Class E basic helix-loop-helix protein 40
HSPB8	Heat shock protein beta-8
UBE2S	Ubiquitin-conjugating enzyme E2
RNF40	RING finger protein 40
CREB3L1	Cyclic AMP-responsive element-binding protein 3-like protein 1
RAP2A	Ras-related protein Rap-2a
MTA3	Metastasis-associated protein MTA3
MAF	Transcription factor Maf
HSPA1A	Heat shock 70 kDa protein 1A
TICAM2	TIR domain-containing adapter molecule 2
CAT	Catalase
UBE2H	Ubiquitin-conjugating enzyme E2 H
UBE2L3	Ubiquitin-conjugating enzyme E2 L3
SERP1	Stress-associated endoplasmic reticulum protein 1
JAK1	Janus kinase 1
MYC	Myc proto-oncogene protein

MAX	MYC Associated Factor X
HMGB1	High mobility group protein B1
TCF7	Transcription factor 7
HSPA8	Heat shock cognate 71 kDa protein
TLR4	Toll-like receptor 4
CYCS	Cytochrome c
UBA52	Ubiquitin A-52 residue ribosomal protein fusion product 1
EIF2S3	Eukaryotic translation initiation factor 2 subunit 3
JAK3	Tyrosine-protein kinase JAK3
CASP3	Caspase-3
PROX1	Prospero homeobox protein 1
NCOR1	Nuclear receptor corepressor 1
IKZF1	Ikaros family zinc finger protein 1
HSP90AB1	Heat shock protein HSP 90-beta
APC	Adenomatous polyposis coli protein
CCS	Copper chaperone for superoxide dismutase
UBA1	Ubiquitin-activating enzyme E1
MAP2K1	MAP kinase kinase 1
HSP90AA1	Heat shock protein HSP 90-alpha
PRDX5	Peroxiredoxin-5
CALR	Calreticulin
MAPK1	Mitogen-activated protein kinase 1
MAPK8	Mitogen-activated protein kinase 8
PRDX6	Peroxiredoxin-6
OSM	Oncostatin-M (OSM)
MLKL	Mixed lineage kinase domain-like protein
PRDX1	Peroxiredoxin-1
GSK3A	Glycogen synthase kinase-3 alpha
RAP1B	Ras-related protein Rap-1b
SOD1	Superoxide dismutase
VIM	Vimentin
CYBB	Cytochrome b-245 heavy chain
STAT1	Signal transducer and activator of transcription 1-alpha/beta
HMGB2	High mobility group protein B2
STAT5B	Signal transducer and activator of transcription 5B
TYK2	Non-receptor tyrosine-protein kinase
RRAS	Ras-related protein R-Ras (p23)
PBMCs	Peripheral blood mononuclear cell
RIA	Radioimmunoassay
THI	Temperature Humidity Index
Cxcr4	C-X-C chemokine receptor type 4

PTEN	Phosphatase And Tensin-Like Protein
EIF2	Eukaryotic translation initiation factor 2
BMP	Bone Morphogenetic Proteins
FMLP	N-formyl-methionyl-leucyl-phenylalanine

References

1. Parsell D, Lindquist S: **The function of heat-shock proteins in stress tolerance: degradation and reactivation of damaged proteins**. *Annual review of genetics* 1993, **27**(1):437-496.
2. Rout P, Kaushik R, Ramachandran N: **Differential expression pattern of heat shock protein 70 gene in tissues and heat stress phenotypes in goats during peak heat stress period**. *Cell Stress and Chaperones* 2016, **21**(4):645-651.
3. Mehla K, Magotra A, Choudhary J, Singh A, Mohanty A, Upadhyay R, Srinivasan S, Gupta P, Choudhary N, Antony B: **Genome-wide analysis of the heat stress response in Zebu (Sahiwal) cattle**. *Gene* 2014, **533**(2):500-507.
4. Armstrong DV: **Heat stress interaction with shade and cooling**. *J Dairy Sci* 1994, **77**(7):2044-2050.
5. Rojas-Downing MM, Nejadhashemi AP, Harrigan T, Woznicki SA: **Climate change and livestock: Impacts, adaptation, and mitigation**. *Climate Risk Management* 2017, **16**:145-163.
6. Hahn G: **Dynamic responses of cattle to thermal heat loads**. *Journal of animal science* 1999, **77**(suppl_2):10-20.
7. Dash S, Chakravarty A, Singh A, Upadhyay A, Singh M, Yousuf S: **Effect of heat stress on reproductive performances of dairy cattle and buffaloes: A review**. *Veterinary world* 2016, **9**(3):235.
8. Renaudeau D, Collin A, Yahav S, De Basilio V, Gourdine J, Collier RJ: **Adaptation to hot climate and strategies to alleviate heat stress in livestock production**. *Animal* 2012, **6**(5):707-728.
9. Berman A: **Invited review: Are adaptations present to support dairy cattle productivity in warm climates?** *Journal of Dairy Science* 2011, **94**(5):2147-2158.
10. Rashamol VP, Sejian V, Bagath M, Krishnan G, Archana PR, Bhatta R: **Physiological adaptability of livestock to heat stress: an updated review**. *J Anim Behav Biometeorol* 2018, **6**(3):62-71.
11. Aritonang S, Yuniati R, Abinawanto, Imron M, Bowolaksono A: **Physiology response of the indigenous cattle breeds to the environment in West Sumbawa, Indonesia**. In: *AIP Conference Proceedings: 2017*: AIP Publishing; 2017: 030098.
12. Cardoso C, Peripolli V, Amador S, Brandão E, Esteves G, Sousa C, França M, Gonçalves F, Barbosa F, Montalvão T: **Physiological and thermographic response to heat stress in zebu cattle**. *Livestock Science* 2015, **182**:83-92.
13. Valente ÉEL, Chizzotti ML, de Oliveira CVR, Galvão MC, Domingues SS, de Castro Rodrigues A, Ladeira MM: **Intake, physiological parameters and behavior of Angus and Nellore bulls subjected to heat stress**. *Semina: Ciências Agrárias* 2015, **36**(2):4565-4574.
14. Salleh M, Mazzoni G, Höglund J, Olijhoek D, Lund P, Løvendahl P, Kadarmideen H: **RNA-Seq transcriptomics and pathway analyses reveal potential regulatory genes and molecular mechanisms in high-and low-residual feed intake in Nordic dairy cattle**. *BMC genomics* 2017, **18**(1):258.

15. Cánovas A, Rincon G, Islas-Trejo A, Wickramasinghe S, Medrano JF: **SNP discovery in the bovine milk transcriptome using RNA-Seq technology**. *Mammalian genome* 2010, **21**(11-12):592-598.
16. Wani SA, Sahu AR, Khan RIN, Pandey A, Saxena S, Hosamani N, Malla WA, Chaudhary D, Kanchan S, Sah V: **Contrasting gene expression profiles of monocytes and lymphocytes from Peste-des-petits-ruminants virus infected goats**. *Frontiers in Immunology* 2019, **10**.
17. Manjunath S, Mishra BP, Mishra B, Sahoo AP, Tiwari AK, Rajak KK, Muthuchelvan D, Saxena S, Santra L, Sahu AR: **Comparative and temporal transcriptome analysis of peste des petits ruminants virus infected goat peripheral blood mononuclear cells**. *Virus research* 2017, **229**:28-40.
18. Mortazavi A, Williams BA, McCue K, Schaeffer L, Wold B: **Mapping and quantifying mammalian transcriptomes by RNA-Seq**. *Nature methods* 2008, **5**(7):621.
19. Wang Z, Gerstein M, Snyder M: **RNA-Seq: a revolutionary tool for transcriptomics**. *Nature reviews genetics* 2009, **10**(1):57.
20. Li P, Piao Y, Shon HS, Ryu KH: **Comparing the normalization methods for the differential analysis of Illumina high-throughput RNA-Seq data**. *BMC bioinformatics* 2015, **16**(1):347.
21. Khanduri A, Sahu AR, Wani SA, Khan RIN, Pandey A, Saxena S, Malla WA, Mondal P, Rajak KK, Mishra B: **Dysregulated miRNAome and proteome of PPRV infected goat PBMCs reveal a coordinated immune response**. *Frontiers in immunology* 2018, **9**:2631.
22. Pandey A, Sahu AR, Wani SA, Saxena S, Kanchan S, Sah V, Rajak KK, Khanduri A, Sahoo AP, Tiwari AK: **Modulation of host miRNAs transcriptome in lung and spleen of Peste des Petits ruminants virus infected sheep and goats**. *Frontiers in microbiology* 2017, **8**:1146.
23. O'Loughlin A, Lynn DJ, McGee M, Doyle S, McCabe M, Earley B: **Transcriptomic analysis of the stress response to weaning at housing in bovine leukocytes using RNA-seq technology**. *BMC genomics* 2012, **13**(1):250.
24. Salama A, Caja G, Hamzaoui S, Badaoui B, Castro-Costa A, Facanha D, Guilhermino M, Bozzi R: **Different levels of response to heat stress in dairy goats**. *Small Ruminant Research* 2014, **121**(1):73-79.
25. Polsky L, von Keyserlingk MA: **Invited review: Effects of heat stress on dairy cattle welfare**. *Journal of dairy science* 2017, **100**(11):8645-8657.
26. Ross J, Hale B, Gabler N, Rhoads R, Keating A, Baumgard L: **Physiological consequences of heat stress in pigs**. *Animal Production Science* 2015, **55**(12):1381-1390.
27. Mayorga EJ, Renaudeau D, Ramirez BC, Ross JW, Baumgard LH: **Heat stress adaptations in pigs**. *Animal Frontiers* 2018, **9**(1):54-61.
28. Dangi SS, Dangi SK, Chouhan V, Verma M, Kumar P, Singh G, Sarkar M: **Modulatory effect of betaine on expression dynamics of HSPs during heat stress acclimation in goat (Capra hircus)**. *Gene* 2016, **575**(2):543-550.
29. Garrett A, Goosens N, Rehrer N, Patterson M, Harrison J, Sammut I, Cotter J: **Short-term heat acclimation is effective and may be enhanced rather than impaired by dehydration**. *American journal of human biology* 2014, **26**(3):311-320.
30. Blackshaw JK, Blackshaw A: **Heat stress in cattle and the effect of shade on production and behaviour: a review**. *Australian Journal of Experimental Agriculture* 1994, **34**(2):285-295.
31. Beede D, Collier R: **Potential nutritional strategies for intensively managed cattle during thermal stress**. *Journal of Animal Science* 1986, **62**(2):543-554.
32. Ganaie A, Ghasura R, Mir N, Bumla N, Sankar G, Wani S: **Biochemical and physiological changes during thermal stress in bovines: A review**. *Iranian Journal of Applied Animal Science* 2013, **3**(3):423-430.
33. Hansen P: **Physiological and cellular adaptations of zebu cattle to thermal stress**. *Animal reproduction science* 2004, **82**:349-360.

34. Magdub A, Johnson H, Belyea R: **Effect of environmental heat and dietary fiber on thyroid physiology of lactating cows**. *Journal of Dairy Science* 1982, **65**(12):2323-2331.
35. Williamson R, Misson B, Davison T: **The effect of exposure to 40 on the heat production and the serum concentrations of triiodothyronine, thyroxine, and corticosterone in immature domestic fowl**. *General and comparative endocrinology* 1985, **60**(2):178-186.
36. Westerheide SD, Anckar J, Stevens SM, Sistonen L, Morimoto RI: **Stress-inducible regulation of heat shock factor 1 by the deacetylase SIRT1**. *Science* 2009, **323**(5917):1063-1066.
37. Prodromou C: **Mechanisms of Hsp90 regulation**. *Biochemical Journal* 2016, **473**(16):2439-2452.
38. Holmberg CI, Hietakangas V, Mikhailov A, Rantanen JO, Kallio M, Meinander A, Hellman J, Morrice N, MacKintosh C, Morimoto RI: **Phosphorylation of serine 230 promotes inducible transcriptional activity of heat shock factor 1**. *The EMBO journal* 2001, **20**(14):3800-3810.
39. Wang X, Grammatikakis N, Siganou A, Calderwood SK: **Regulation of molecular chaperone gene transcription involves the serine phosphorylation, 14-3-3ε binding, and cytoplasmic sequestration of heat shock factor 1**. *Molecular and cellular biology* 2003, **23**(17):6013-6026.
40. Ryu KY, Maehr R, Gilchrist CA, Long MA, Bouley DM, Mueller B, Ploegh HL, Kopito RR: **The mouse polyubiquitin gene UbC is essential for fetal liver development, cell-cycle progression and stress tolerance**. *The EMBO journal* 2007, **26**(11):2693-2706.
41. Lyzenga WJ, Stone SL: **Abiotic stress tolerance mediated by protein ubiquitination**. *Journal of experimental botany* 2011, **63**(2):599-616.
42. Sulistio YA, Heese K: **The ubiquitin-proteasome system and molecular chaperone deregulation in Alzheimer's disease**. *Molecular neurobiology* 2016, **53**(2):905-931.
43. Fernández-Fernández MR, Gragera M, Ochoa-Ibarrola L, Quintana-Gallardo L, Valpuesta JM: **Hsp70–a master regulator in protein degradation**. *FEBS letters* 2017, **591**(17):2648-2660.
44. Pabarcus MK, Hoe N, Sadeghi S, Patterson C, Wiertz E, Correia MA: **CYP3A4 ubiquitination by gp78 (the tumor autocrine motility factor receptor, AMFR) and CHIP E3 ligases**. *Archives of biochemistry and biophysics* 2009, **483**(1):66-74.
45. David Y, Ziv T, Admon A, Navon A: **The E2 ubiquitin-conjugating enzymes direct polyubiquitination to preferred lysines**. *Journal of Biological Chemistry* 2010, **285**(12):8595-8604.
46. Sheng Y, Hong JH, Doherty R, Srikumar T, Shloush J, Avvakumov GV, Walker JR, Xue S, Neculai D, Wan JW: **A human ubiquitin conjugating enzyme (E2)-HECT E3 ligase structure-function screen**. *Molecular & Cellular Proteomics* 2012, **11**(8):329-341.
47. Kaiser P, Seufert W, Höfferer L, Kofler B, Sachsenmaier C, Herzog H, Jentsch S, Schweiger M, Schneider R: **A human ubiquitin-conjugating enzyme homologous to yeast UBC8**. *Journal of Biological Chemistry* 1994, **269**(12):8797-8802.
48. Li M, Chen D, Shiloh A, Luo J, Nikolaev AY, Qin J, Gu W: **Deubiquitination of p53 by HAUSP is an important pathway for p53 stabilization**. *Nature* 2002, **416**(6881):648.
49. Zhao Y, Majid MC, Soll JM, Brickner JR, Dango S, Mosammaparast N: **Noncanonical regulation of alkylation damage resistance by the OTUD4 deubiquitinase**. *The EMBO journal* 2015, **34**(12):1687-1703.
50. Kadowaki H, Nagai A, Maruyama T, Takami Y, Satrimafitrah P, Kato H, Honda A, Hatta T, Natsume T, Sato T: **Pre-emptive quality control protects the ER from protein overload via the proximity of ERAD components and SRP**. *Cell reports* 2015, **13**(5):944-956.
51. Hori O, Miyazaki M, Tamatani T, Ozawa K, Takano K, Okabe M, Ikawa M, Hartmann E, Mai P, Stern DM: **Deletion of SERP1/RAMP4, a component of the endoplasmic reticulum (ER) translocation sites, leads to ER stress**. *Molecular and cellular biology* 2006, **26**(11):4257-4267.
52. Nauseef WM, McCormick SJ, Clark RA: **Calreticulin functions as a molecular chaperone in the biosynthesis of myeloperoxidase**. *Journal of Biological Chemistry* 1995, **270**(9):4741-4747.

53. O'Connor L, Strasser A, O'Reilly LA, Hausmann G, Adams JM, Cory S, Huang DC: **Bim: a novel member of the Bcl-2 family that promotes apoptosis**. *The EMBO journal* 1998, **17**(2):384-395.
54. Marani M, Tenev T, Hancock D, Downward J, Lemoine NR: **Identification of novel isoforms of the BH3 domain protein Bim which directly activate Bax to trigger apoptosis**. *Molecular and cellular biology* 2002, **22**(11):3577-3589.
55. Muneta Y, Shimoji Y, Inumaru S, Mori Y: **Molecular cloning, characterization, and expression of porcine Fas ligand (CD95 Ligand)**. *Journal of Interferon & Cytokine Research* 2001, **21**(5):305-312.
56. Ruiz-García R, Mora S, Lozano-Sánchez G, Martínez-Lostao L, Paz-Artal E, Ruiz-Contreras J, Anel A, González-Granado LI, Moreno-Pérez D, Allende LM: **Decreased activation-induced cell death by EBV-transformed B-cells from a patient with autoimmune lymphoproliferative syndrome caused by a novel FASLG mutation**. *Pediatric research* 2015, **78**(6):603.
57. Nicholson DW, Ali A, Thornberry NA, Vaillancourt JP, Ding CK, Gallant M, Gareau Y, Griffin PR, Labelle M, Lazebnik YA: **Identification and inhibition of the ICE/CED-3 protease necessary for mammalian apoptosis**. *Nature* 1995, **376**(6535):37.
58. Dai R, Frejtag W, He B, Zhang Y, Mivechi NF: **c-Jun NH2-terminal kinase targeting and phosphorylation of heat shock factor-1 suppress its transcriptional activity**. *Journal of Biological Chemistry* 2000, **275**(24):18210-18218.
59. Cao W-X, Li T, Tang Z-H, Zhang L-L, Wang Z-Y, Guo X, Su M-X, Chen X, Lu J-J: **MLKL mediates apoptosis via a mutual regulation with PERK/eIF2α pathway in response to reactive oxygen species generation**. *Apoptosis* 2018, **23**(9-10):521-531.
60. Lu X-X, Cao L-Y, Chen X, Xiao J, Zou Y, Chen Q: **PTEN inhibits cell proliferation, promotes cell apoptosis, and induces cell cycle arrest via downregulating the PI3K/AKT/hTERT pathway in lung adenocarcinoma A549 cells**. *BioMed research international* 2016, **2016**.
61. Chen C-Y, Chen J, He L, Stiles BL: **PTEN: tumor suppressor and metabolic regulator**. *Frontiers in endocrinology* 2018, **9**.
62. Lee YS, Kim AY, Choi JW, Kim M, Yasue S, Son HJ, Masuzaki H, Park KS, Kim JB: **Dysregulation of adipose glutathione peroxidase 3 in obesity contributes to local and systemic oxidative stress**. *Molecular Endocrinology* 2008, **22**(9):2176-2189.
63. Bierl C, Voetsch B, Jin RC, Handy DE, Loscalzo J: **Determinants of human plasma glutathione peroxidase (GPx-3) expression**. *Journal of Biological Chemistry* 2004, **279**(26):26839-26845.
64. Ighodaro O, Akinloye O: **First line defence antioxidants-superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX): Their fundamental role in the entire antioxidant defence grid**. *Alexandria Journal of Medicine* 2018, **54**(4):287-293.
65. Tsuji K, Copeland N, Jenkins N, Obinata M: **Mammalian antioxidant protein complements alkylhydroperoxide reductase (ahpC) mutation in Escherichia coli**. *Biochemical Journal* 1995, **307**(2):377-381.
66. Yamashita H, Avraham S, Jiang S, London R, Van Veldhoven PP, Subramani S, Rogers RA, Avraham H: **Characterization of human and murine PMP20 peroxisomal proteins that exhibit antioxidant activity in vitro**. *Journal of Biological Chemistry* 1999, **274**(42):29897-29904.
67. Knoops B, Clippe A, Bogard C, Arsalane K, Wattiez R, Hermans C, Duconseille E, Falmagne P, Bernard A: **Cloning and characterization of AOEB166, a novel mammalian antioxidant enzyme of the peroxiredoxin family**. *Journal of Biological Chemistry* 1999, **274**(43):30451-30458.
68. Kang SW, Baines IC, Rhee SG: **Characterization of a mammalian peroxiredoxin that contains one conserved cysteine**. *Journal of Biological Chemistry* 1998, **273**(11):6303-6311.
69. Chen J-W, Dodia C, Feinstein SI, Jain MK, Fisher AB: **1-Cys peroxiredoxin, a bifunctional enzyme with glutathione peroxidase and phospholipase A2 activities**. *Journal of Biological Chemistry* 2000, **275**(37):28421-28427.

70. Sodhi M, Mukesh M, Prakash B, Ahlawat S, Sobti R: **Microsatellite DNA typing for assessment of genetic variability in Tharparkar breed of Indian zebu (Bos indicus) cattle, a major breed of Rajasthan**. *Journal of genetics* 2006, **85**(3):165-170.
71. Rachagani S, Gupta ID, Gupta N, Gupta S: **Genotyping of β-Lactoglobulin gene by PCR-RFLP in Sahiwal and Tharparkar cattle breeds**. *BMC genetics* 2006, **7**(1):31.
72. Singh RR, Dutt T, Kumar A, Tomar A, Singh M: **On-farm characterization of Vrindavani cattle in India**. *Indian Journal of Animal Sciences* 2011, **81**(3):267-271.
73. Schmieder R, Edwards R: **Quality control and preprocessing of metagenomic datasets**. *Bioinformatics* 2011, **27**(6):863-864.
74. Cunningham F, Amode MR, Barrell D, Beal K, Billis K, Brent S, Carvalho-Silva D, Clapham P, Coates G, Fitzgerald S: **Ensembl 2015**. *Nucleic acids research* 2014, **43**(D1):D662-D669.
75. Li B, Dewey CN: **RSEM: accurate transcript quantification from RNA-Seq data with or without a reference genome**. *BMC bioinformatics* 2011, **12**(1):323.
76. Langmead B, Salzberg SL: **Fast gapped-read alignment with Bowtie 2**. *Nature methods* 2012, **9**(4):357.
77. Robinson MD, McCarthy DJ, Smyth GK: **edgeR: a Bioconductor package for differential expression analysis of digital gene expression data**. *Bioinformatics* 2010, **26**(1):139-140.
78. Reimand J, Kull M, Peterson H, Hansen J, Vilo J: **g: Profiler—a web-based toolset for functional profiling of gene lists from large-scale experiments**. *Nucleic acids research* 2007, **35**(suppl_2):W193-W200.
79. Reimand J, Arak T, Adler P, Kolberg L, Reisberg S, Peterson H, Vilo J: **g: Profiler—a web server for functional interpretation of gene lists (2016 update)**. *Nucleic acids research* 2016, **44**(W1):W83-W89.
80. Gu Z, Li L, Wu F, Zhao P, Yang H, Liu Y, Geng Y, Zhao M, Su L: **Heat stress induced apoptosis is triggered by transcription-independent p53, Ca 2+ dyshomeostasis and the subsequent Bax mitochondrial translocation**. *Scientific reports* 2015, **5**:11497.
81. Li L, Tan H, Gu Z, Liu Z, Geng Y, Liu Y, Tong H, Tang Y, Qiu J, Su L: **Heat stress induces apoptosis through a Ca2+-mediated mitochondrial apoptotic pathway in human umbilical vein endothelial cells**. *PloS one* 2014, **9**(12):e111083.
82. Kelly SM, VanSlyke JK, Musil LS: **Regulation of Ubiquitin-Proteasome System–mediated Degradation by Cytosolic Stress**. *Molecular biology of the cell* 2007, **18**(11):4279-4291.
83. Flick K, Kaiser P: **Protein degradation and the stress response**. In: *Seminars in cell & developmental biology: 2012*: Elsevier; 2012: 515-522.
84. Homma T, Fujii J: **Heat stress promotes the down-regulation of IRE1α in cells: An atypical modulation of the UPR pathway**. *Experimental cell research* 2016, **349**(1):128-138.
85. Belhadj Slimen I, Najar T, Ghram A, Dabbebi H, Ben Mrad M, Abdrabbah M: **Reactive oxygen species, heat stress and oxidative-induced mitochondrial damage. A review**. *International journal of hyperthermia* 2014, **30**(7):513-523.
86. Yang L, Tan G-Y, Fu Y-Q, Feng J-H, Zhang M-H: **Effects of acute heat stress and subsequent stress removal on function of hepatic mitochondrial respiration, ROS production and lipid peroxidation in broiler chickens**. *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology* 2010, **151**(2):204-208.
87. Fabregat A, Jupe S, Matthews L, Sidiropoulos K, Gillespie M, Garapati P, Haw R, Jassal B, Korninger F, May B: **The reactome pathway knowledgebase**. *Nucleic acids research* 2017, **46**(D1):D649-D655.
88. Szklarczyk D, Gable AL, Lyon D, Junge A, Wyder S, Huerta-Cepas J, Simonovic M, Doncheva NT, Morris JH, Bork P: **STRING v11: protein–protein association networks with increased coverage,**

supporting functional discovery in genome-wide experimental datasets. *Nucleic acids research* 2018, **47**(D1):D607-D613.
89. Shannon P, Markiel A, Ozier O, Baliga NS, Wang JT, Ramage D, Amin N, Schwikowski B, Ideker T: **Cytoscape: a software environment for integrated models of biomolecular interaction networks**. *Genome research* 2003, **13**(11):2498-2504.
90. Krämer A, Green J, Pollard Jr J, Tugendreich S: **Causal analysis approaches in ingenuity pathway analysis**. *Bioinformatics* 2013, **30**(4):523-530.
91. Schmittgen TD, Livak KJ: **Analyzing real-time PCR data by the comparative C T method**. *Nature protocols* 2008, **3**(6):1101.

Legends

Figure 1: Overview of the work done : Two genetic groups (Tharparkar and Crossbred) of cattle were exposed to a temperature of 42 °C for 7 days. Heat stress indicators - Respiration rate (RR), Rectal temperature and T3 level before exposure to heat (0day – control group) and at 7th day of exposure (treated) were measured to evaluate heat stress. At these time points, RNA was isolated from PBMCs for high throughput sequencing. Transcriptome analysis was done to identify differentially expressed genes (DEGs) under heat treatment in both genetic groups. Genes involved in physiological processes (heat stress response, apoptosis, ubiquitination, unfolded protein response and antioxidant level) that are commonly associated with heat stress were compared between the two genetic groups. Further, functional annotation of DEGs was done using IPA.

Figure 2: Respiration rate, Rectal Temperature and T3 level measured at 0 day (control) and 7 day post-heat exposure (treated) in Crossbred and Tharparkar (n=5) . Levels not connected by the same superscript are significantly different ($p \leq 0.05$)..

Figure 3: Expression of DEGs in Crossbred and Tharparkar under heat stress: (a) Venn diagrams showing unique/common DEGs between Crossbred and Tharparkar (b) Number of upregulated and downregulated in both genetic groups (c) Contrast in the expression of common DEGs

Figure 4: Contrast in the expression of genes involved in heat stress response, apoptosis, ubiquitination, unfolded protein response and balance in the production of ROS and antioxidants between two genetic groups.

Figure 5: Predicted Protein-protein interaction network of expressed genes common to Tharparkar and Crossbred. The diameter of the node represents the connectivity/degree of the node among the genes.

Figure 6: Canonical pathways activated/inactivated in (a) Crossbred (b) Tharparkar under heat stress generated in the core analysis of Ingenuity pathway analysis tool. Orange color pathways are activated (Z > 2) and blue color pathways are inactivated (Z< -2). Height of the bar graphs indicates -log (p-value) and line graph showing the ratio of list genes found in each pathway over the total number of genes in that pathway.

Figure 7: Comparison of activated/inactivated pathways in Crossbred and Tharparkar. Activated pathways have Z score > 2 and indicated by red color while inactivated pathways are having Z score < - 2 and indicated by green color.

Figure 8: Canonical pathways generated in Ingenuity Pathway Analysis of Oncostatin M signaling pathway of DEGs in (A) Crossbred, (B) Tharparkar. Genes that were upregulated are shown in red and downregulated in green. The intensity of red and green corresponds to an increase and decrease, respectively, in Log2 fold change. Genes in grey were not significantly dysregulated and those in white are not present in the dataset but have been incorporated in the network through the relationship with other molecules by IPA.

Figure 9: Canonical pathways generated in Ingenuity Pathway Analysis of PTEN signaling pathway of DEGs in (A) Crossbred, (B) Tharparkar. Genes that were upregulated are shown in red and downregulated in green. The intensity of red and green corresponds to an increase and decrease, respectively, in Log2 fold change. Genes in grey were not significantly dysregulated and those in white are not present in the dataset but have been incorporated in the network through the relationship with other molecules by IPA.

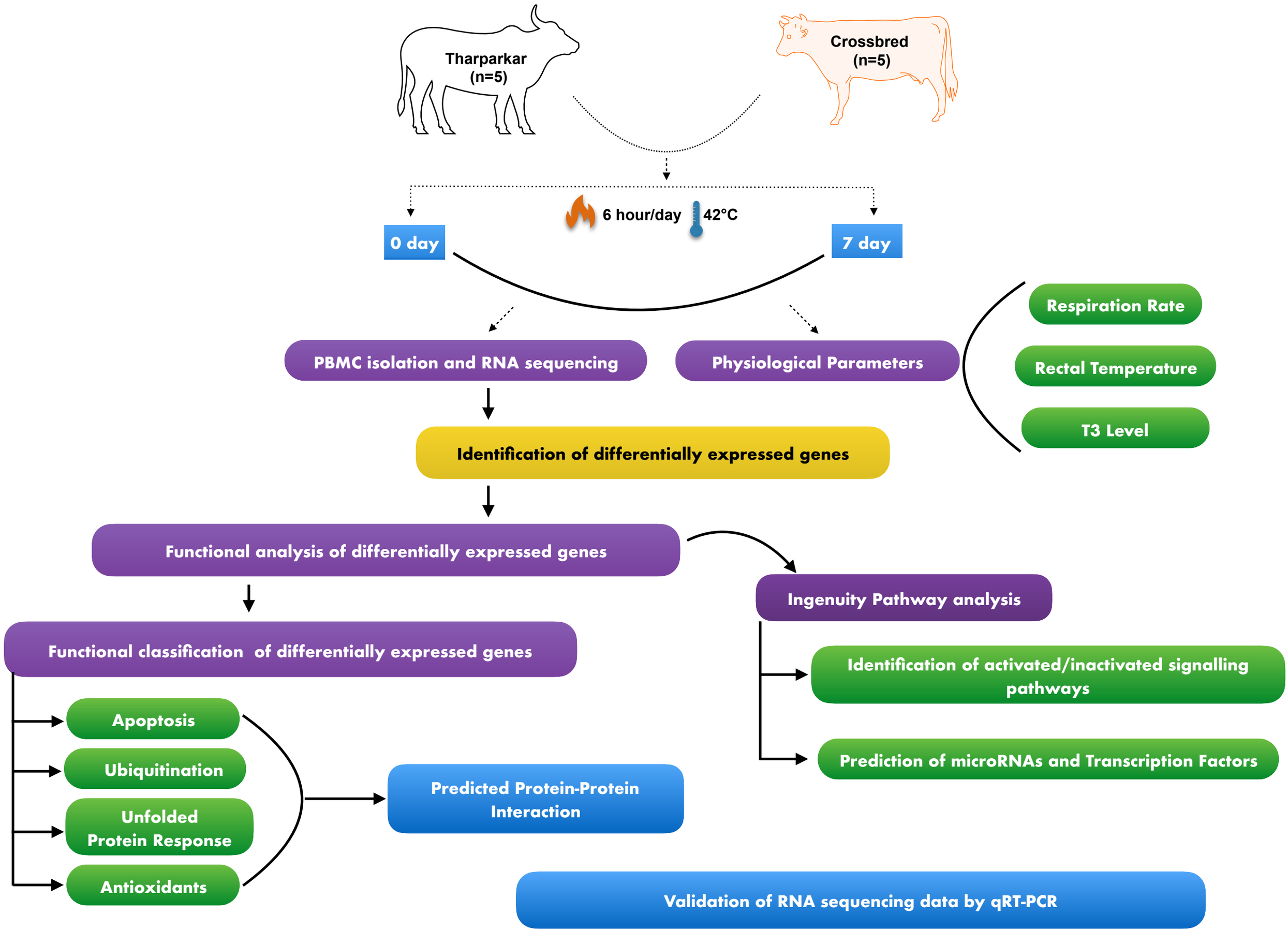
Figure 10: Comparison of activated/inactivated miRNAs in Crossbred and Tharparkar as predicted by IPA upstream analysis. Activated pathways have Z score > 2 and indicated by red color while inactivated pathways are having Z score < - 2 and indicated by green color.

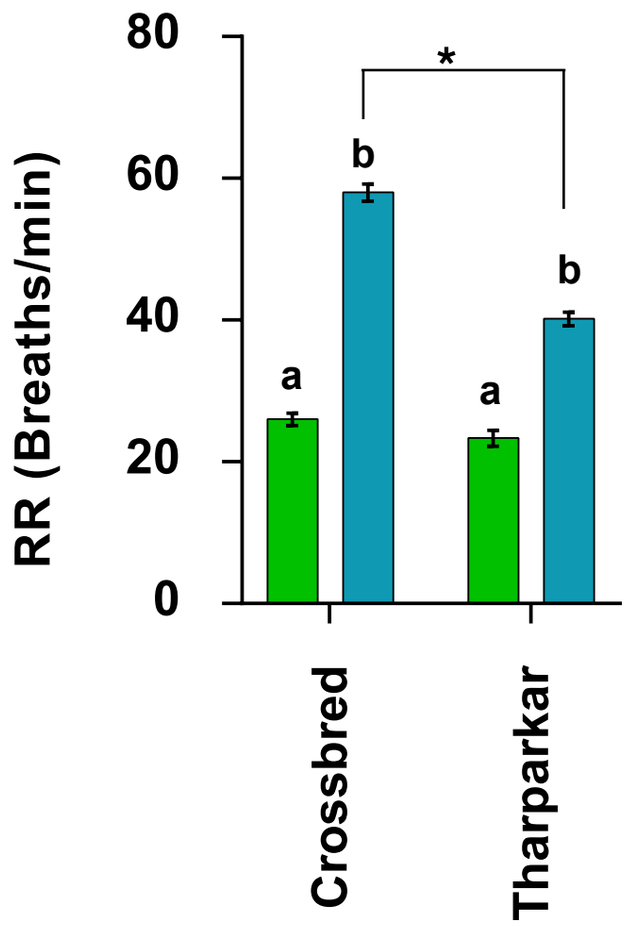
Figure 11: Comparison of activated/inactivated Transcription factors as predicted by IPA upstream analysis (Transcription factors of Crossbred are red-colored and Tharparkar are blue

colored) vis-à-vis their Log_2FC in both genetic groups. The encircled ones are common to both groups

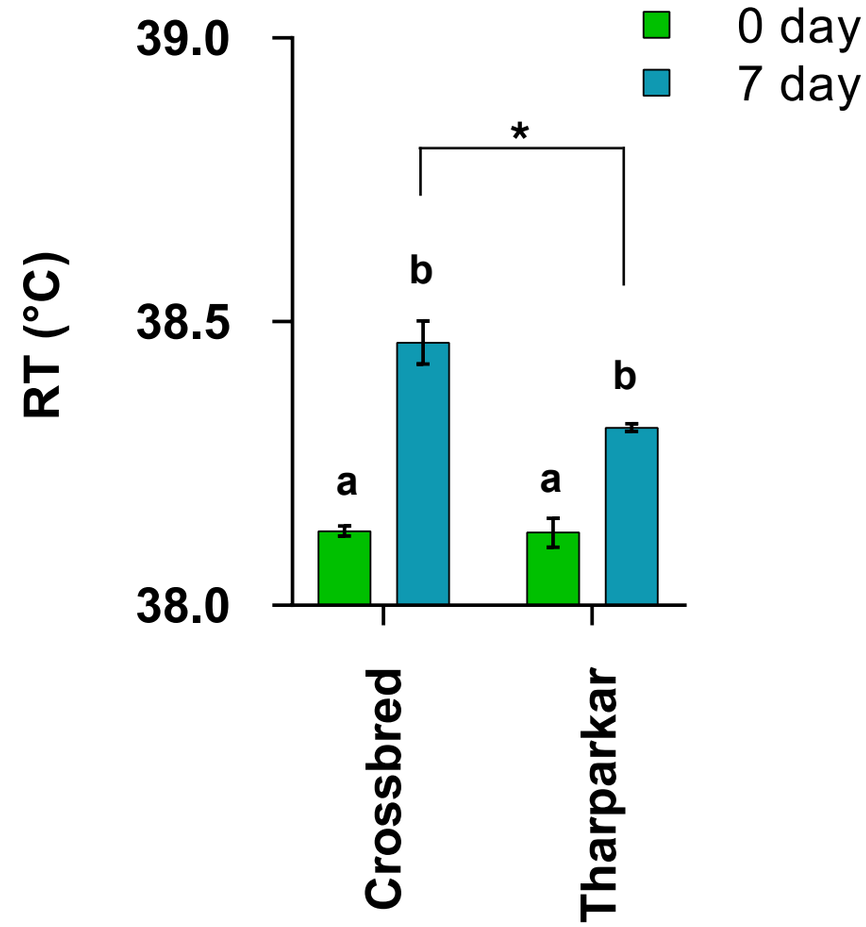
Figure 12: Validation of RNA sequencing data by Real-Time data in Crossbred (a) and Tharparkar (b). The expression of 10 selected genes was found in concordance with RNA Sequencing data. The correlation ($r^2 = 0.9942$ in (a) and 0.9972 in (b)) was found to be significant ($P < .01$) in both cases .

Figure 13: Predicted interplay of molecules that is underway during heat stress in both groups : Heat stress causes unfolding of native proteins. HSP70 acts as a chaperone to facilitate refolding to restore the structure of unfolded proteins. Under normal condition, HSP70 is bound to HSF1 thereby preventing HSF1 to promote transcription of HSP70. Under heat stress ATP binds to the HSP70 and HSF1 complex to release HSF1, promoting the binding of the unfolded protein to HSP70 and ATP. CAMK2D that induces the transcription of HSP70 via HSF1 was found downregulated in Tharparkar. GSK3A that inhibits the trimerization of HSF1 that is needed for the induction of HSP70 expression was found upregulated in Tharparkar. The decreased level of HSP70 in Crossbred makes it inevitable that such negative feedbacks would further reduce its level and GSK3A was found downregulated and CAMK2D, upregulated. Further, in Tharparkar, HSP70 tends to activate ubiquitination pathway to decrease the accumulation of unfolded proteins that can't be refolded by it. This pathway activation is supported by upregulation of E3 ligases (UBE2G1, UBE2S, and UBE2H) in Tharparkar. However, the E3 ligase in Crossbred was found downregulated. With HSP70 being upregulated and having cytoprotection activity, Tharparkar shows the decline in apoptosis as compared to Crossbred. This is supported by downregulation of BCL2L11, FASLG, MLKL, CASP3, MAPK8 and VIM in Tharparkar and vice-versa. Besides, higher expression of the antioxidants (SOD, CAT, GPX) in Tharparkar enables it to cope up with higher levels of free radicals generated as a result of heat stress while Crossbred is unable to do so.

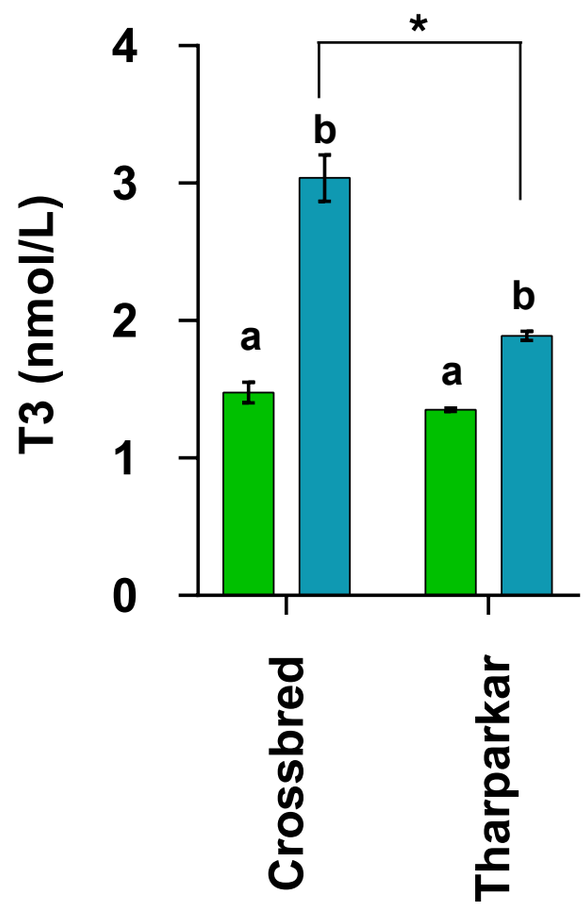

Tharparkar
(n=5)
Crossbred
(n=5)
6 hour/day
42°C
0 day
7 day
PBMC isolation and RNA sequencing
Physiological Parameters
Respiration Rate
Rectal Temperature
T3 Level
Identification of differentially expressed genes
Functional analysis of differentially expressed genes
Ingenuity Pathway analysis
Identification of activated/inactivated signalling pathways
Prediction of microRNAs and Transcription Factors
Functional classification of differentially expressed genes
Apoptosis
Ubiquitination
Unfolded Protein Response
Antioxidants
Predicted Protein-Protein Interaction
Validation of RNA sequencing data by qRT-PCR


RR (Breaths/min)
80
60
40
20
0
*
a
b
a
b
Crossbred
Tharparkar

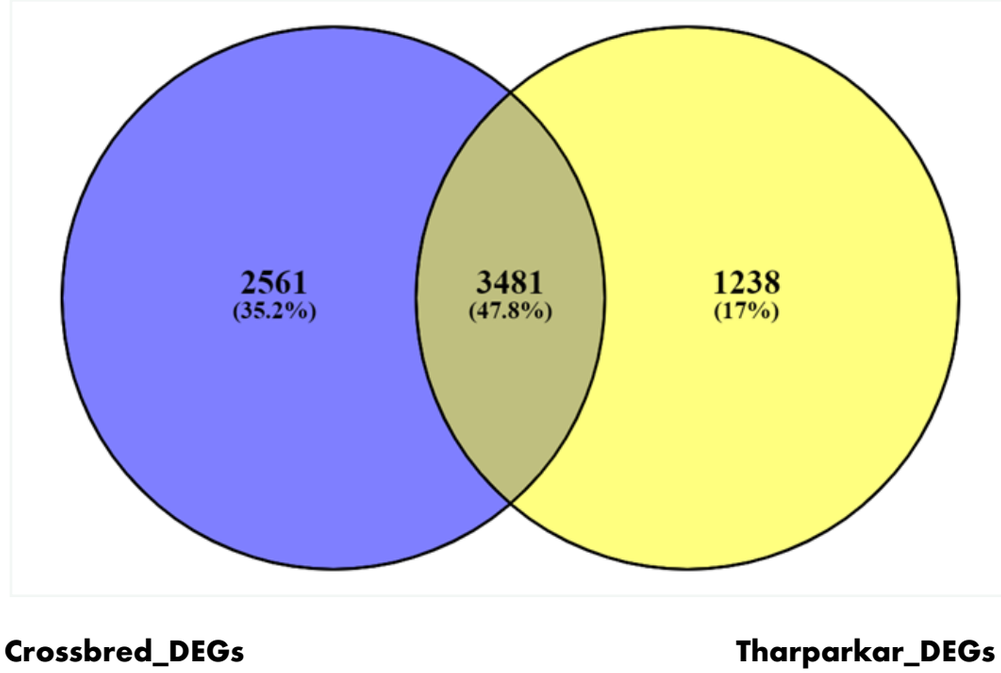
(a)


0 day
7 day
RT (°C)
39.0
38.5
38.0
*
a
b
a
b
Crossbred
Tharparkar

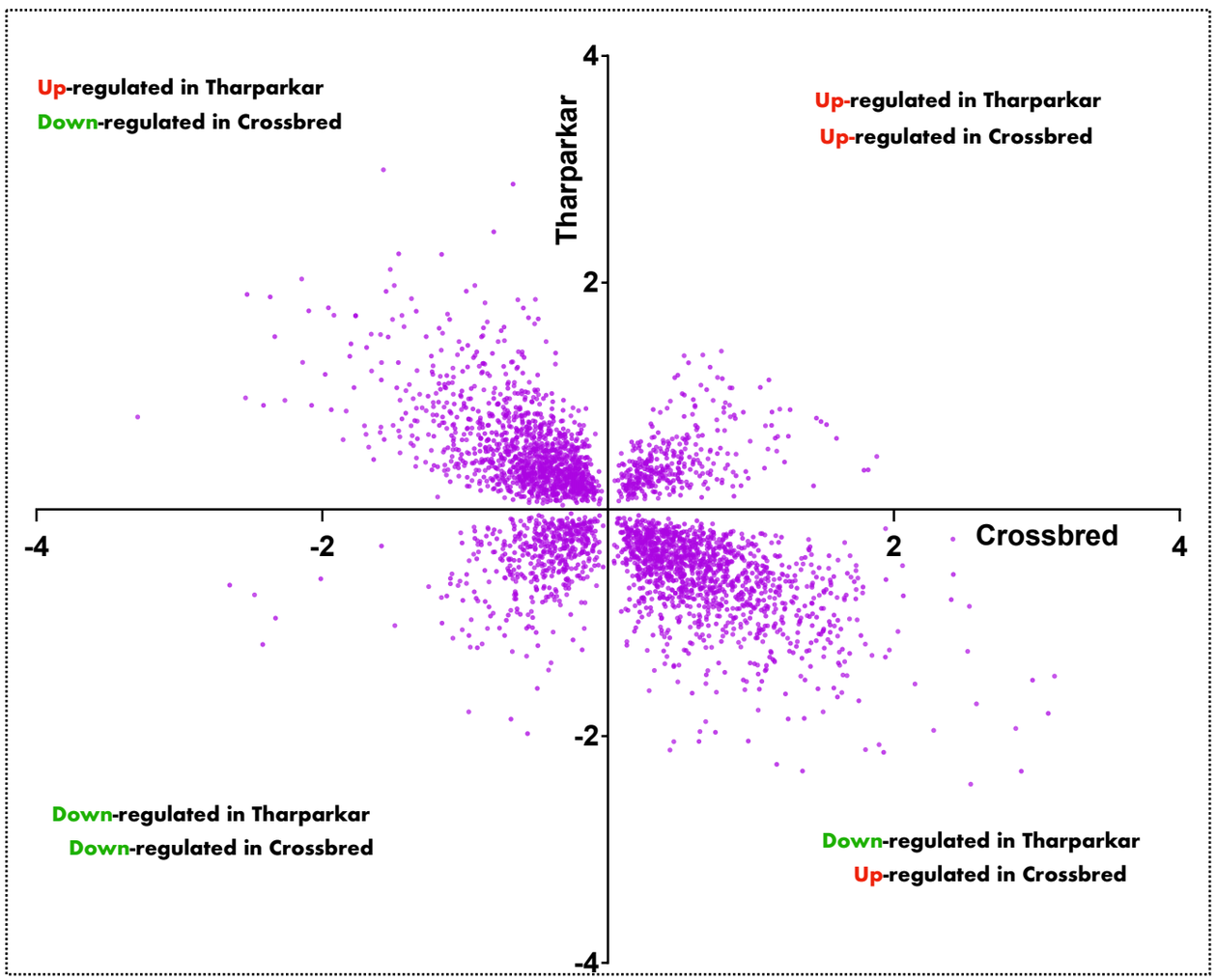
(b)


T3 (nmol/L)
4
3
2
1
0
*
a
b
a
b
Crossbred
Tharparkar

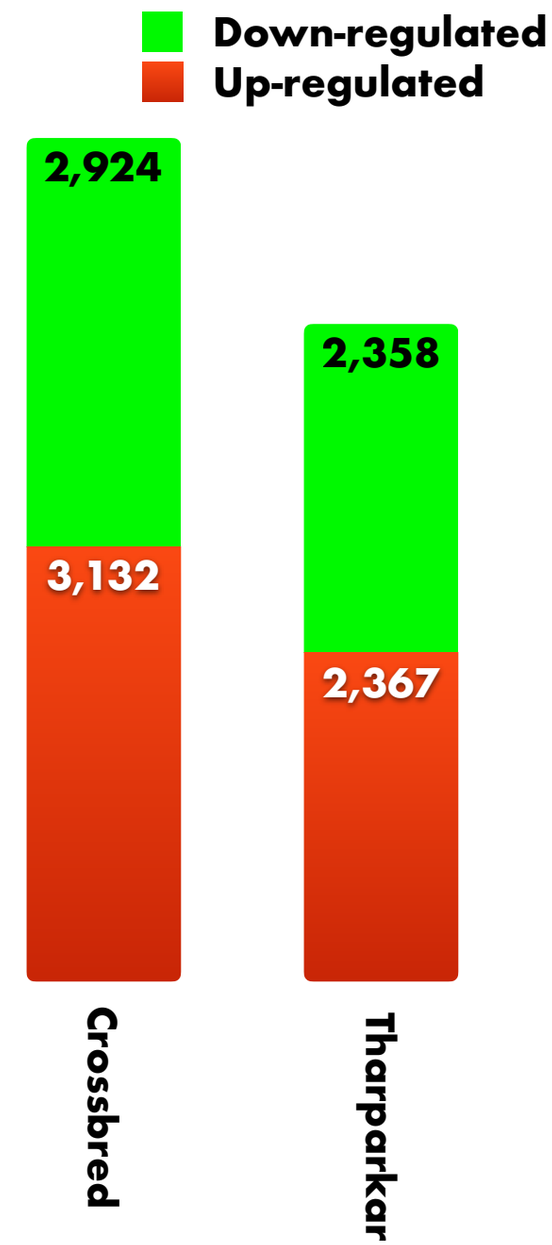
(c)


2561
(35.2%)
3481
(47.8%)
1238
(17%)
Crossbred_DEGs
Tharparkar_DEGs

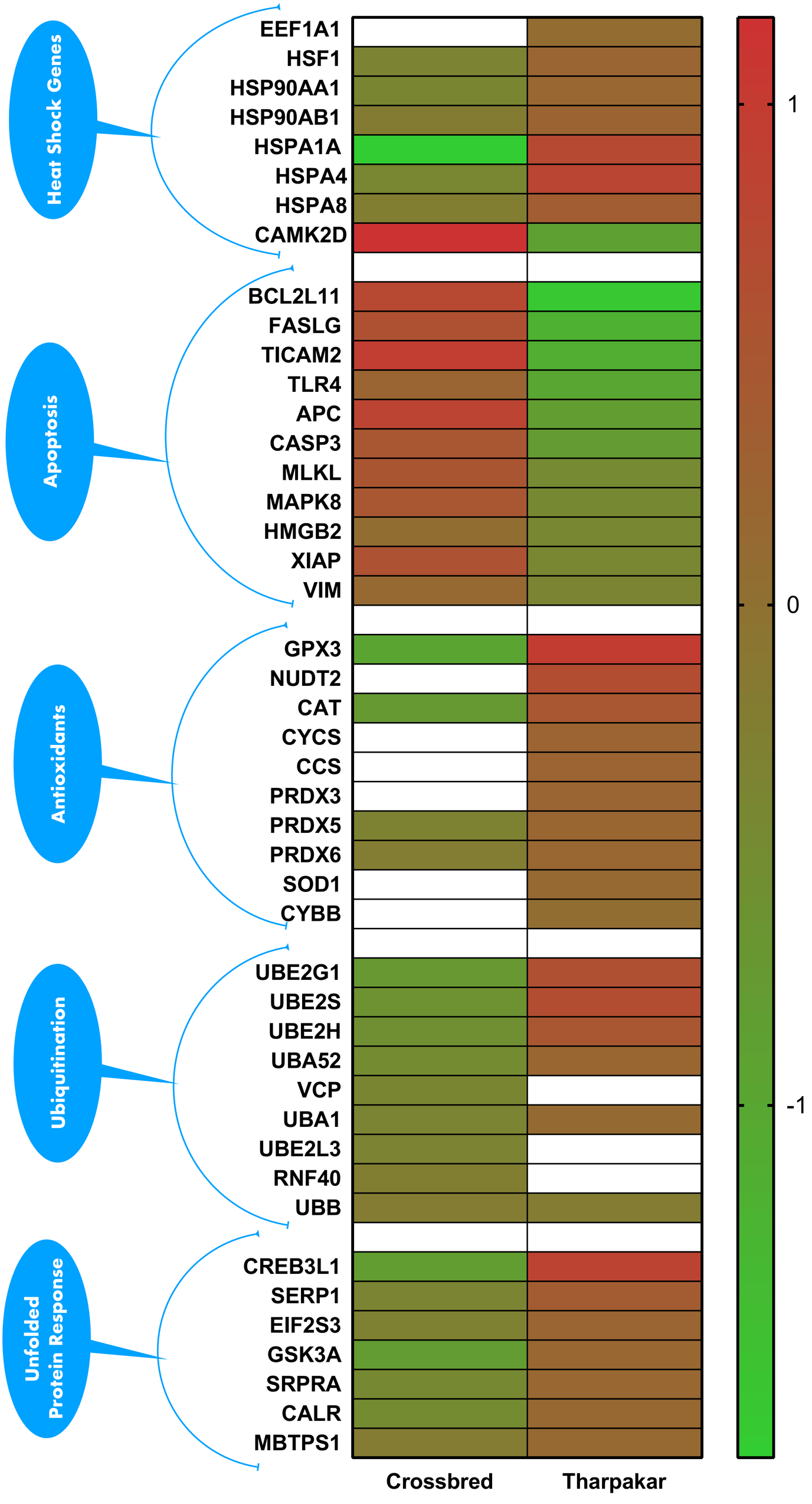
(a)

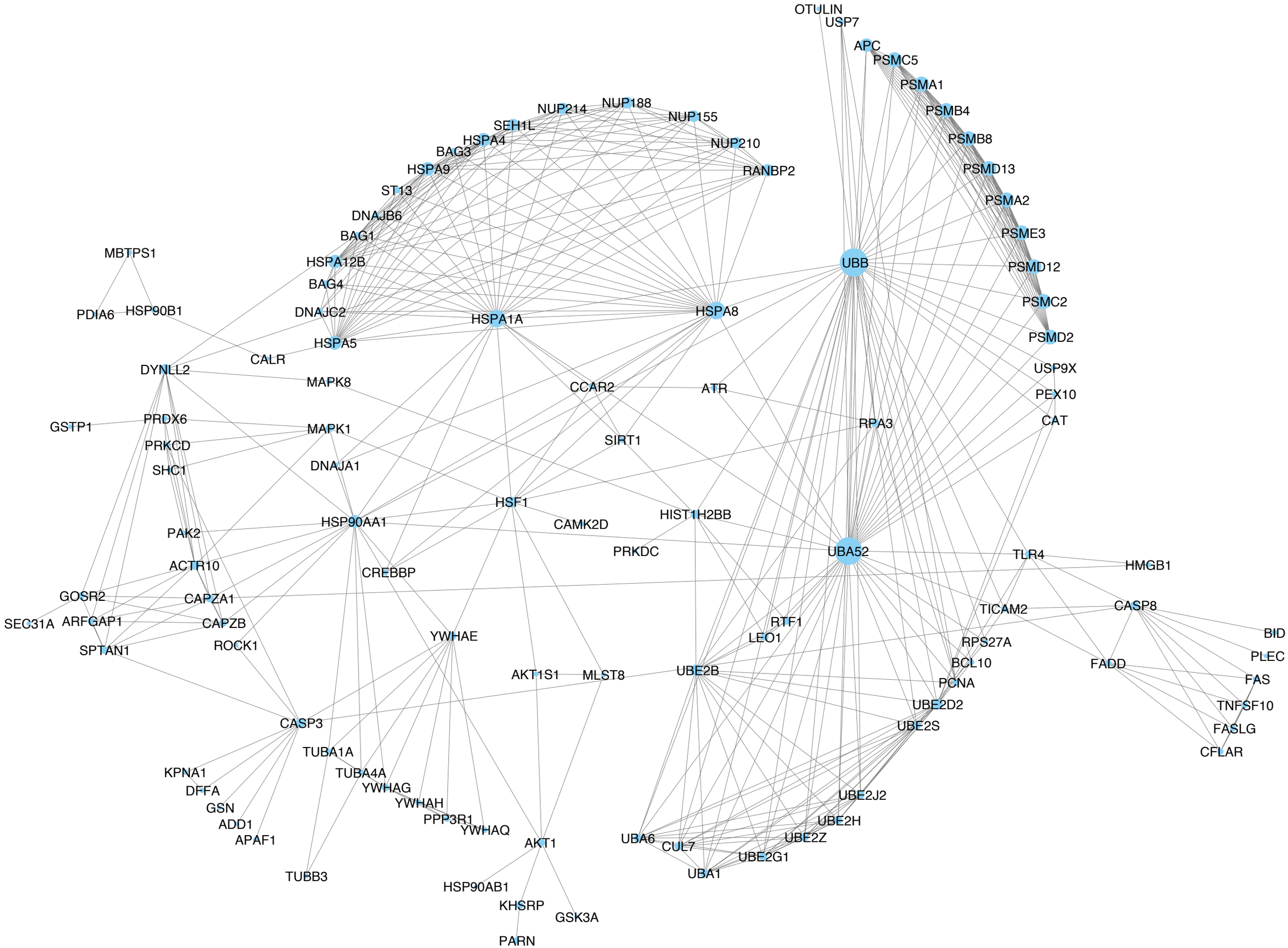

Up-regulated in Tharparkar
Down-regulated in Crossbred
Up-regulated in Tharparkar
Up-regulated in Crossbred
Down-regulated in Tharparkar
Down-regulated in Crossbred
Down-regulated in Tharparkar
Up-regulated in Crossbred
Tharparkar
Crossbred
4
2
-2
-4
-4
-2
2
4

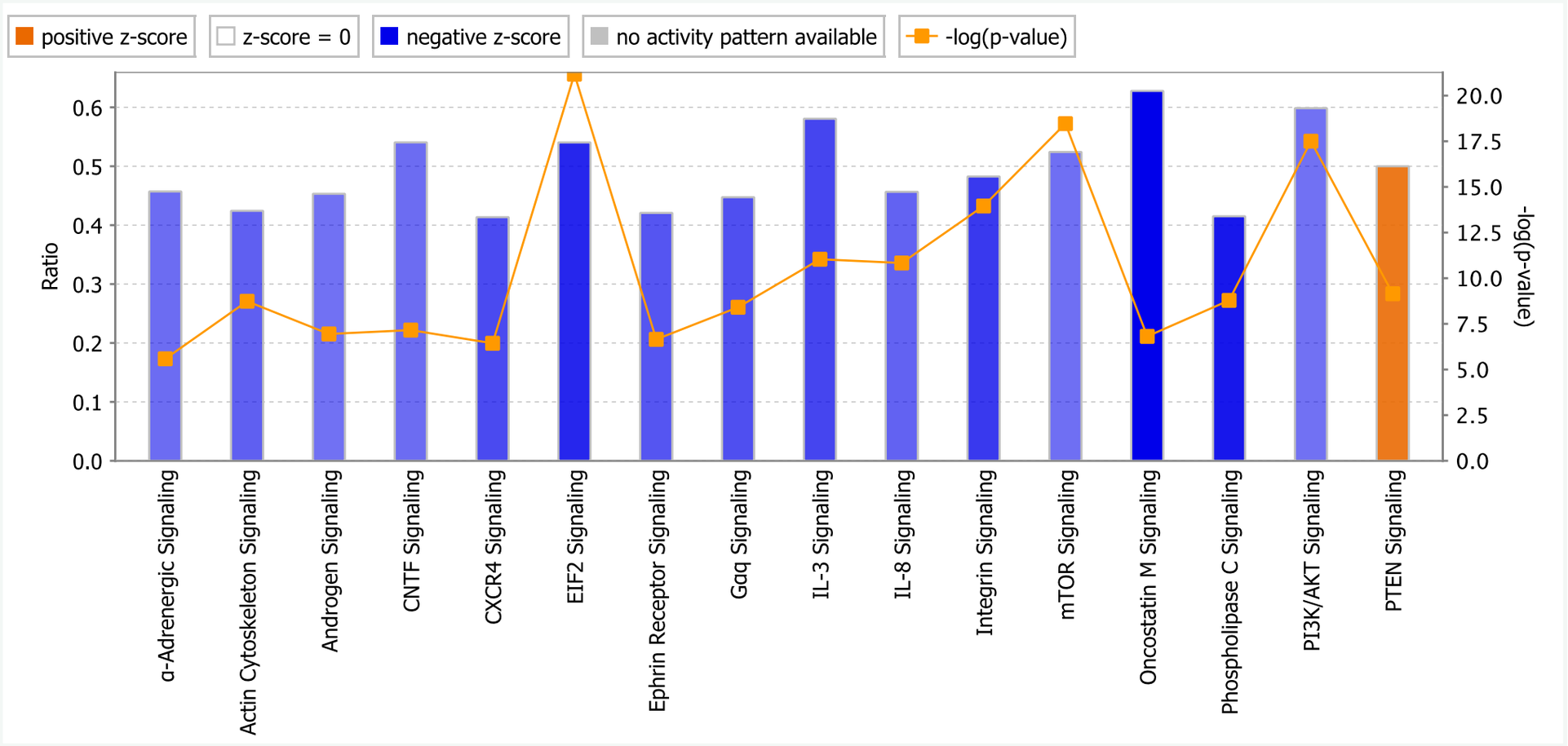
(c)


Down-regulated
Up-regulated
2,924
3,132
2,358
2,367
Crossbred
Tharparkar

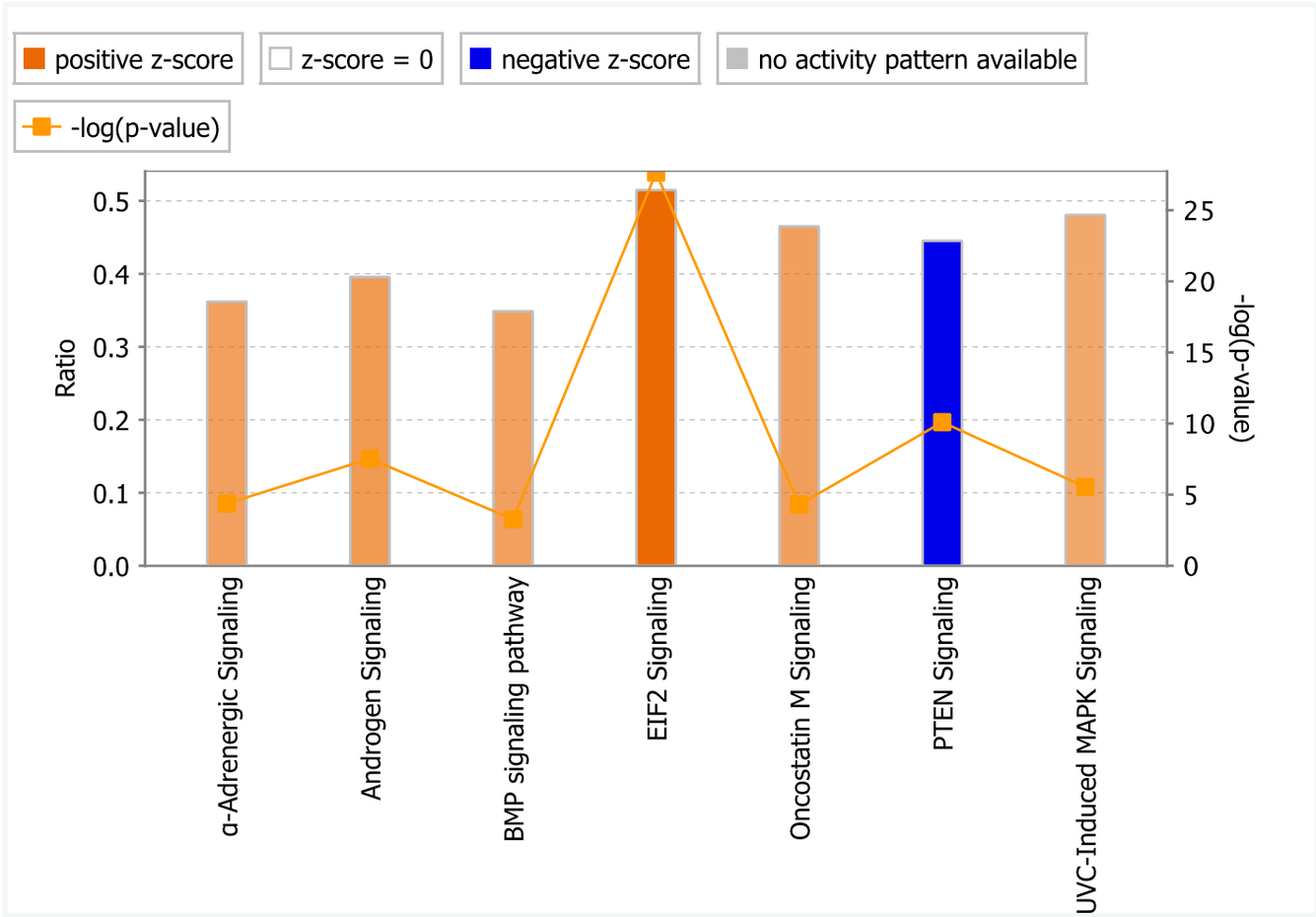
(b)


Heat Shock Genes
EEF1A1
HSF1
HSP90AA1
HSP90AB1
HSPA1A
HSPA4
HSPA8
CAMK2D
Apoptosis
BCL2L11
FASLG
TICAM2
TLR4
APC
CASP3
MLKL
MAPK8
HMGB2
XIAP
VIM
Antioxidants
GPX3
NUDT2
CAT
CYCS
CCS
PRDX3
PRDX5
PRDX6
SOD1
CYBB
Ubiquitination
UBE2G1
UBE2S
UBE2H
UBA52
VCP
UBA1
UBE2L3
RNF40
UBB
Unfolded Protein Response
CREB3L1
SERP1
EIF2S3
GSK3A
SRPRA
CALR
MBTPS1
Crossbred
Tharpakar
1
0
-1

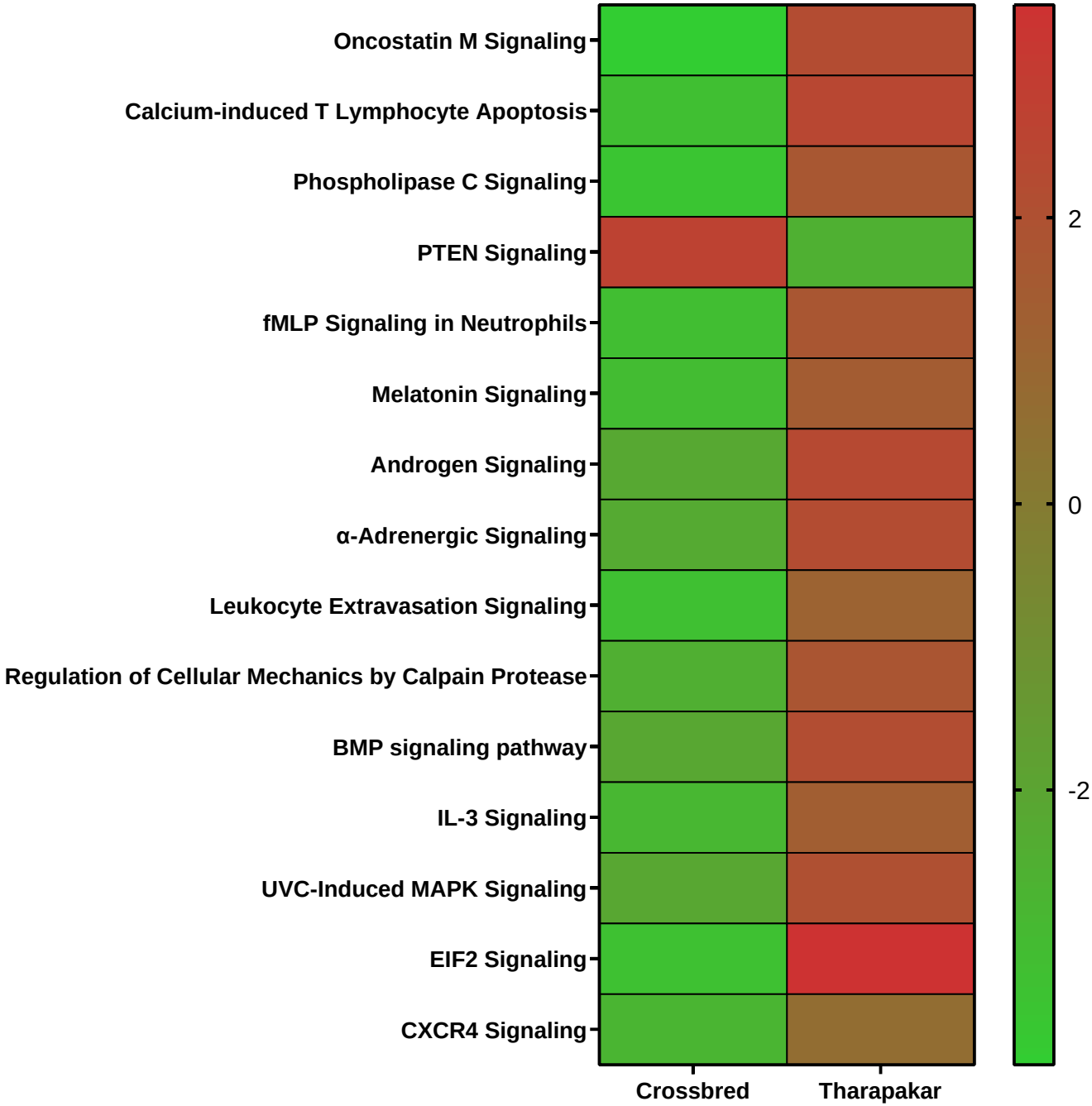

OTULIN
USP7
APC
PSMC5
PSMA1
PSMB4
PSMB8
PSMD13
PSMA2
PSME3
PSMD12
PSMC2
PSMD2
USP9X
PEX10
CAT
NUP214
NUP188
NUP155
SEH1L
HSPA4
NUP210
BAG3
HSPA9
RANBP2
ST13
DNAJB6
BAG1
MBTPS1
HSPA12B
UBB
BAG4
PDIA6
HSP90B1
DNAJC2
HSPA1A
HSPA8
HSPA5
CALR
DYNLL2
MAPK8
CCAR2
ATR
GSTP1
PRDX6
MAPK1
RPA3
PRKCD
SIRT1
SHC1
DNAJA1
HSF1
HIST1H2BB
HSP90AA1
CAMK2D
PAK2
PRKDC
UBA52
TLR4
HMGB1
ACTR10
CREBBP
GOSR2
CAPZA1
TICAM2
CASP8
SEC31A
ARFGAP1
CAPZB
RTF1
BID
YWHAE
LEO1
RPS27A
SPTAN1
ROCK1
PLEC
BCL10
FADD
AKT1S1
MLST8
UBE2B
PCNA
FAS
UBE2D2
TNFSF10
CASP3
UBE2S
FASLG
TUBA1A
CFLAR
KPNA1
TUBA4A
DFFA
YWHAG
UBE2J2
GSN
YWHAH
UBE2H
ADD1
PPP3R1
YWHAQ
UBE2Z
APAF1
UBA6
CUL7
AKT1
UBE2G1
UBA1
TUBB3
HSP90AB1
KHSRP
GSK3A
PARN

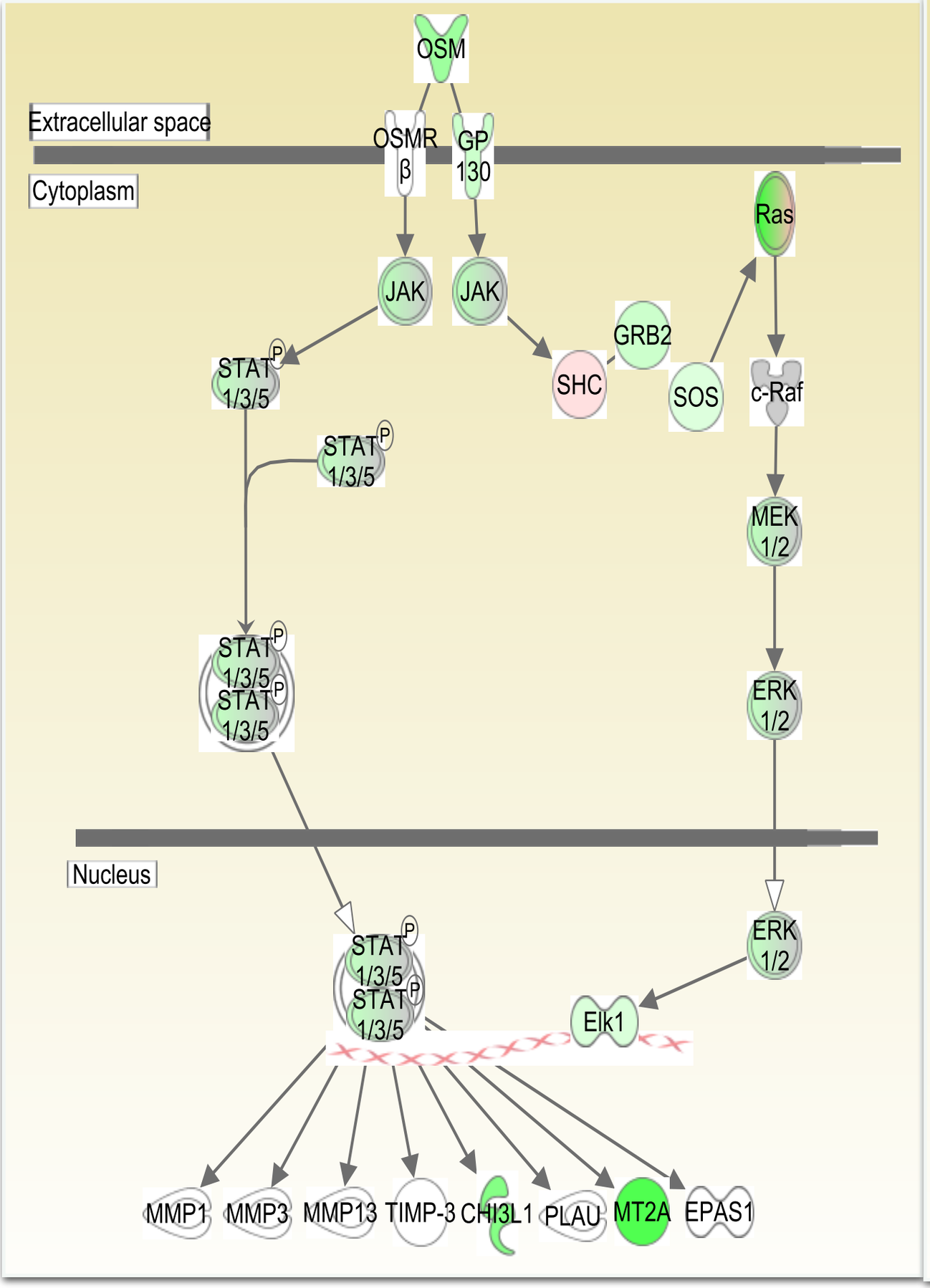

positive z-score
z-score = 0
negative z-score
no activity pattern available
-log(p-value)
Ratio
-log(p-value)
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.0
2.5
5.0
7.5
10.0
12.5
15.0
17.5
20.0
α-Adrenergic Signaling
Actin Cytoskeleton Signaling
Androgen Signaling
CNTF Signaling
CXCR4 Signaling
EIF2 Signaling
Ephrin Receptor Signaling
Gαq Signaling
IL-3 Signaling
IL-8 Signaling
Integrin Signaling
mTOR Signaling
Oncostatin M Signaling
Phospholipase C Signaling
PI3K/AKT Signaling
PTEN Signaling

(a)

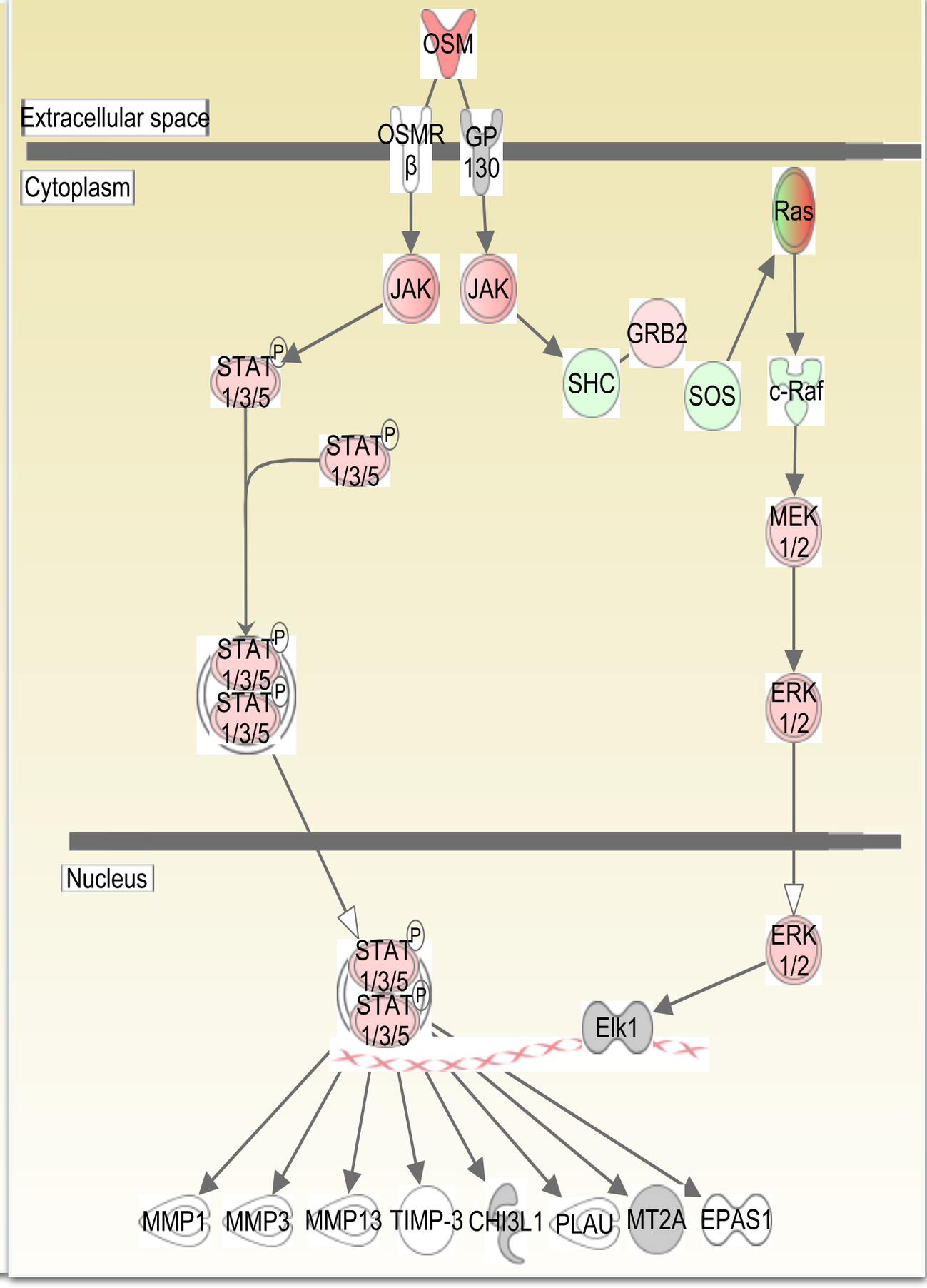

positive z-score
z-score = 0
negative z-score
no activity pattern available
-log(p-value)
Ratio
-log(p-value)
0.0
0.1
0.2
0.3
0.4
0.5
0
5
10
15
20
25
α-Adrenergic Signaling
Androgen Signaling
BMP signaling pathway
EIF2 Signaling
Oncostatin M Signaling
PTEN Signaling
UVC-Induced MAPK Signaling

(b)

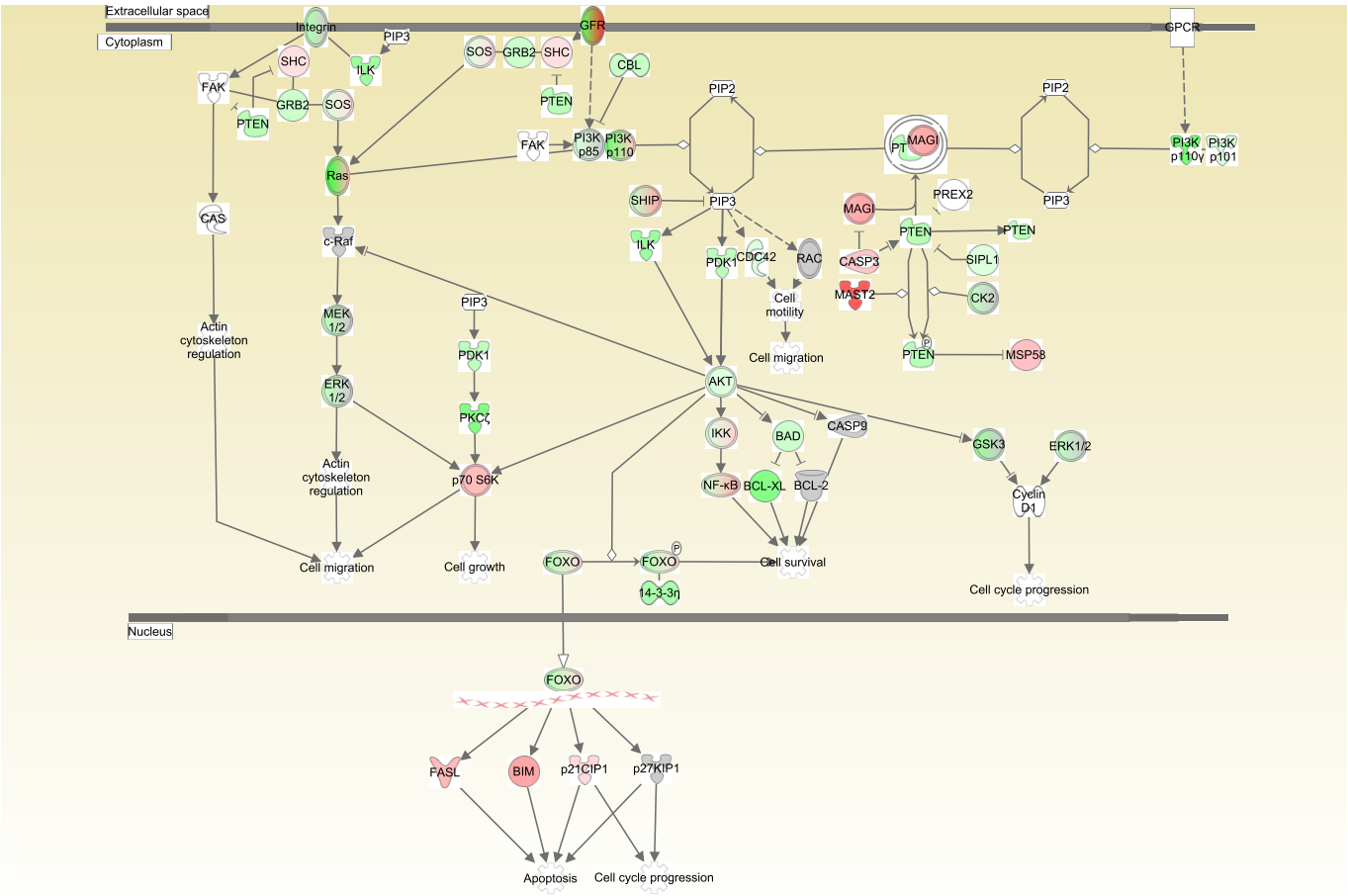

Oncostatin M Signaling
Calcium-induced T Lymphocyte Apoptosis
Phospholipase C Signaling
PTEN Signaling
fMLP Signaling in Neutrophils
Melatonin Signaling
Androgen Signaling
α-Adrenergic Signaling
Leukocyte Extravasation Signaling
Regulation of Cellular Mechanics by Calpain Protease
BMP signaling pathway
IL-3 Signaling
UVC-Induced MAPK Signaling
EIF2 Signaling
CXCR4 Signaling
Crossbred
Tharapakar
2
0
-2


OSM
Extracellular space
OSMR β
GP 130
Cytoplasm
Ras
JAK
JAK
GRB2
STAT 1/3/5
SHC
SOS
c-Raf
STAT 1/3/5
MEK 1/2
STAT 1/3/5
STAT 1/3/5
ERK 1/2
Nucleus
STAT 1/3/5
STAT 1/3/5
ERK 1/2
Elk1
MMP1
MMP3
MMP13
TIMP-3
CHI3L1
PLAU
MT2A
EPAS1

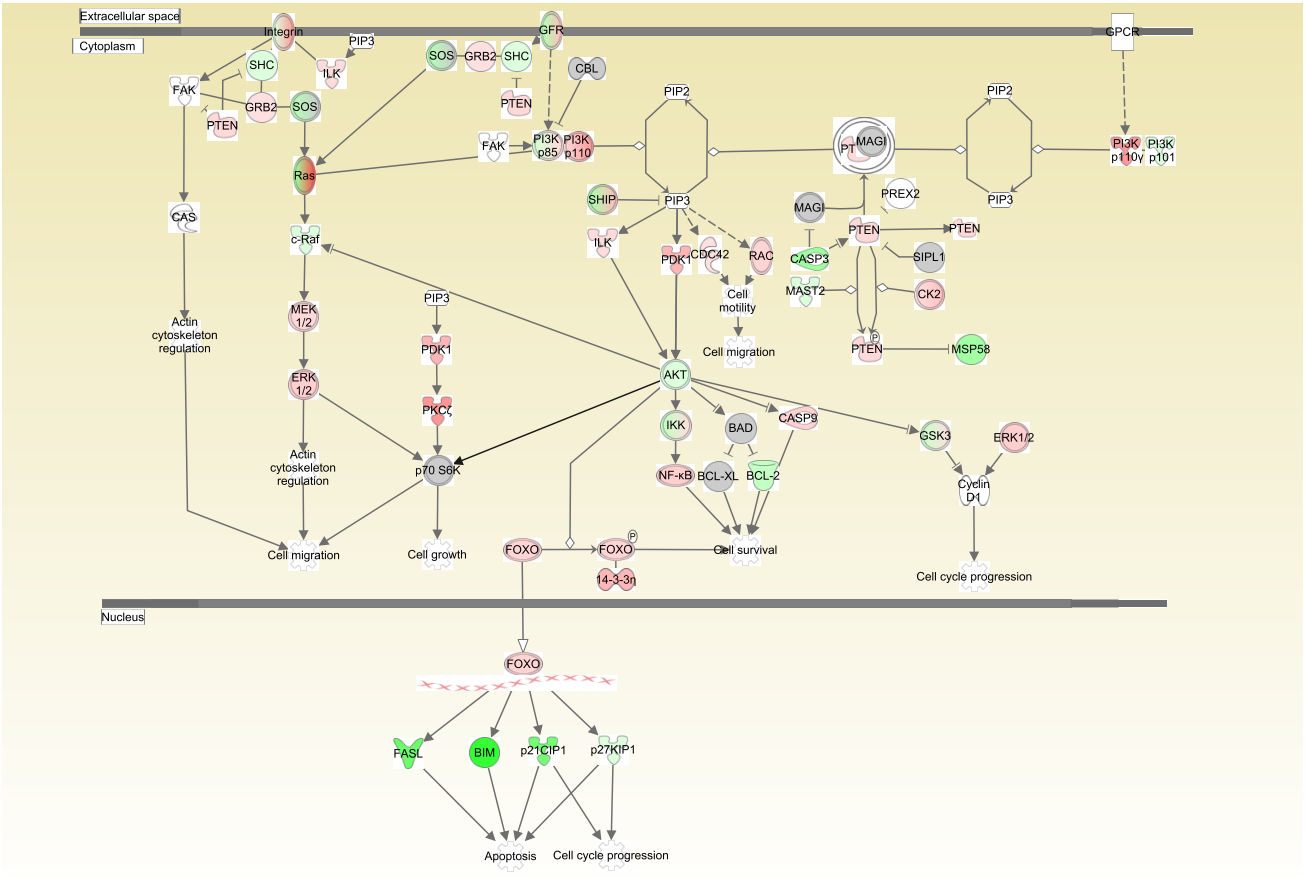
(a)


OSM
Extracellular space
OSMR β
GP 130
Cytoplasm
Ras
JAK
JAK
GRB2
STAT 1/3/5
SHC
SOS
c-Raf
STAT 1/3/5
MEK 1/2
STAT 1/3/5
STAT 1/3/5
ERK 1/2
Nucleus
STAT 1/3/5
STAT 1/3/5
ERK 1/2
Elk1
MMP1
MMP3
MMP13
TIMP-3
CHI3L1
PLAU
MT2A
EPAS1

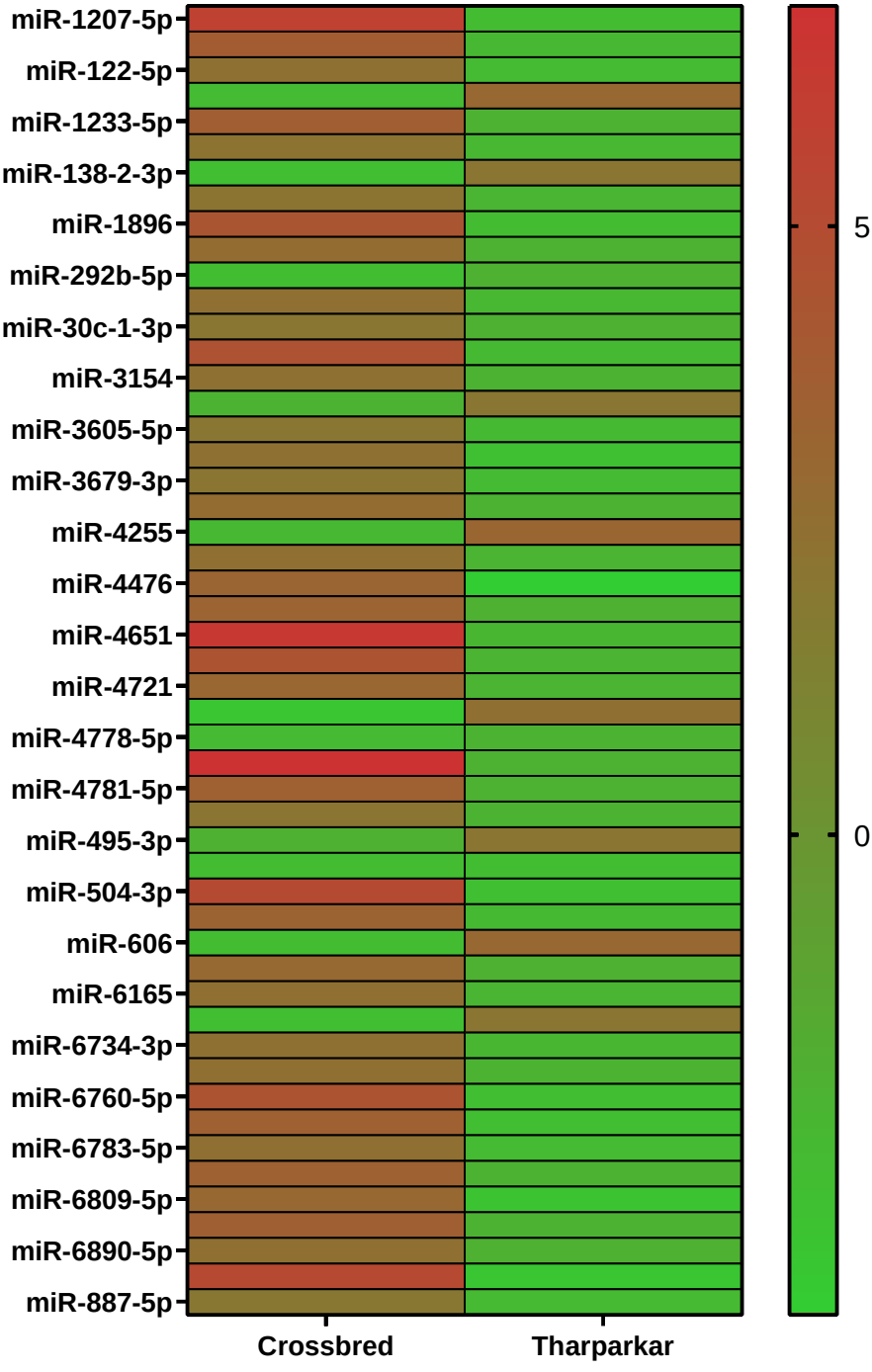
(b)

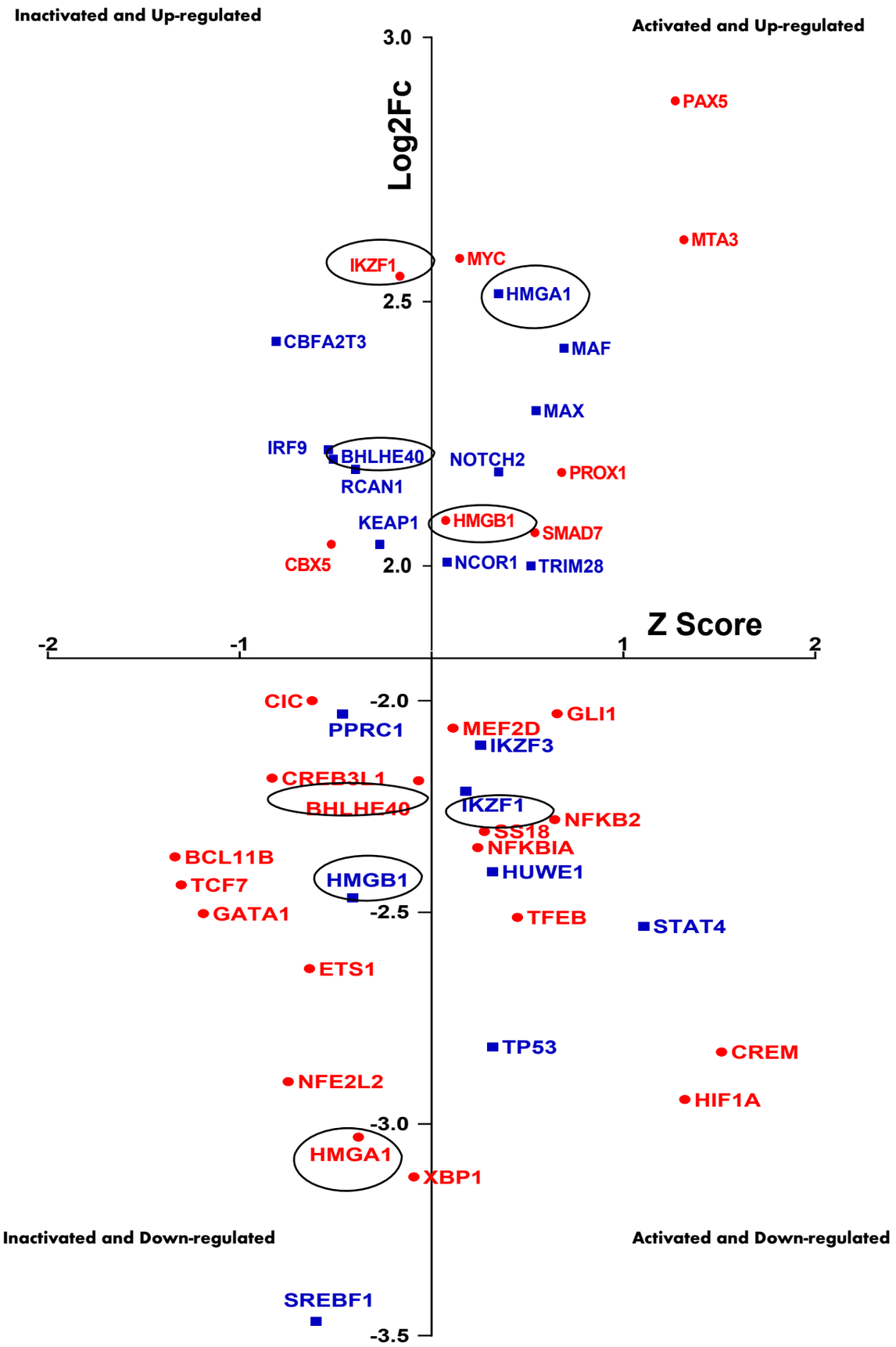

Extracellular space
Cytoplasm
Integrin
PIP3
GFR
GPCR
SHC
ILK
SOS
GRB2
CBL
FAK
PTEN
PI3K p85
PI3K p110
PIP2
PT
MAGI
PI3K p110γ
PI3K p101
Ras
SHIP
PREX2
CAS
c-Raf
PDK1
CDC42
RAC
CASP3
SIPL1
Cell motility
MAST2
CK2
MEK 1/2
Actin cytoskeleton regulation
Cell migration
MSP58
ERK 1/2
AKT
PKCζ
IKK
BAD
CASP9
GSK3
ERK1/2
NF-κB
BCL-XL
BCL-2
Cyclin D1
p70 S6K
Cell growth
FOXO
14-3-3η
Cell survival
Cell cycle progression
Nucleus
FASL
BIM
p21CIP1
p27KIP1
Apoptosis

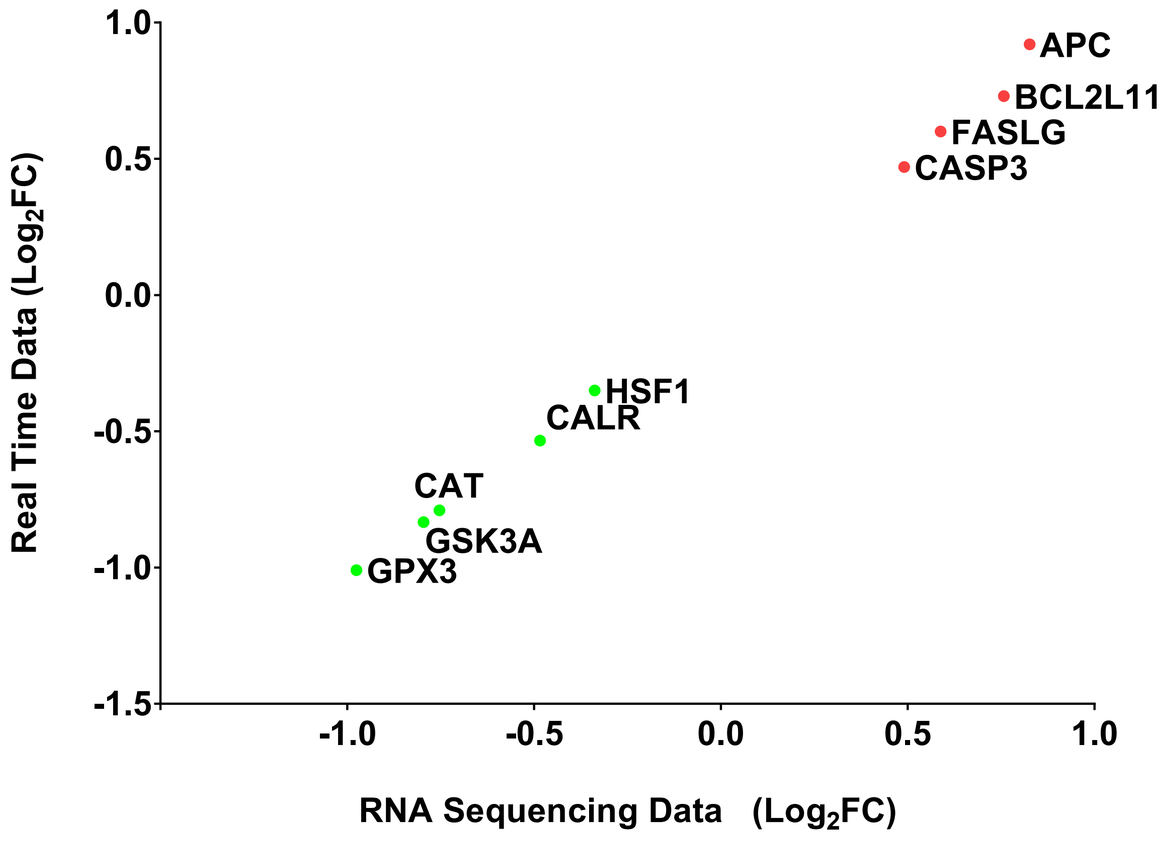
(a)


Extracellular space
Cytoplasm
Integrin
PIP3
GFR
GPCR
SHC
ILK
SOS
GRB2
CBL
FAK
PTEN
PI3K p85
PI3K p110
PIP2
PT
MAGI
PI3K p110γ
PI3K p101
Ras
SHIP
PREX2
CAS
c-Raf
PDK1
CDC42
RAC
CASP3
SIPL1
Cell motility
MAST2
CK2
MEK 1/2
Actin cytoskeleton regulation
Cell migration
MSP58
ERK 1/2
AKT
PKCζ
IKK
BAD
CASP9
GSK3
ERK1/2
NF-κB
BCL-XL
BCL-2
Cyclin D1
p70 S6K
Cell growth
FOXO
14-3-3η
Cell survival
Cell cycle progression
Nucleus
FASL
BIM
p21CIP1
p27KIP1
Apoptosis

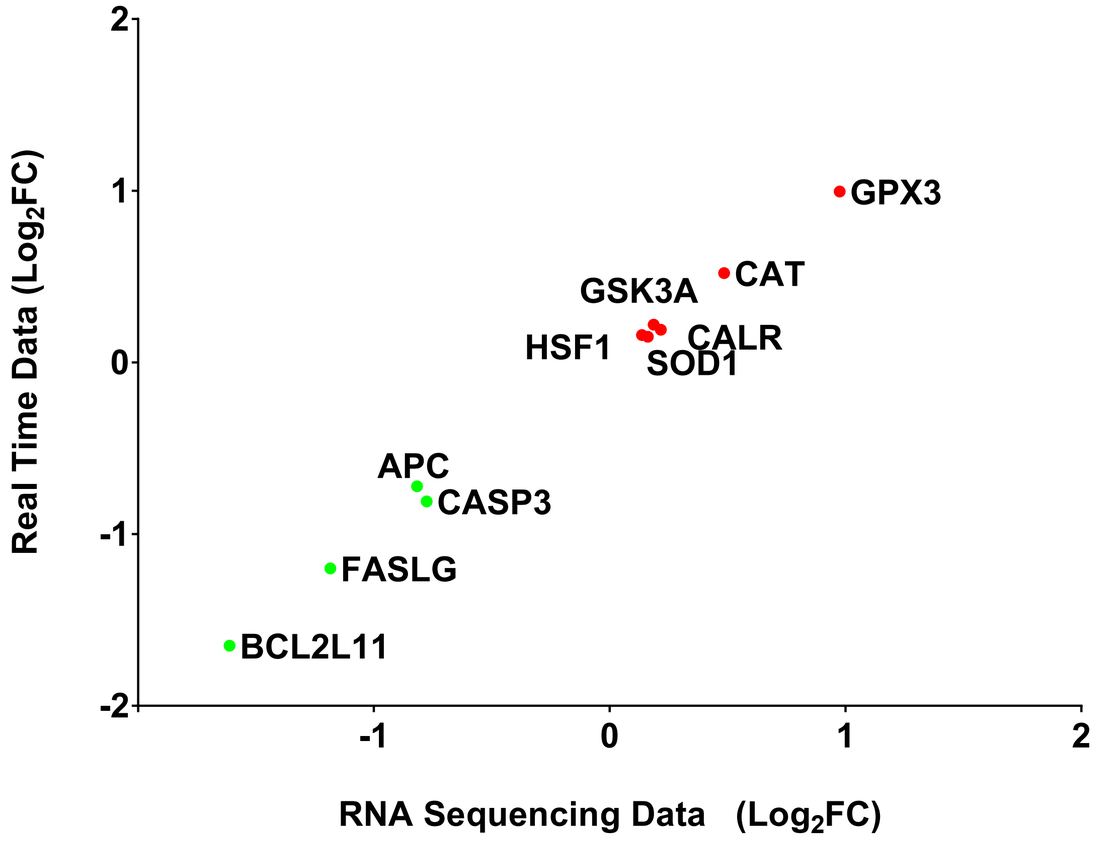
(b)


miR-1207-5p
miR-122-5p
miR-1233-5p
miR-138-2-3p
miR-1896
miR-292b-5p
miR-30c-1-3p
miR-3154
miR-3605-5p
miR-3679-3p
miR-4255
miR-4476
miR-4651
miR-4721
miR-4778-5p
miR-4781-5p
miR-495-3p
miR-504-3p
miR-606
miR-6165
miR-6734-3p
miR-6760-5p
miR-6783-5p
miR-6809-5p
miR-6890-5p
miR-887-5p
Crossbred
Tharparkar
5
0

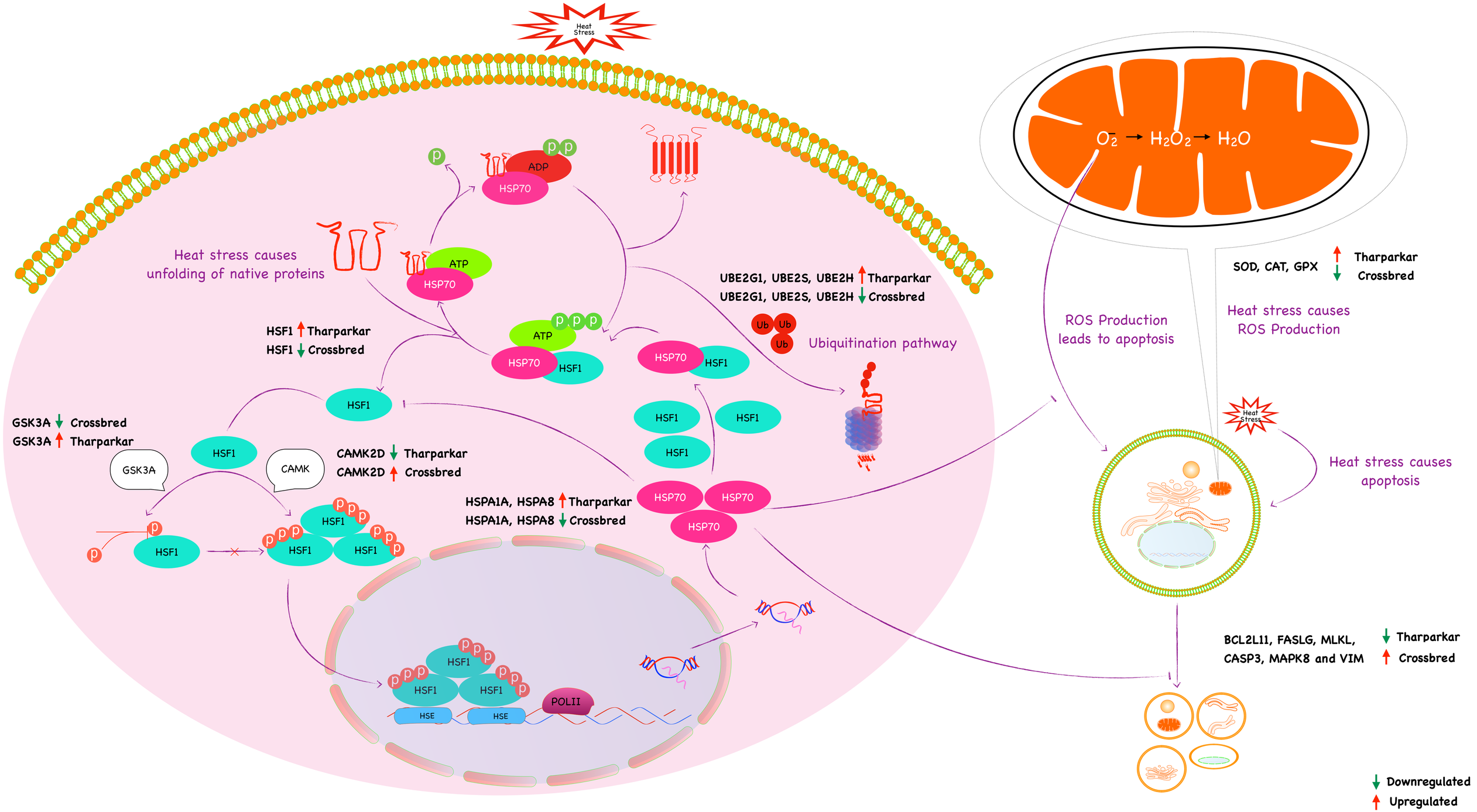

Inactivated and Up-regulated
Activated and Up-regulated
Log2Fc
Z Score
3.0
2.5
2.0
-2.0
-2.5
-3.0
-3.5
-2
-1
1
2
PAX5
MTA3
MYC
IKZF1
HMGA1
CBFA2T3
MAF
MAX
IRF9
BHLHE40
NOTCH2
PROX1
RCAN1
KEAP1
HMGB1
SMAD7
CBX5
NCOR1
TRIM28
CIC
GLI1
PPRC1
MEF2D
IKZF3
CREB3L1
BHLHE40
IKZF1
NFKB2
SS18
NFKBIA
BCL11B
HUWE1
TCF7
HMGB1
GATA1
TFEB
STAT4
ETS1
TP53
CREM
NFE2L2
HIF1A
HMGA1
XBP1
Inactivated and Down-regulated
Activated and Down-regulated
SREBF1


1.0
0.5
0.0
-0.5
-1.0
-1.5
Real Time Data (Log2FC)
-1.0
-0.5
0.0
0.5
1.0
RNA Sequencing Data (Log2FC)
APC
BCL2L11
FASLG
CASP3
HSF1
CALR
CAT
GSK3A
GPX3

(a)


2
1
0
-1
-2
Real Time Data (Log2FC)
-1
0
1
2
RNA Sequencing Data (Log2FC)
GPX3
CAT
GSK3A
HSF1
CALR
SOD1
APC
CASP3
FASLG
BCL2L11

(b)



Heat Stress
Heat stress causes unfolding of native proteins
ADP
HSP70
ATP
HSP70
ATP
HSP70
HSF1
HSF1
HSF1
HSF1
HSF1
HSF1
HSF1
HSF1
HSF1
HSF1
HSF1
HSF1
HSF1
HSF1
HSF1
HSF1
HSF1
HSF1
HSF1
HSF1
HSP70
HSP70
HSP70
HSP70
HSP70
HSF1
HSF1
HSF1
HSE
HSE
POLII
GSK3A
CAMK
GSK3A ↓ Crossbred
GSK3A ↑ Tharparkar
CAMK2D ↓ Tharparkar
CAMK2D ↑ Crossbred
HSF1 ↑ Tharparkar
HSF1 ↓ Crossbred
HSPA1A, HSPA8 ↑ Tharparkar
HSPA1A, HSPA8 ↓ Crossbred
UBE2G1, UBE2S, UBE2H ↑ Tharparkar
UBE2G1, UBE2S, UBE2H ↓ Crossbred
Ub
Ubiquitination pathway
$O_2^- \rightarrow H_2O_2 \rightarrow H_2O$
SOD, CAT, GPX ↑ Tharparkar ↓ Crossbred
ROS Production leads to apoptosis
Heat stress causes ROS Production
Heat Stress
Heat stress causes apoptosis
BCL2L11, FASLG, MLKL, CASP3, MAPK8 and VIM ↓ Tharparkar ↑ Crossbred
↓ Downregulated
↑ Upregulated