Short heat shock has a long-term effect on mesenchymal stem cells' transcriptome

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Abstract

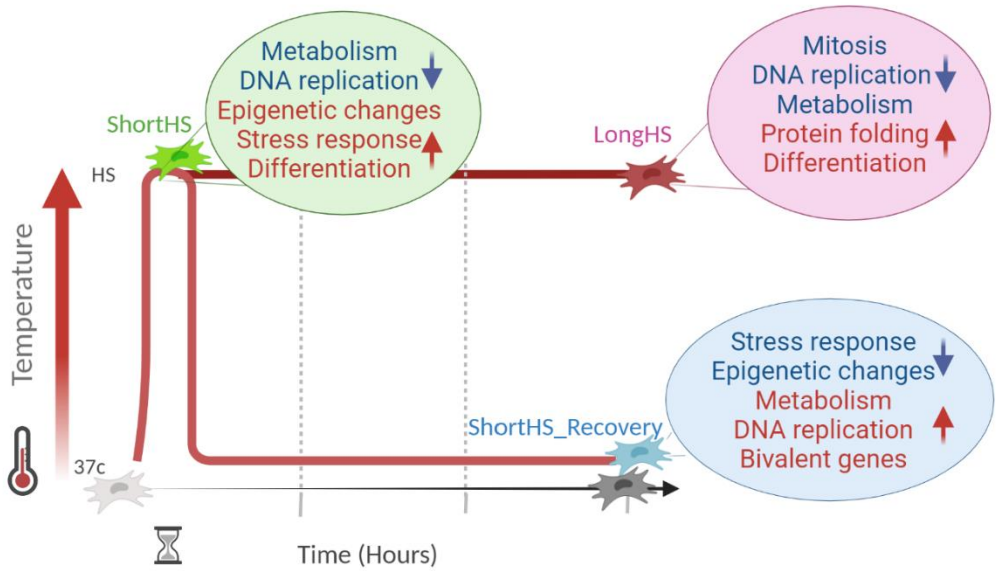
Background: Mesenchymal stem cells (MSCs) are multipotent stromal, non-hematopoietic cells with self-renewal and differentiation properties and are therefore a preferred source for cellular therapies. However, a better understanding of culture techniques is required to harness their full potential. Here we aim to compare the effects of short and long heat shock (HS) on the transcriptomic landscape of MSCs.

Methods: MSCs were extracted from the umbilical cord of a bovine fetus, cultured, and validated as MSCs. Early passage cells were exposed to 40.5°C for six hours or three days. RNA sequencing and bioinformatics analysis were performed to systematically examine the transcriptional changes following each treatment and to identify specific biological features and processes.

Results: The data indicates that while long heat stress influences many cell processes, such as immune response, cell cycle, and differentiation, the short HS mostly upregulates the cellular stress response. Once normothermia is resumed the long-term effects of the short HS can be revealed: although most genes revert to their original expression levels, a subgroup of epigenetically marked genes termed bivalent genes, maintains high expression levels. These genes are known to support cell lineage specification and are carefully regulated by a group of chromatin modifiers. One family of those chromatin modifiers, called MLL genes, is highly over-represented in the cluster of genes that are transiently upregulated following six hours of HS. Therefore, our data provide a mechanistic explanation for the long-term phenotype of short HS on development-related genes and could be used to predict the long-term effect of HS on cell identity.

Conclusions: Understanding the influence of culture conditions on morphology, phenotype, proliferative capacity, and fate decision of MSCs is needed to optimize culture conditions suitable for clinical or commercial use. Here, we suggest that simple and short stress can alter the cell's proliferation and differentiation capacities and therefore, following future optimizations, be used to shift the cells toward a more desirable functionality.

Graphical abstract



Keywords

Heat shock, bovine, mesenchymal stem cells, transcriptome, stress, bivalent genes, umbilical cord.

Background

Mesenchymal stromal cells (also called mesenchymal stem cells, MSCs) are a heterogeneous group of non-hematopoietic multipotent stem cells that assist in the preservation of homeostasis in many organs and tissues [1]. Additionally, physiological MSC stores are essential for the regeneration of many tissues in the body, acting through common signaling pathways such as Wnt, BMP and Notch [2]. MSCs in vitro are most frequently derived from adult tissue sources such as bone marrow and adipose tissue or birth associated tissue such as placental tissue, amniotic membranes and umbilical cord [3,4]. When grown in culture, MSCs can self-renew, differentiate into several tissues, and modulate the immune response in their surroundings [5–7]. When activated, MSCs secrete biologically active compounds and generate exosomes that modify the function of their cellular microenvironment [8–10]. Due to these characteristics, MSCs are commonly suggested as

candidates for cell-based therapy or as the cell source for tissue engineering and cultured meat products. However, despite their promise, it is still unclear how the properties and key biological functions of MSCs *in vitro* are influenced by parameters such as cell source, culture conditions, and varied pretreatments or enrichment protocols.

MSCs have been investigated and applied in cell-based therapy as immunomodulatory agents or as the cell source for tissue regeneration. To enhance their therapeutic efficacy, numerous chemical and physical agents have been assessed for their ability to speed cell expansion or enhance differentiation to the required cell types. These pretreatments, also termed priming, of MSCs with cytokines, drugs, or environmental stress conditions, were proven highly beneficial in many clinical settings [11]. For example, MSCs cultured under hypoxic conditions usually maintain their 'stemness' better, as illustrated by their increased proliferation capacity, better survival rates, and increased glucose consumption [12]. Therefore, the extent to which MSCs fulfill their immunomodulatory or regenerative potential can be altered by changing their extracellular environment. Hence, understanding the mechanisms through which the external conditions of the cells direct their biological functions is necessary. Nevertheless, the characterization and elucidation of these distinct effects remain challenging.

Lately, research has focused on the response of MSCs to sublethal exposure to cellular stressors such as hypoxia, heat, and nutrient depletion. These stressors resemble either ischemic or inflammatory microenvironments and are considered the major challenges to cell survival in vivo [13]. These preconditioning strategies induce hormesis - a phenomenon in which low doses of a harmful stressor produce a cascade of beneficial biological effects - and have been shown to improve migration, regenerative and proangiogenic potential, and stemness of MSCs [14,15]. Specifically, priming MSCs by heat shock (HS) is an effective way to protect cells undergoing transplantation, as

it enhances the survival rate and reduces the apoptotic rate of transplanted MSCs [16–19] On the cellular level, thermal stress is associated with oxidative stress, endoplasmic reticulum (ER) stress, and neurochemical stress, and impairs protein folding, cell cycle, and mitochondrial function [16,20–22].

On the transcriptional level, cells exposed to HS generally activate a cascade of heat shock proteins and factors which in turn regulate downstream pathways that protect the stressed cells. The effect of external temperature has been examined in various animals and tissues such as bovine PBMCs [23], mammary tissues [24,25] and granulosa cells [26]. Another study exposed rats to heat stress and examined the transcriptional changes in 3 different tissues [27]. Although many key genes and pathways that respond to acute thermal stress were identified in these studies, only a few genes were common across tissues, suggesting a high level of tissue specificity in the cellular response to thermal stress. Additionally, although short preconditioning with temperatures above the standard has been shown to prevent or reverse age related impairment and significantly improve the regenerative potential and therapeutic potential of human MSCs [16,28–32], other studies have shown reduced proliferative and regenerative abilities [22,33,34]. This inconsistency is possibly due to the different treatment protocols (namely, different temperatures and durations of heat stress) and the origin of MSCs used but could also be attributed to the intrinsic heterogeneity of MSCs. Studies using single-cell RNA-seq have shown that MSCs are a heterogeneous population containing pre-MSCs and lineage committed MSCs [35–37]. Hence, the effect of environmental factors, including heat, on MSCs transcriptome and function is not yet fully understood. Furthermore, the long-term epigenetic effects of variations in culture conditions are only beginning to be uncovered [38,39]. An example of such an effect is hypoxia-preconditioning, which was shown to significantly

reduce global 5hmC in swine MSCs but had no effects on H3K4me3, H3K9me3, or H3K27me3 [39]. Overall, the long-term effects of stress-preconditioning on MSCs are barely characterized.

Here, we set out to evaluate the immediate and long-term effects of HS treatment on the bovine umbilical cord (bUC)-derived MSCs transcriptome. In our previous study, we demonstrated that HS treatment changed the proliferation, differentiation, and immunomodulatory potential of bUC-MSCs [22]. Several HS protocols were used and shown to have a common effect of reducing proliferation and inducing oxidative stress and premature senescence. However, different HS protocols showed profound variation in gene expression and immunomodulatory potential. Surprisingly, even one hour of treatment at 42°C had a long-term effect on bUC-MSCs function and transcriptional pattern 3 days after the HS, which partially persisted even after eleven passages (about 40 days) in culture [22]. However, at temperatures higher than 41°C, the survival rate of mammalian cells decreases with increasing exposure time; to avoid this bias we performed our HS at 40.50C [40,41]. Here, to gain an unbiased view of the cell-intrinsic response to heat shock, we performed gene expression profiling using RNA sequencing (RNA-Seq) following exposure of the bUC-MSCs to moderate HS at different times. We hypothesize that transcriptional response to heat stress has two manifestations: a major immediate reaction of stress response related genes, and a minor response of permanently altered transcripts. This work will advance our understanding of the cellular response to thermal stress and the long-term effects on the functionality of MSCs and the enduring impact of short and mild HS event on the organismal stem cell pool.

Methods

Cell Culture

Bovine mesenchymal stem cells were isolated, cultured and characterized based on generally accepted criteria [6,42] as we have previously reported [22,43]. Briefly, umbilical cords of Holstein

dairy cows were obtained from abattoirs located in the north of Israel. In the lab, the umbilical cords were digested and plated as previously described in [44], followed by expansion in low glucose Dulbecco's Modified Eagle's Medium (Gibco, 31885-023 or Biological Industries (BI), 04-001-1A), containing 10% Fetal Bovine Serum (FBS) (BI, 04-001-1A), penicillin-streptomycin solution 1% (BI, 03-031-1B), Glutamine 1% (BI, #03-020-1B) and cryopreserved at different passages using FBS with 10% DMSO (Sigma-Aldrich, W387520). The media was changed every 2-3 days and all cells were cultured in a humidified incubator with a controlled environment of 5% CO2 and a temperature of 37°C, unless mentioned otherwise.

Cell Characterization

Three bovine MSCs lines were used in this study. Two were previously examined and verified [22,43] and the third was examined and verified in this study (Supplementary Figure 1 (S1A-S1C)) before subsequent heat shock experiments. One line was used for the RNA-seq experiment, while all three lines were used for RNA-seq validation and further examinations. All the experiments described were done on cells in passages 2-4, and at least 3 biological replicas were used unless specified otherwise.

Cell Death Quantification Using Propidium Iodide (PI)

For quantification of cell death in culture, cells were harvested by trypsinization, washed with PBS, and re-suspended in 10 g/mL PI for 5 min on ice. The percentage of live/dead cells was determined within 1 hour of staining by flow cytometry (CytoFLEX, Beckman Coulter, Indianapolis, IN, USA). 10,000 events were collected per sample. At least three biological repeats were used for each treatment. Data analysis was performed using Flow-Jo software.

Heat-shock Induction

MSCs at early passages (P2–P4) were seeded in 6-well plates (Costar, 3516) at different concentrations to allow similar confluence at the end of the experiment, as follows:

Time of treatment	Temperature	# of cells seeded	abbreviation
six hours	37°C	250,000	ShortNT
	40.5°C	250,000	ShortHS
Three days	37°C	42,000	LongNT
	Six hours at 40.5°C, three days recovery at 37°C	72,000	ShortHS_Recovery
	40.5°C	72,000	LongHS

The cells were incubated for 24 hours in normal conditions before treatment onset (time 0).

Cell Counting

Following the HS, the cells were washed with Dulbecco's Phosphate Buffered Saline (BI, 02-021-IA) and trypsinized (BI, 03-053-1A). 6µL of trypsinized cells were mixed with 6µL of trypan-blue (Sigma, T8154), loaded into an appropriate slide, and counted using an automated cell counter (TC20, Bio-Rad Laboratories Hercules, CA, USA). Three to five biological repeats were performed.

RNA-sequencing

Total RNA from the heat shocked cells was extracted via PureLink RNA Minikit (Invitrogen, 12183018A). RNA concentrations were measured using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies) and the quality of extracted RNA was confirmed (RNA integrity number (RIN) > 9) using an Agilent 2100Bioanalyzer before further processing. Library preparation was done using the INCPM mRNA library of the Weizmann Institute, Rehovot. The quality of cDNA libraries was determined using a tape-station. Sequencing was done using Nextseq Illumina in the INCPM of Weizmann Institute, with single-end reads of 75bp. Sequencing was done on 14 samples

from the same MSC line as in [22], and after initial quality analysis one sample was removed and we were left with 13 samples to perform the in-depth analysis: 3 biological replicas for ShortNT, ShortHS and LongHS treatments, and 2 biological replicas for LongNT and ShortHS_Recovery treatments.

Data Analysis

Following sequencing, reads were trimmed using Cutadapt, mapped to genome (Bos_taurus.ARS-UCD1.2) and counted using STAR and HTSeq. Further quality control of alignment sequencing data was done using the Qualimap. Normalization of the counts and differential expression analysis were performed using DESeq2. Hierarchical and K-means clustering as well as data visualizations were performed using R packages. Gene names were taken from the Ensemble BioMart ARS-UCD1.2 dataset. Gene Ontology (GO), KEGG and Reactome pathways enrichment analyses were conducted using both ranked gene list analyses using Gene Set Enrichment Analysis (GSEA) software version 4.1.0 and DEGs list analysis using g:Profiler version e103_eg50_p15_eadf141 and ClueGO v2.5.7 (Cytoscape plug-in) with default parameters. KEGG pathway maps were downloaded from KEGG pathway database. String datasets were used for functional protein association networks. Epigenetic complexes analysis was performed using the EpiFactors Database [45] as well GO annotations of the MGI database (Mouse Genome Informatics). The cytokine pathway list was taken from PathCards v5.7.551.0. Bivalent enrichment was done by comparing our list to "3,868 bonafide bivalent genes" in supplementary table 1 [46]. For detailed information see Supplementary Materials and Methods.

RNA extraction, Reverse transcription, and Real-time PCR

RNA extraction from cells was carried out using GenElute™ Total RNA Purification Kit (Sigma, RNB100-50RXN). RNA was then reverse-transcribed into cDNA using qScriptTM cDNA Synthesis Kit (Quanta-bio, 95047-100). Real-Time PCR (RT-qPCR) reactions were performed using Fast SYBR

Green Master Mix (Applied Biosystem, 4385614) in an ABI Step-One Plus Real-Time PCR system. All primers were verified by standard curve evaluation and are shown in Supplementary Table S1. Primers for expression analysis were designed on exon-exon junctions and a -RT control was performed each time. Relative mRNA fold change was calculated with the ΔΔCT method, using 1-2 control genes (PSMB2 and RPS9) as reference.

Statistical Analysis

GraphPad Prism (La Jolla, CA, United States) was used for statistical analysis and visualization of RT-qPCR. $p < 0.05$ was considered statistically significant. For enrichment analysis hypergeometric p-value was calculated using The Graeber Lab online calculator 2009 © (https://systems.crump.ucla.edu/hypergeometric/). See Supplementary Materials and Methods for more detailed information.

Results

RNA-seq analysis of heat shock treated and control cells.

To examine the effects of thermal stress on the cell's transcriptome, we evaluated the transcriptional response of MSCs to *in vitro* heat shock (HS). MSCs were extracted from the UC of a preterm fetus as previously described [22]. Prior to the HS treatment, MSCs marker expression (Supplementary Figure S1A), proliferation (Supplementary Figure S1B), and differentiation capacities (Supplementary Figure S1C) were examined and validated as in [22,43].

HS treatment protocols were designed to examine the short- or long-term effects of exposure to mild HS conditions (Figure 1A). One day after seeding MSCs from early passages (P2–P4), were moved from 37°C to 40.5°C for six-hour (ShortHS) or three days (LongHS) or a six hours HS followed by three days recovery back at 37°C (ShortHS_Recovery). Each HS treatment had a matching 37°C normothermic (NT) control (ShortNT and LongNT). The two time points were selected to capture

the immediate transcriptional effects of HS vs. the stable and delayed ones. Immediately after the completion of the six hours or three days treatments, the cells were harvested, viability was checked and pellets were stored at -80º for later matched RNA extraction and sequencing. The viability of the cells and their MSC marker expression levels remained high following the various treatment protocols (Supplementary Figure S1D, S1E). Two or three biological repeats were done for each treatment (Supplementary Table S2). All RNA samples were run together on Nextseq Illumina and showed a high percentage and quality of mapping to exons and low levels of rRNA and mitochondrial DNA contaminations (Supplementary Figure S2A, S2B). Principal component analysis shows that the HS treatment determined the transcriptome profile as samples from the same treatment clustered together and apart from the other treatment groups (Figure 1B). Hierarchical clustering uncovered a secondary factor affecting the transcriptome which could be attributed to the time passed from seeding (Supplementary Figure S2C) or the population doubling (Supplementary Table S3). Several genes were also evaluated by RT-qPCR to validate the sequencing results (Supplementary Figure S2D).

We found that the various HS treatments significantly changed the expression levels of a total of 3667 genes (padj ≤0.05 and log2(FC)≥1) (Figure 1C). After six hours of HS, 712 differentially expressed genes (DEGs) were found, whereas after three days of HS 1,371 DEGs were found, most of them downregulated (Supplementary Figure S2E). In the cells that were allowed to recover at 37ºC for three days after the short HS, 2,037 genes were changed versus the short HS. However, only a slight difference is found when compared to the long NT sample. This suggests that after a short HS there is a major recovery from the stress, at least on the transcriptional level. The results indicate that short thermal shock affects MSCs' gene expression rapidly and widely but mostly transiently.

To discern the dominant systematic changes following the different HS treatments, we performed k-means clustering on all 3,667 DEGs (Figure 1D and Supplementary Figure S3). The resulting clusters revealed three major modes of change. Clusters 2 and 4 show the common effect of both long and short HS on the transcriptional pattern. Conversely, in clusters 3 and 7 the change is only observed after the long HS, portraying that more genes were induced after three days, possibly due to a slower transcriptional response rate (Figure 1C). Clusters 1 and 6 show the effect of culturing time on the transcriptional pattern.

While changes in expression patterns are evident from the k-means clustering analysis, we wanted to systematically examine the transcriptional changes following each treatment alone and identify specific biological features and processes.

Cell cycle and immune response are downregulated as developmental pathways are activated following a long heat shock

To get a comprehensive understanding of all the changes that occurred following the HS, we delved into the analysis of each treatment using the gene set enrichment analysis (GSEA) and gene ontology (GO) tools (as described in the supplementary materials and methods). First, we focused on the changes observed in cells exposed to long HS vs. long NT control (Figure 2A). On the morphological level, cells appeared flattened, with expanded cytoplasm and detectable stress granules (Figure 2B, stress granules marked with arrows). On the transcriptional level, 1,192 DEGs (padj ≤0.05 & log2(FC)≥1) were found (Figure 1C). GSEA analysis show downregulation of cell proliferation and metabolism; namely cell cycle and DNA repair, glycolysis and oxidative phosphorylation (Figure 2C and Supplementary Figure 4A) and upregulated response to stress (Figure 2C). Interestingly, many differentiation and cell-fate related pathways (Figure 2D, Supplementary Figure 4B, C) were also upregulated, as previously suggested [47]. For example, general terms of cell growth and

morphogenesis were enriched, as well as more specific terms like connective tissue, chondrocytes, osteoblasts, kidney, and neuronal system are upregulated. In addition, there is apparent upregulation of cell adhesion molecules, which represents cell-cell interactions and the interaction between the cell and the extracellular matrix (ECM). These molecules promote a broad spectrum of cell signaling that directly or indirectly modulates stem cell proliferation, self-renewal property, adhesion, and multilineage differentiation [48,49]. There is downregulation of gene sets related to inflammation and the immune system, as well as DNA damage and ROS response. This could be explained by the long duration of the HS, which might have required the cells to attenuate their stress response. What is more, these changes were accompanied by the upregulation of post-translational modifiers, specifically histone demethylases and Polycomb group (PcG) complex members (Figure 2E). Additionally, KEGG analysis showed enrichment and upregulation of pathways such as MAPK, PI3K-Akt, RAS and RAP1 (Figure 2F). In general, those pathways are known to regulate cell size, survival, differentiation, adaptation to growth conditions, and stress responses [33]. PI3K-Akt activates the mTOR signaling pathway [33,50], which is also elevated following heat stress in bovine granulosa cells [47]. Activation of PI3K-Akt and mTOR pathways plays a central role in cellular senescence and organismal aging and thus acts as a driver of stem cell depletion and reduced tissue regenerative capacity [51–53].

In summary, our data demonstrate that three days of HS treatment are not detrimental to cell viability but have a major effect on cell fate and aging. Although the impact of long HS on differentiation might prove useful as a pretreatment protocol for MSC transplantations, this duration of HS is scarcely experienced in physiological settings. Hence, we wished to examine the effect of short (six hours) HS on the MSCs.

Stress responses are induced while cellular functions decline following a short heat shock.

Initially, we focused on the immediate effects of short HS (Figure 3A) using GSEA analysis. Five out of the ten most upregulated genes in this treatment (HSPA1A, HSPA6, HSPA4L, HSPB1, HSPH1, Figure 3B) were heat stress genes, along with genes that regulate redox maintenance (SLC27A5), insulin levels (MAFA) and mitotic progression (MISP). The most downregulated genes following short HS are involved in RNA interference (RNAi) process (TSNAX), post-splicing multiprotein complex (UPF3A), cell polarity (DAAM2), metabolism regulation (DHRSI2) and chromatin and transcription process (H1-2). These genes are mostly included in the Hallmark gene sets that are enriched for up- and down- regulated processes following six hours of HS (Figure 3C and Supplementary Figure 5A, 5B). Short heat stress induced stress response and differentiation pathways while halting oxidative phosphorylation and cell cycle. Upregulated biological processes enriched in GSEA were stress response, autophagy, development, and mRNA transcription (Figure 3D, Supplementary Table S4), while downregulated processes were all related to DNA replication (Figure 3E and Supplementary Figure 5B). In addition to GSEA, we performed the analysis in g:Profiler, KEGG and Reactome which also suggests that stress response and ER activity were induced whereas DNA replication, with an emphasis on initiation, was decreased (Figure 3F). After discovering dramatic short-term changes, we proceeded to check how many of these changes remained stable. To elucidate these stable changes, the treated cells were allowed to recover in normothermia for three days before the transcriptional analysis.

Long term effects of short heat stress.

To identify the transcriptional changes which occurred after short HS but remained stable even after 3 days of recovery, we compared the three different time points: control (as the zero-time point), six hour HS and six hour HS followed by three days of recovery (ShortNT vs. ShortHS vs.

ShortHS_Recovery, Figure 4A). Thus, we re-analyzed the data (see Supplementary materials and methods) using K-means clustering to visualize transcriptomic differences (Figure 4B). While after recovery most genes regained similar expression levels to that of the control sample, two clusters in which the differential expression persisted were identified. Cluster 2 genes were lowly expressed in the control sample but were then upregulated after the short HS and remained active after recovery. GO annotations and KEGG pathways enriched in this cluster are mostly related to stress and protein degradation (Figure 4C). This implies that some elements of the stress response to acute stress last for more than three days. Cluster 3 is the mirror image of cluster 2, presenting stably downregulated genes related to proliferation and metabolism but also senescence and DNA damage (Figure 4D). This might suggest that following short HS, the cells enter a state of quiescence to maintain their stemness. This data is in agreement with our population doubling time analysis, which shows slower proliferation after both the long and the short HS (Figure 4E).

Similar analysis done on the other two clusters, 1 and 4, raised an interesting point. Of the most 25 significantly enriched GO terms in cluster 4, only 3 are not related to transcriptional regulation, mostly epigenetic modification (Figure 4F). This raised the intriguing hypothesis that the epigenetic landscape is altered following the short HS, possibly with long term consequences. To examine how a short period of stress could have that kind of persistent effect, we used the EpiFactors Database [45] and related MGI annotations to identify enriched epigenetic modifiers in the data. Several epigenetic complexes were found highly enriched in Cluster 4, i.e., upregulated after six hour HS and then back to low expression after recovery (Figure 4G). Interestingly, those complexes are all related to the mixed lineage leukemia (MLL) proteins which catalyze the trimethylation of histone H3 Lys 4 (H3K4me3), a mark associated with active or poised transcription and found in the promoters of most active or poised genes [54]. Bivalent genes are lineage-specification genes that

carry both H3K4me3 and H3K27me3 histone marks and are regulated by the balance between the two histone marks. This balance is mediated by PcG members and the MLL coactivator complex [55]. We searched for known targets of the MLL complex in our list and found cluster 2 (i.e. upregulated in both short- and long-term) to be significantly enriched for bivalent genes [46] crucial to cell fate regulation: several FGF family members, FZD3, JUN, FOXF1, VEGFA and KDM6B, to state a few (Supplementary Figure S6). This was in contrast to the other clusters, where these and other bivalent genes were significantly underrepresented (Figure 4H). Overall, this data sheds light on the long-term effects of short HS and suggests that a short stress event can modulate the epigenetic regulation of key cell fate genes.

Next, we wished to uncover the transcriptional changes shared by all HS-treated cells.

Key characteristics of MSC vary with heat stress duration.

So far, we have shown considerable effects of short and long HS *in vitro*. To examine to what extent the DEGs are shared between the timepoints, we had to eliminate the effect of culturing time and the circadian clock and focus solely on the effect of HS. To that end, we removed all DEGs that changed between the short and long NT controls (see Supplementary materials and methods for the analysis) and examined which DEGs were commonly upregulated (Figure 5A) or downregulated (Figure 5B) in all HS. A total of 102 upregulated and 93 downregulated DEGs were shared by short and long HS treatments, a highly significant enrichment over what could be expected by chance. While the commonly upregulated genes were mostly related to stress response, the commonly downregulated genes were not significantly enriched to any specific pathway or process. Hence, we examined if the different DEGs are annotated to shared biological processes and pathways which are therefore affected by both short and long HS. Important cellular functions like oxidative phosphorylation and cell cycle were found to be impaired in all HS; immune system was

downregulated, and the hedgehog signaling pathway was significantly upregulated following HS, even after the recovery period (Figure 5C, Supplementary Figures S7A-D).

To understand the effect of HS on the biological processes related to the key capacities of MSCs, namely self-renewal, differentiation and immunomodulation, we compared the relevant biological processes and functions between different treatments. For cell cycle we saw that while in all three treatments the downregulated DEGs were enriched for the general term 'cell cycle', only the short HS is downregulating specifically DNA replication pre-initiation (e.g., genes like MCM2 and 7, CDC6 and E2F1). The three days samples showed downregulation of genes required for the G2/M phase (namely cyclin A and B and CDC25), suggesting a block before mitosis (Figure 5D). Interestingly, the cells that got the short HS but were let to recover for three days in normothermia show both the G1/S and the G2/M effects although to a lesser degree, suggesting that even short thermal stress might disrupt cell cycle regulation and proliferation. These results are supported by the population doubling we saw in the culture following the experiments (Figure 4E) as well as in our previously described cell cycle analysis [22].

As for differentiation, it appears that several developmental pathways were activated, depending on the heat shock treatment (Figure 5E, Supplementary Figure S7E-F). The long HS induced various differentiation pathways; the most enriched were the differentiation to mesoderm/mesenchymal fate. Notably, both canonical and non-canonical WNT pathways were upregulated after long HS, suggesting a general shift in cell identity. The short HS upregulates the canonical WNT but downregulates the non-canonical (WNT5a related) pathway (Figure 5E, Supplementary Figures S7E, F). In line with the fact that KEGG definitions like "canonical WNT pathway" includes both activators and suppressors, this and other pathways are enriched in both the down and upregulated DEGs. Interestingly, HOX genes related to skeletal morphogenesis and several epigenetic factors (e.g.,

Setd2, Kdm6a, Jarid1) were upregulated following both long and short HS; suggesting that heat shock treatment may slow proliferation while promoting MSC's commitment and differentiation. Immunomodulatory properties of MSCs have previously been shown to decline following stress [22,56]. Major downregulation of immune related pathways was detected after HS (Figure 5F). GO analysis in GSEA revealed considerable effects on the production of interleukins (ILs), TNFs and INF-ɤ. In addition, we observed the downregulation of antigen processing, acute inflammatory response, response to chemokines, and the migration and chemotaxis of macrophages, neutrophils, and granulocytes (Supplementary Table S5). The list of 760 genes in the "Cytokine Signaling in Immune system SuperPath" (https://pathcards.genecards.org) was analyzed and 38 of them (for example IL1Rs, IL7 and IL18) were to be downregulated after long HS while 17 (like FGF2 and FGFR2 which are related to differentiation [57,58]), are upregulated (Figure 5G). Overall, genes related to IL production are significantly downregulated (hypergeometric p-value = 2.49e-04, Figure 5G).

Discussion

Here, we show that the transcriptional changes following heat shock *in vitro* are broad and can alter every functional aspect of MSC identity. We analyzed RNA-seq from bUC-MSCs in normothermia vs. short or long exposures to HS, to investigate the subsequent changes in the transcriptional landscape that might lead to a shift in the cell's identity and fate. We were particularly interested in identifying the long-term effect of thermal stress on cell proliferation, differentiation, and immunomodulation capacities; as those key MSC features were amongst the most abundant GO term groups. To examine the relevance of our data to physiological heat stress, we compared our DEGs to a list of 55 genes defined as thermal stress responsive in a bovine study done *in vivo* [60]: 36 of the 55 genes were found to be differentially expressed following short, long or both HS groups. The genes EIF2A, HSPA1A, HSP90AA1, and HSF1 were considered by the authors key genes that

responded to thermal stress of Holstein dairy cattle. From our experiments, HSPA1A, and HSP90AA1 were indeed upregulated DEGs for short and long HS groups, and EIF2A for shortHS group only. HSF1 was not differentially expressed in our treatments, maybe due to our use of 40.5°C which is mild relative to the 42°C used by the authors. In addition, Fang et al. bioinformatics analyses pinpointed biological processes and pathways associated with thermal stress that are very similar to those we have identified, e.g., protein folding, transcription factor binding, immune effector process, negative regulation of cell proliferation, PI3K- Akt signaling pathway, and MAPK signaling pathway. The immediate transcriptional changes found following short HS include the onset of several cellular stress responses and cell cycle arrest in G1/S. This checkpoint arrest is a known adaptive cellular response to heat stress, in which cells slowdown proliferation rate while rapidly upregulate the transcription and translation of HSPs to maintain cell homeostasis and retain their cellular functions [60]. Our previous results, which showed cell cycle arrest at G1/S after short HS [22] also support this finding. Two possible interpretations arise, each with a different implication for stemness and differentiation: in one, following the short HS the cells enter a quiescent state; this is in alignment with the observed metabolic changes (reduced amino acid metabolism, reduced oxidative phosphorylation, increased glycolysis, and mitophagy induction of mitochondrial renewal, etc., as reviewed in [61]. This suggest that short HS treatment encourages the retention of MSC stemness. In the other interpretation, a long G1 may allow for the accumulation of the epigenetic changes needed for the initiation of fate decisions [62]. This interpretation might explain the transient upregulation of many epigenetic factors and the stable changes in many lineage commitment genes observed after three days recovery. To mechanistically challenge those two interpretations and to determine whether HS promotes stemness or differentiation. Further study, preferably on the single cell level, is required to mechanistically challenge these two interpretations.

Evident changes were observed in the morphology and proliferation rate of the cells three days after the Short or long HS treatment. However, on the transcriptional level, the majority of ShortHS DEGs reverted to normal expression levels after three days recovery, and only a subset of the DEGs remained. In this subset of stable DEGs we see the upregulation of several developmental genes known as "bivalent" for their dual histone lysine tri-methylation marking in both K4 (active mark) and K27 (repressive mark). Those genes are known targets of the MLL complex, which carries out the methylation of histone H3 lysine 4 and was transiently overexpressed after the initial HS event. So, a sequence of events can be hypothesized. First, the short HS upregulates the MLL complex members, thus shifting the balance between the active and repressive histone marks. Consequently, the chromatin of the previously poised (and silent) bivalent genes opens to enable transcription of developmental genes, priming the cells toward cell fate determination. Evaluation of this hypothesis will require follow-up studies examining changes in epigenetic marking following HS.

The idea that cells after HS pretreatment are more prone to differentiation is tempting. If indeed priming MSCs by specific stress can help us direct their fate, it could provide us with another tool in the cellular therapy toolbox. Moreover, as the idea of cellular agriculture, or cultured meat, gains increasing attention, more cost effective and clean ways to differentiate the source cells toward the intended fate (usually muscle or fat) are needed. Current differentiation protocols require large amounts of expensive and unstable growth factors, which raise costs and hinder commercialization of cultured meat. Every pretreatment protocol that is easy, cheap, and reduces the time and resources necessary for differentiation, will have huge economic and environmental consequences.

On the clinical level it is interesting to note the negative effect of long HS on MSC immune activity, possibly compromising the cells' immunomodulatory functions. Indeed, reduced immunomodulation was previously observed when we co-cultured MSCs subjected to HS with

macrophages [22]. The reduced immunomodulation capacity of the stressed MSCs might account for the increased production of inflammatory cytokines found in mammary epithelial cells following HS [63]. Hence, the increased rates of inflammatory diseases in dairy cows during the summer months could be partially attributed to the failure of the malfunctioning MSCs to modulate the inflammatory response of the surrounding cells to HS. Overall, identifying the long-term effect of HS on MSC capacities suggest an explanation for the seeming contradiction between the in vitro experiments which show beneficial effects to HS priming and the in vivo data that demonstrate harmful physiological consequences to thermal stress (reviewed by [64]).

Although we have demonstrated that HS affects MSCs' transcriptome, our study has some limitations. Population heterogeneity, circadian clock, culturing time, and culture conditions all have effects on RNA-seq results that need to be controlled for (Supplementary Figure S8). To counter this drawback we (1) made sure that at the end of the experiment (either six hours or 3 days) all the treatment groups had similar culture density (Supplementary Table S2) (2) sequenced two or three independent biological repeats from each treatment group (Supplementary Table S3) (3) to reduce heterogeneity that might originate in genetic background, the same MSC line was used in all three repeats. To counter the effects of culturing time, we used untreated (NT) controls, one for the short HS, and the other for the long HS. When we compared gene expression at the short and long time points, all DEGs that were apparent between the NT control time points (and were assumed to be the result of culturing effect) were ignored. Interestingly, two transcription factors regulating the circadian clock, BHLHE40 and TIMELESS, were found among those ignored DEGs. These two transcription factors together regulate the expression of 202 other DEGs in this group (Supplementary Figure S8B). These oscillating genes may partly explain the relatively high number of DEGs due to culturing effect since the ShortHS and ShortNT samples were taken at noon (24h

after seeding + six hours treatment) while the three day samples (LongHS, LongNT and ShortHS_Recovery) were collected in the morning (exactly 96h after seeding).

Conclusions

The use of MSCs for cell therapy or cultured meat has several advantages, such as high availability, low production price, and fast and easy differentiation. Nonetheless, our limited understanding of their heterogeneity, fate determination, and immunomodulation holds back further MSC applications. It is therefore essential to improve the consistency and efficacy of MSCs. Such enhancement can be achieved using in-vitro preconditioning treatments such as HS [14]. Here we show that the correct application of HS allows for the enhancement of desired MSC characteristics and induction of a wide range of stem cell fates and differentiation pathways.

List of abbreviations

MSCs:
Mesenchymal stem cells
bUC-MSCs:
Bovine umbilical cord-derived mesenchymal stem cells
ER :
endoplasmic reticulum
FBS:
Fetal Bovine Serum
RNA-seq:
RNA sequencing
RT-qPCR:
Reverse transcriptase- quantitative polymerase chain reaction
HS:
Heat shock
NT:

Normothermic

DEGs:

Differentially expressed genes

GSEA:

Gene set enrichment analysis

GO:

Gene ontology

ECM:

Extracellular matrix

PcG:

Polycomb group

MLL:

Mixed lineage leukemia

H3K4me3:

The trimethylation of histone H3 Lys 4

ILs:

Interleukins

Ethics approval and consent to participate

The procedures of isolated UC-MSCs were conducted abiding by the Institutional Animal Care and Use Committee of the Israel Ministry of Agriculture and Rural Development, permit #11380.

Consent for publication

Not applicable.

Availability of data and materials

The datasets supporting the conclusions of this article are available in the GEO repository, GSE214467 study at: https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE214467

Some datasets supporting the conclusions of this article are included within the article additional files.

Competing interests

The authors declare that they have no competing interests.

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Authors contributions

IRH: conception and design, designing and performed the experiments, bioinformatics analysis and data interpretation, and manuscript writing. GS: validation of MSC lines by flow and RT-qPCR. CS: support with MSC extraction and culturing. SS: conception and design, assembly of data and data analysis, manuscript writing. The authors read and approved the final manuscript.

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Additional information

Additional file 1.pdf. A detailed list of additional files, Supplementary Figure 1-8, Supplementary figure legends 1-8, Supplementary Materials and Methods, and Supplementary Table 1-3.

Additional file 2. xlsx. Supplementary Table 4

Additional file 3.xlsx. Supplementary Table 5

Additional file 4.xlsx. Supplementary Table 6

Additional file 5.xlsx. Supplementary Table 7

Figure legends:

Figure 1: Transcriptome changes in response to heat shock. (A) A schematic timeline showing the HS treatments from which RNA-Seq libraries were prepared. MSCs were plated and 24h later exposed to heat shock (HS, 40.5°C) for: 6h (ShortHS), 6h + 3 days recovery at 37°C (ShortHS_Recovery), 72h (LongHS), followed by collection for RNA extraction. Control cells were parallelly plated and maintained in normal temperature (NT, 37°C). Each treatment has colour code which will be used throughout the article: ShortNT (red), ShortHS (green), LongNT (olive), ShortHS_Recovery (blue), LongHS (purple). (B) Scatter plot for principal component analysis of global gene expression (RNA-seq) demonstrates clear separation of gene transcripts between groups. (C) Number of differentially expressed genes (DEGs) with padj ≤0.05 and log2(FC)≥1 that change between the treatment groups. (D) K-means clustering of 3667 DEGs.

Figure 2: Downregulation of cell cycle and immune response genes while developmental pathways genes are induced following long heat shock. (A) Illustration of long heat shock treatment. (B) Cells after LongHS get enlarged and flattened. Bright field x4 (up) and x10 (down). Arrows point to stress granules. (C) Results of GSEA Hallmark analysis showing enriched gene

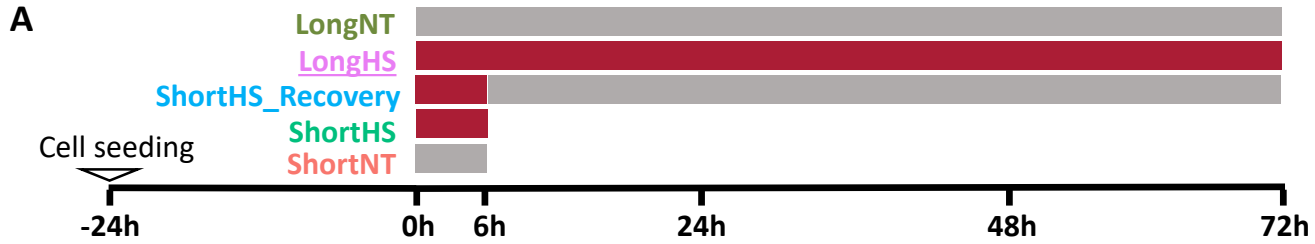
sets (FDR q-val < 25% and p-val < 0.05). A positive normalized enrichment score (NES) values, mark in red, indicates upregulation in the LongHS phenotype. (D) Differentiation pathways significantly upregulated in LongHS vs. LongNT as per g:Profiler analysis. Plot was generated using GOplot R package. (E) Upregulated histone modifications and PcG complex following LongHS as per GO analysis with GSEA. (F) KEGGs significantly upregulated in differentially expressed genes of LongHS vs LongNT using ClueGo. Colors represented p-value: light red 0.05, red 0.01, brown 0.001. The node size represents the term enrichment significance.

Figure 3: Induced stress response and reduced cellular functions following short heat shock. (A) Illustration of short heat shock treatment. (B-E) Gene set enrichment analysis (GSEA) results of 40.5c6h vs. control comparison. RNA-Seq was performed on samples collected after 6 hours at 40.5c compared to cells cultured in 37c (control). (B) Heat map of the top 30 marker genes upregulated and downregulated in short heat stress. Expression values are represented as colors and range from red (high expression), pink (moderate), light blue (low) to dark blue (lowest expression). (C) Results of GSEA Hallmark analysis showing enriched gene sets (FDR q-val < 25% and p-val < 0.05). A positive normalized enrichment score (NES) values, marked in red, indicate enrichment in the 40.5c6h phenotype. (D) Upregulated GSEA biological processes significantly enriched (FDR q-val < 0.25 and p-val< 0.01) in the comparison of ShortHS vs. ShortNT, ordered by the increasing NES (dark color is the most enriched). Due to high number of GOs, the GOs with similar process were combined under one general phrase, which is represented by one part of the pie chart (Supplementary Table S4). The number defines average normalized enrichment score. (E) Downregulated GSEA biological processes significantly decreased (FDR q-val < 0.25, p-val < 0.01) in the comparison of ShortHS vs. ShortNT, ordered by the increasing NES. (F) Functional enrichment analysis of the upregulated (red) and downregulated (blue) differentially expressed genes (DEGs) between ShortHS and ShortNT cells using g:Profiler.

Figure 4: Transient upregulation of chromatin modifiers after short heat shock and stable activation of lineage commitment genes, maintained even after three days recovery. (A) Illustration of ShortHS_Recovery vs ShortHS treatment. (B) Heat map of genes differentially expressed (padj < 0.05) between ShortHS and ShortNT. (C) Biological processes enriched in cluster 2 (pink) (D) Biological processes enriched in cluster 3 (blue) (E) Population doubling time for prolonged treated groups. (F) Biological processes enriched in cluster 4 (purple) (G) MLL (GO:0044665, GO:0044666), HAT (GO:0000123) and HMT (GO:0035097) are upregulated following ShortHS but return to normal/downregulated during recovery period (H) Bivalent genes are upregulated following ShortHS and remain in this state even after recovery period.

Figure 5: Cell cycle and metabolism are commonly reduced following all heat shock protocols, while other processes related to key characteristics of MSC are differently regulated. (A) Venn diagram of upregulated genes (padj < 0.05) between the treatment groups. (B) Venn diagram of downregulated genes (padj < 0.05) between the treatment groups. (C) Heat shock common hallmarks and their enrichment score. Above hyphenated line are hallmarks common to all 3 HS groups. (D) Cell cycle related biological processes, effected by HS, and their enrichment score. N00455: CDC25-Cell cycle G2/M (KEGG pathway), GO:0007049: Cell cycle (MGI database), R-BTA-69002 – DNA Replication Pre-Initiation (Reactome). (E) Enrichment of

biological processed related to development effected by HS. GO:0060070: Canonical WNT signalling, GO 0048863: Stem cell differentiation, GO:0048468: Cell development, GO:0009888: Tissue development, GO:0007492: Endoderm development. GO:0007498: Mesoderm development, GO:0048762: mesenchymal cell differentiation. (F) Enrichment of biological processed related to immune system effected by HS. GO:0002526: Acute Inflammatory response, GO:1905517: Macrophage migration, GO:0032635: IL-6 production, Cytokine pathway list was taken from PathCards v5.7.551.0. (G) Cytokine pathways genes that are differentially expressed in LongHS vs. LongNT.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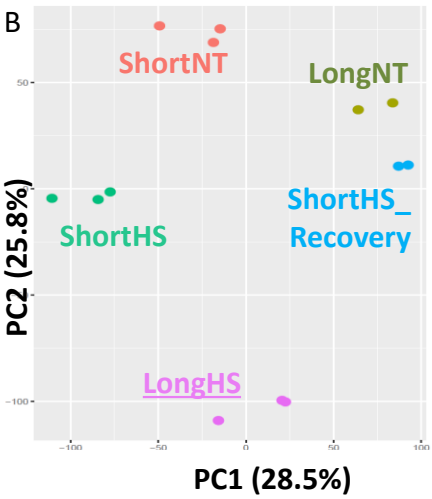
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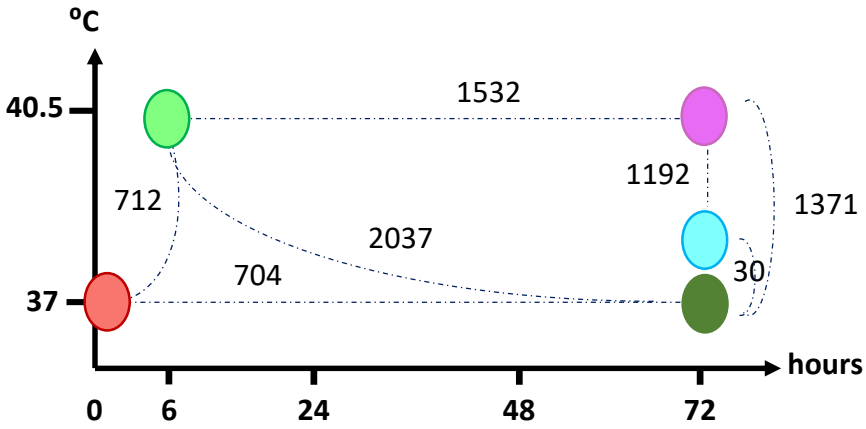
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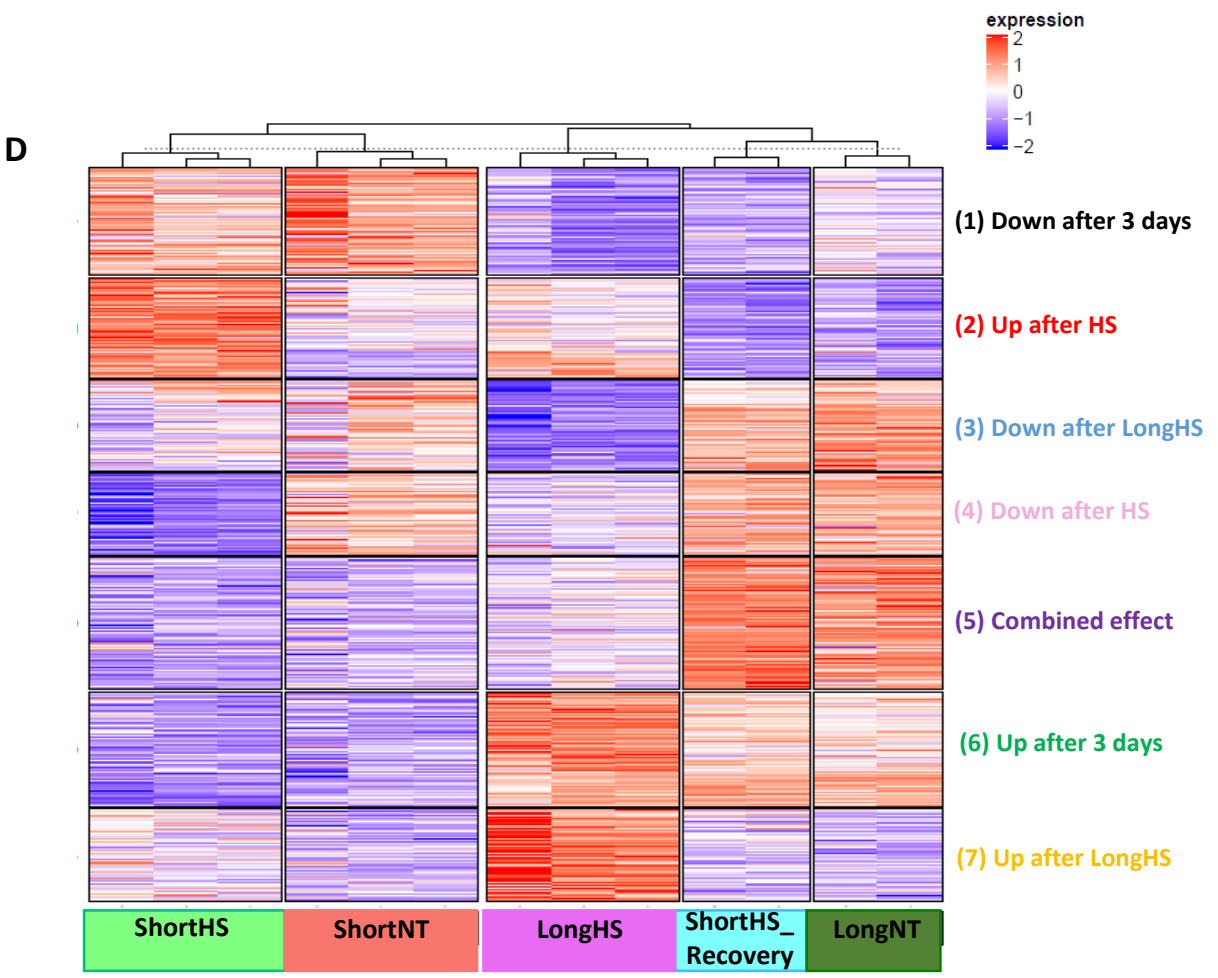
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(2) Up after HS
(3) Down after LongHS
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ShortHS
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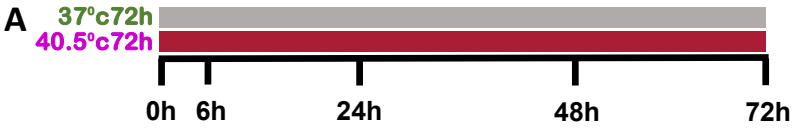
ShortHS_
Recovery
LongNT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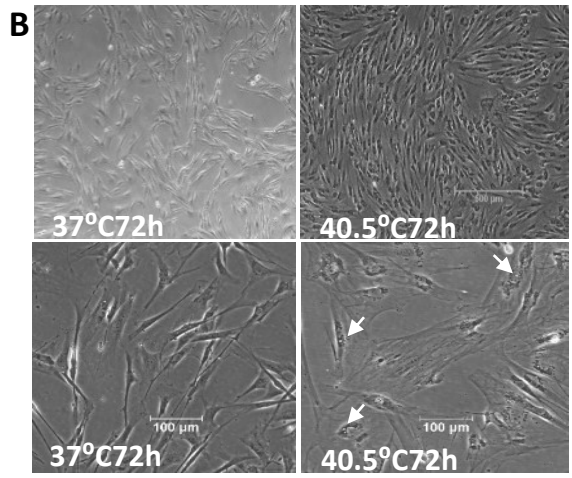
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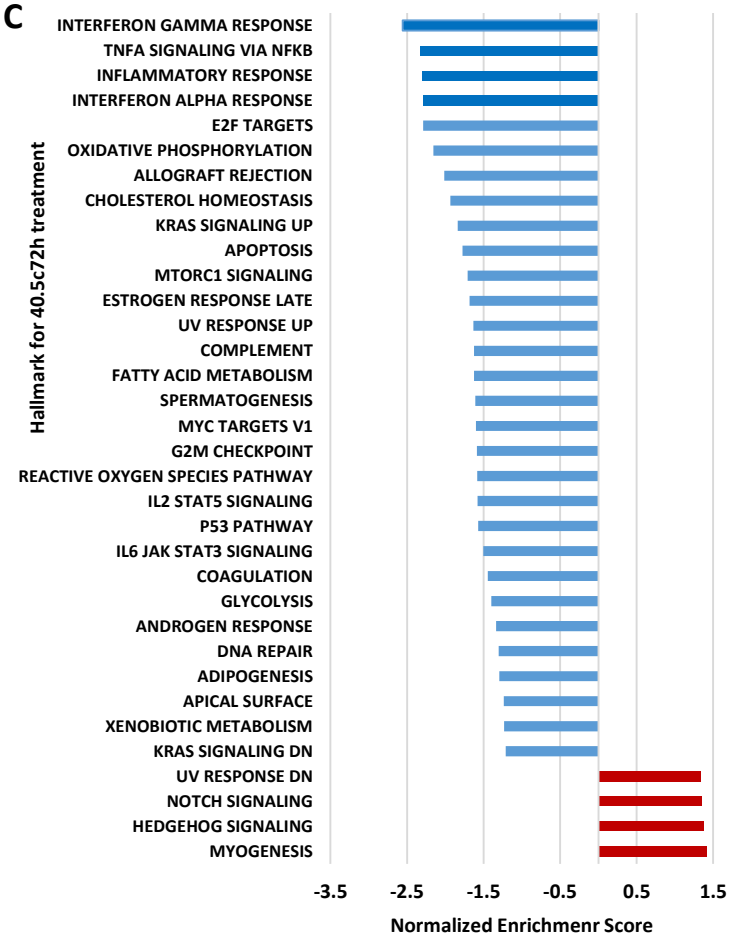
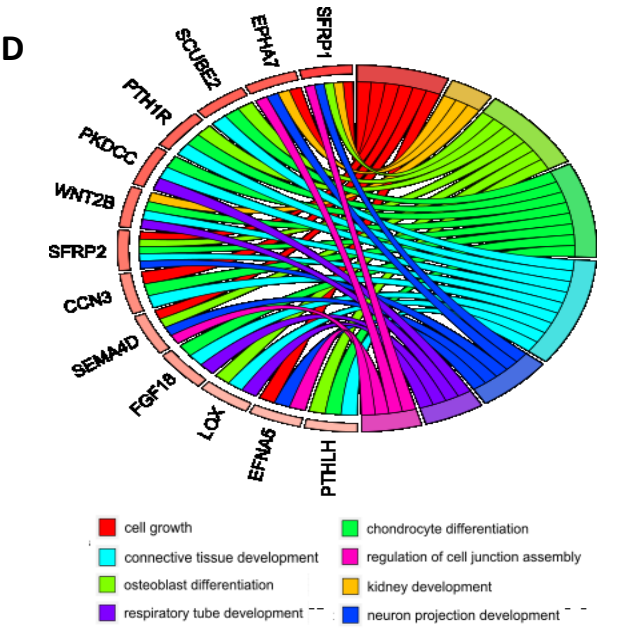
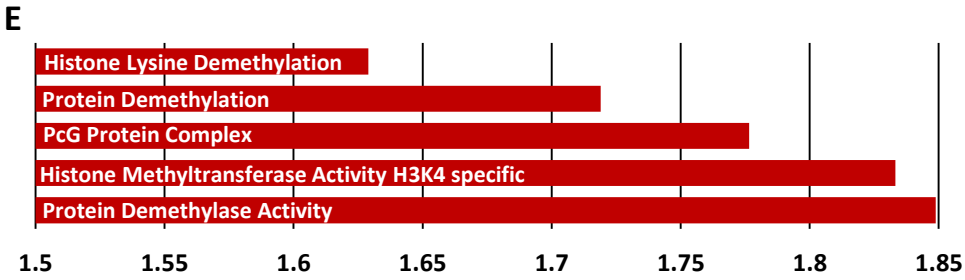
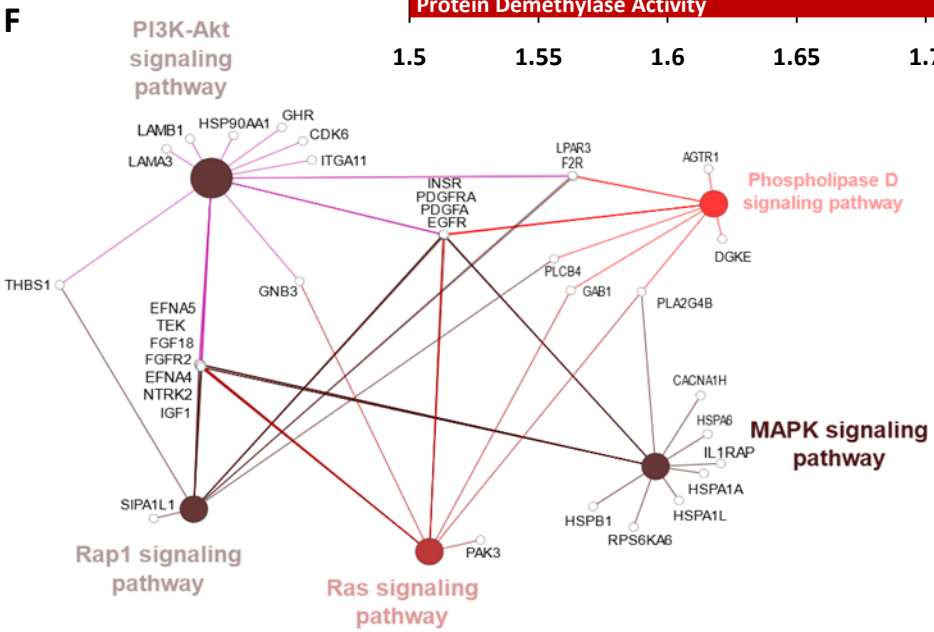
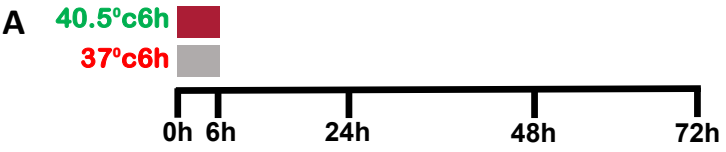

C
Hallmark for 40.5c72h treatment
INTERFERON GAMMA RESPONSE
TNFA SIGNALING VIA NFKB
INFLAMMATORY RESPONSE
INTERFERON ALPHA RESPONSE
E2F TARGETS
OXIDATIVE PHOSPHORYLATION
ALLOGRAFT REJECTION
CHOLESTEROL HOMEOSTASIS
KRAS SIGNALING UP
APOPTOSIS
MTORC1 SIGNALING
ESTROGEN RESPONSE LATE
UV RESPONSE UP
COMPLEMENT
FATTY ACID METABOLISM
SPERMATOGENESIS
MYC TARGETS V1
G2M CHECKPOINT
REACTIVE OXYGEN SPECIES PATHWAY
IL2 STAT5 SIGNALING
P53 PATHWAY
IL6 JAK STAT3 SIGNALING
COAGULATION
GLYCOLYSIS
ANDROGEN RESPONSE
DNA REPAIR
ADIPOGENESIS
APICAL SURFACE
XENOBIOTIC METABOLISM
KRAS SIGNALING DN
UV RESPONSE DN
NOTCH SIGNALING
HEDGEHOG SIGNALING
MYOGENESIS
-3.5
-2.5
-1.5
-0.5
0.5
1.5
Normalized Enrichmenr Score


D
SFRP1
EPHA7
SCUBE2
PTH1R
PKDCC
WNT2B
SFRP2
CCN3
SEMA4D
FGF18
LOX
EFNA5
PTHLH
cell growth
connective tissue development
osteoblast differentiation
respiratory tube development
chondrocyte differentiation
regulation of cell junction assembly
kidney development
neuron projection development

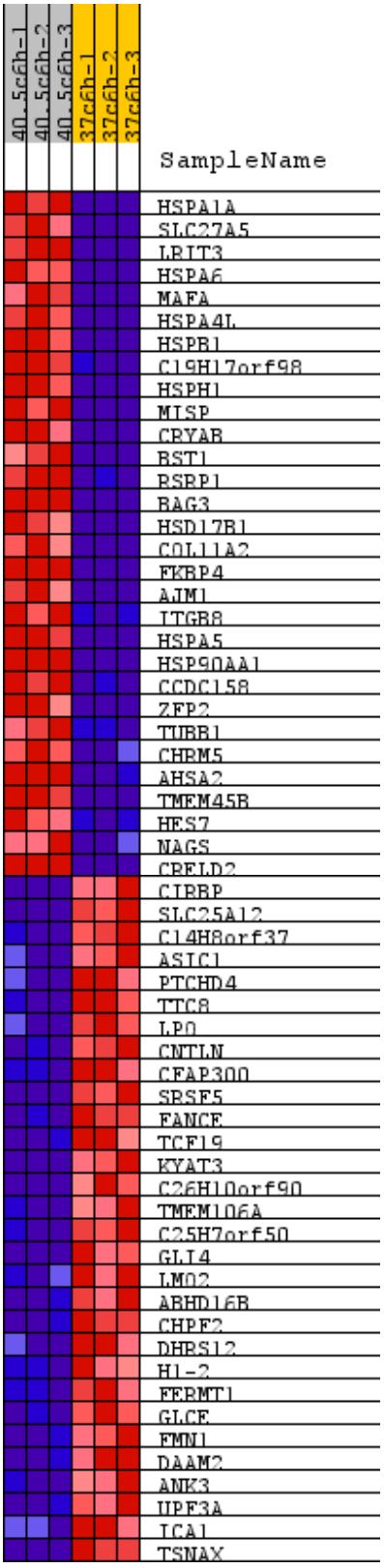

E
Histone Lysine Demethylation
Protein Demethylation
PcG Protein Complex
Histone Methyltransferase Activity H3K4 specific
Protein Demethylase Activity
1.5
1.55
1.6
1.65
1.7
1.75
1.8
1.85


F
PI3K-Akt signaling pathway
LAMB1
HSP90AA1
GHR
CDK6
LAMA3
ITGA11
LPAR3
F2R
AGTR1
Phospholipase D signaling pathway
INSR
PDGFRA
PDGFA
EGFR
DGKE
PLCB4
GAB1
PLA2G4B
THBS1
GNB3
EFNA5
TEK
FGF18
FGFR2
EFNA4
NTRK2
IGF1
CACNA1H
HSPA6
IL1RAP
MAPK signaling pathway
HSPA1A
HSPA1L
HSPB1
RPS6KA6
SIPA1L1
PAK3
Rap1 signaling pathway
Ras signaling pathway

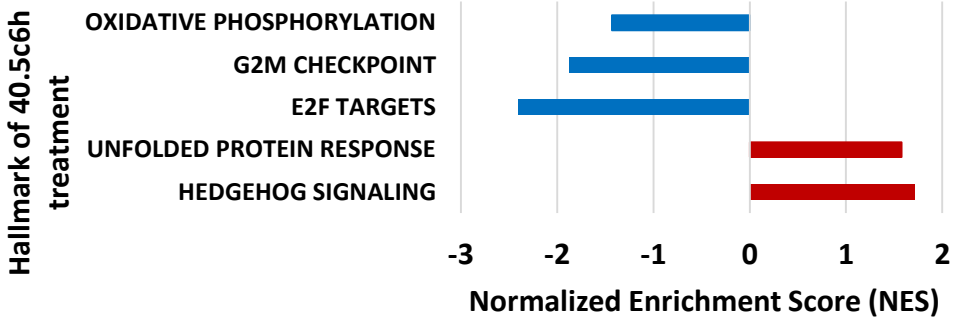
A



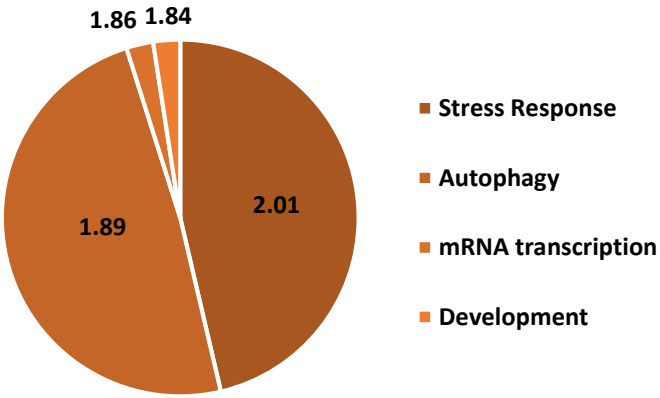
B



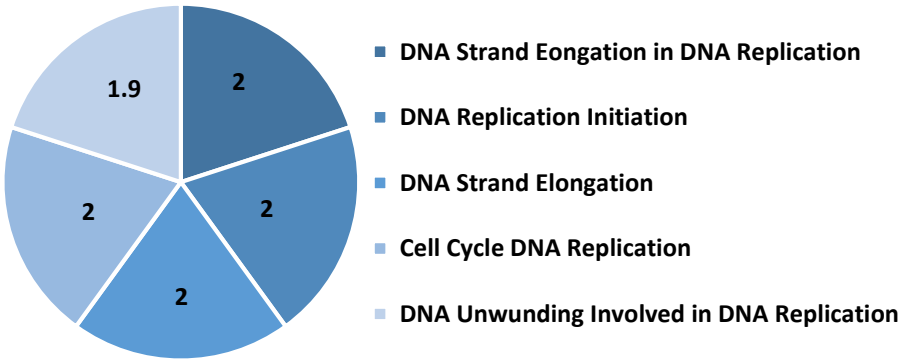
C



D

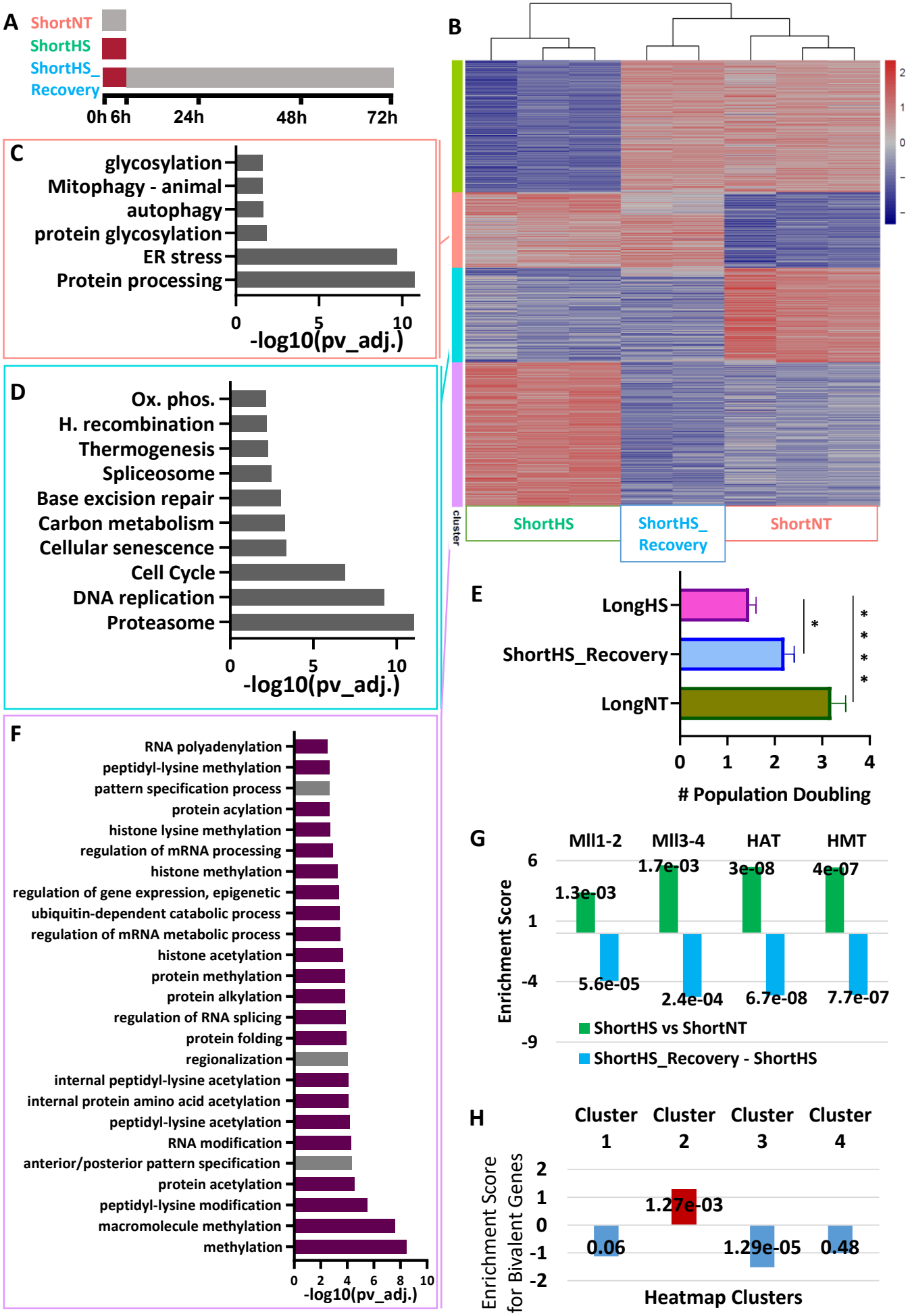


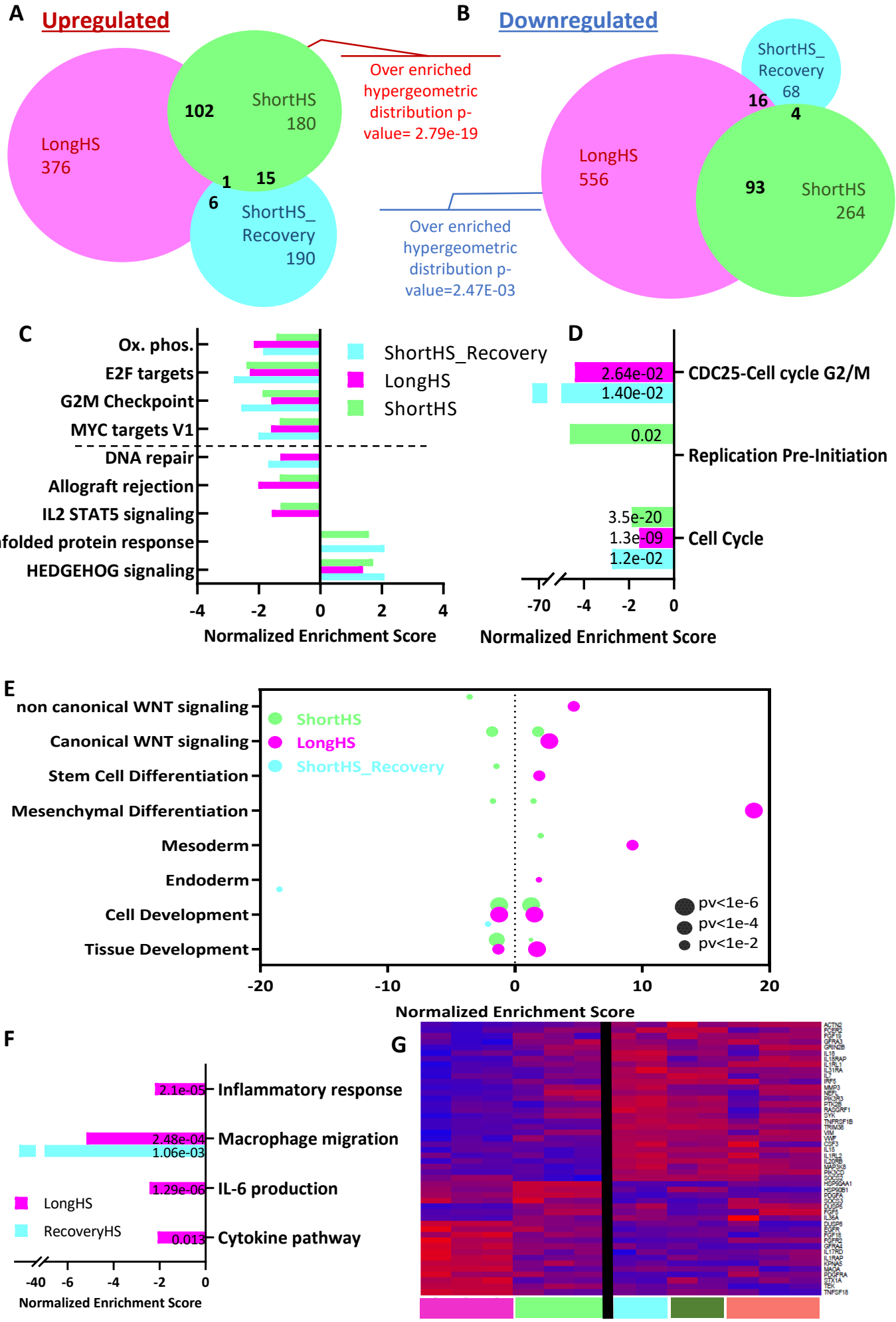
E



F

Term name	Term ID	p_{adj}
Protein processing in endoplasmic reticulum	KEGG:04141	1.720×10^{-9}
DNA-binding transcription activator activity	GO:0001216	1.222×10^{-2}
HSP90 chaperone cycle for steroid hormone receptors (SHR)	REAC:R-BTA-337...	2.349×10^{-4}
Regulation of HSF1-mediated heat shock response	REAC:R-BTA-337...	3.540×10^{-4}
Cellular response to heat stress	REAC:R-BTA-337...	3.540×10^{-4}
DNA replication initiation	GO:0006270	2.933×10^{-5}
organic acid catabolic process	GO:0016054	3.993×10^{-3}
carboxylic acid catabolic process	GO:0046395	1.330×10^{-2}
DNA-dependent DNA replication	GO:0006261	1.708×10^{-2}
small molecule catabolic process	GO:0044282	2.304×10^{-2}
DNA replication	KEGG:03030	1.031×10^{-3}
Cell cycle	KEGG:04110	1.126×10^{-3}
Tyrosine metabolism	KEGG:00350	2.121×10^{-2}
Glycine, serine and threonine metabolism	KEGG:00260	4.270×10^{-2}
DNA Replication Pre-Initiation	REAC:R-BTA-690...	2.363×10^{-3}
Metabolism of amino acids and derivatives	REAC:R-BTA-712...	3.758×10^{-3}
DNA Replication	REAC:R-BTA-693...	1.445×10^{-2}
Phenylalanine and tyrosine metabolism	REAC:R-BTA-896...	2.615×10^{-2}
Assembly of the pre-replicative complex	REAC:R-BTA-688...	2.615×10^{-2}


A
ShortNT
ShortHS
ShortHS_
Recovery
0h 6h
24h
48h
72h
B
2
1
0
-
-
cluster
ShortHS
ShortHS_
Recovery
ShortNT
C
glycosylation
Mitophagy - animal
autophagy
protein glycosylation
ER stress
Protein processing
0
5
10
-log10(pv_adj.)
D
Ox. phos.
H. recombination
Thermogenesis
Spliceosome
Base excision repair
Carbon metabolism
Cellular senescence
Cell Cycle
DNA replication
Proteasome
0
5
10
-log10(pv_adj.)
E
LongHS
ShortHS_Recovery
LongNT
*
*
*
*
0
1
2
3
4
Population Doubling
F
RNA polyadenylation
peptidyl-lysine methylation
pattern specification process
protein acylation
histone lysine methylation
regulation of mRNA processing
histone methylation
regulation of gene expression, epigenetic
ubiquitin-dependent catabolic process
regulation of mRNA metabolic process
histone acetylation
protein methylation
protein alkylation
regulation of RNA splicing
protein folding
regionalization
internal peptidyl-lysine acetylation
internal protein amino acid acetylation
peptidyl-lysine acetylation
RNA modification
anterior/posterior pattern specification
protein acetylation
peptidyl-lysine modification
macromolecule methylation
methylation
0
2
4
6
8
10
-log10(pv_adj.)
G
Mll1-2
Mll3-4
HAT
HMT
6
1
-4
-9
Enrichment Score
1.3e-03
5.6e-05
1.7e-03
2.4e-04
3e-08
6.7e-08
4e-07
7.7e-07
ShortHS vs ShortNT
ShortHS_Recovery - ShortHS
H
Cluster 1
Cluster 2
Cluster 3
Cluster 4
2
1
0
-1
-2
Enrichment Score for Bivalent Genes
0.06
1.27e-03
1.29e-05
0.48
Heatmap Clusters


A
Upregulated
LongHS
376
102
ShortHS
180
1
15
6
ShortHS_
Recovery
190
Over enriched
hypergeometric
distribution p-
value= 2.79e-19
B
Downregulated
ShortHS_
Recovery
68
16
4
LongHS
556
93
ShortHS
264
Over enriched
hypergeometric
distribution p-
value=2.47E-03
C
Ox. phos.
E2F targets
G2M Checkpoint
MYC targets V1
DNA repair
Allograft rejection
IL2 STAT5 signaling
Unfolded protein response
HEDGEHOG signaling
ShortHS_Recovery
LongHS
ShortHS
-4
-2
0
2
4
Normalized Enrichment Score
D
2.64e-02
1.40e-02
CDC25-Cell cycle G2/M
0.02
Replication Pre-Initiation
3.5e-20
1.3e-09
1.2e-02
Cell Cycle
-70
-4
-2
0
Normalized Enrichment Score
E
non canonical WNT signaling
Canonical WNT signaling
Stem Cell Differentiation
Mesenchymal Differentiation
Mesoderm
Endoderm
Cell Development
Tissue Development
ShortHS
LongHS
ShortHS_Recovery
pv<1e-6
pv<1e-4
pv<1e-2
-20
-10
0
10
20
Normalized Enrichment Score
F
2.1e-05
Inflammatory response
2.48e-04
1.06e-03
Macrophage migration
1.29e-06
IL-6 production
0.013
Cytokine pathway
LongHS
RecoveryHS
-40
-6
-4
-2
0
Normalized Enrichment Score
G