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Global gene expression analysis reveals specific and redundant roles for H1 variants, H1c and H1°, in gene expression regulation

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Abstract

In mammals, the functional significance of the presence of evolutionarily conserved, multiple non-allelic H1 variants remains unclear. We used a unique overproduction approach coupled with cell cycle synchronization and early time point assays to assess differential effects of H1 variants, H1c and H1°, on global gene expression in the absence of compensatory events that may mask variant-specific effects. We found that H1c and H1° act primarily as specific rather than global regulators of gene expression. Many of the genes affected were uniquely targeted by either H1c or H1°, affirming that H1 variants have some unique roles. We also identified genes that were affected by both variants, in which cases the expression of these genes was, for the most part, affected similarly by both the variants. This observation suggests that as well as having specific functions, the H1 variants share common roles in the organization of chromatin. We further noted that H1° repressed more genes than did H1c, which may underlie the prevailing notion that H1° is a stronger repressor of transcription.

Keywords

Chromatin; overexpression; linker histone; variants

1. INTRODUCTION

Histone H1 is an essential component of chromatin that binds to the linker DNA between nucleosomes and facilitates the formation of a compact chromatin fiber (van Holde, 1998; Thoma et al., 1979). In mammals, eleven non-allelic variants of histone H1 have been identified. These include the somatic variants H1a-e, H1°, H1x (Happel et al., 2005), testes-specific variants H1T2 (Martianov et al., 2005; Tanaka et al., 2005), H1LS1 (Yan et al., 2003) and H1t, and oocyte-specific variant H1oo. Extensive literature suggests that these variants are functionally heterogeneous and play distinct roles in the organization of chromatin (Cole, 1987; Brown, 2001). The variants differ from each other in their expression in dividing

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versus non-dividing cells (Lennox and Cohen, 1983) and also in their expression pattern during development and differentiation (Khochbin and Wolffe, 1994). They differ from each other in their relative rates of synthesis, turnover rates, evolutionary and metabolic stability (Lennox, 1984) and phosphorylation patterns during cell division (Talas et al., 1996). Lennox proposed that the H1 variants differ in their ability to condense chromatin and that different regions of chromatin are maintained at different degrees of condensation due to non-random distribution of the H1 variants (Lennox, 1984). This view was supported by Huang *et.al.* who demonstrated that regions of chromatin exhibiting different degrees of condensation differ from each other with respect to both content and composition of H1 subtypes (Huang and Cole, 1984). Despite the plethora of evidence suggesting functional heterogeneity of H1 variants, it is not yet clear what unique functional roles these variants serve.

That H1s, as a group, function as global transcriptional repressors (Weintraub, 1984; Zlatanova and van Holde, 1992) has been challenged by reports that suggest a more specific role for H1s in the regulation of gene expression (Hellauer et al., 2001; Panetta et al., 1998; Sera and Wolffe, 1998; Shen and Gorovsky, 1996). Very few studies have focused on the effects of specific variants on gene expression. The first direct evidence for the differential effects of H1 variants on gene expression came from a study done in our laboratory in which two mouse variants, H1c and H1°, were inducibly overproduced to about 70% of all H1s (Brown et al., 1996). Overproduction of H1° caused the downregulation of the genes that were tested. In contrast, overproduction of H1c either had no effect or caused expression to go up. Another study showed that the deletion of specific histone variants differentially affects the expression of transgenes in mice (Alami et al., 2003). In the DT40 chicken B-cell line, analyses of mutants deficient in individual H1 variants revealed unique proteome patterns, indicating that each of the variants of H1 affected genes' expression differentially (Takami et al., 2000). Knockout studies in mouse also indicate that H1 variants are involved in controlling specific gene expression (Lin et al., 2004; Fan et al., 2005). However, the long term nature of these experiments (due to the necessity of selecting for cells or animals that can survive the knockout of these genes), allowed for cellular compensatory mechanisms, such as changes in transcription factor levels that may compete with H1 binding, to come into play, thus masking the true effects of the variants.

The original study of the overexpression of the two variants H1c and H1° looked at effects on expression of only seven genes (Brown et al., 1996). The H1 knockout experiments looked at many more genes and suggested that H1s, in general, regulate only a small number of genes. We revisited our experimental system of overproducing particular variants in part to resolve whether or not the result of the assessment of H1 variant function by knockouts was a compensatory artifact. In our system the chromatin contains 70% of one variant or another. Compensation can take place during the overproduction of individual variants during quiescence. However, with the induction of a programmed, multi-gene expression event, i.e. synchronous entry into the cell cycle, assessment of variant specific differences can be made before any further compensation can occur. The differences that are observed must be due to changes in the chromatin that occurred during overproduction of the specific variant while cells were quiescent. We infer that the differences observed are due to the abundance of a particular variant. We found that expression of numerous genes was affected by overproduction of the H1 variants, both positively and negatively. Some of the genes were affected by both variants, while some were unique targets of either one of the variants, suggesting both redundant and unique roles for these H1 variants in regulation of gene expression. Identification of genes differentially affected by H1s is crucial to deciphering the functional differences between the H1 variants as well as specific roles played by the variants within this class of proteins.

2. MATERIALS AND METHODS

2.1 Cell culture and synchronization in G1 phase with induction

BALB/c 3T3 cells (clone A31 from American Type Culture Collection) and the derived transformants, MTH1c (H1c overproducing cells) and MTH1° (H1° overproducing cells) were grown as described (Brown et al., 1996). Cells were density-arrested in G0 and concomitantly induced with ZnCl₂: 2.5 million (MTH1c) or 3.5 million (3T3 and MTH1°) cells were seeded into 150 cm² flasks. 24 hours after seeding, the cells were fed fresh medium and induced with either 100 μM (3T3 and MTH1c) or 75 μM (MTH1°) ZnCl₂, to achieve comparable levels of induction, for 72 hours at which time cells were density arrested. After induction, cells were trypsinized, diluted 9.5 times and plated in fresh medium. Three and six hours after release from G0, the cells were washed with cold phosphate-buffered saline (PBS) followed by incubation in CDS (CDS, Sigma-Aldrich, St. Louis, MO) at 37°C for 20 minutes. Cells were pelleted, washed and resuspended with PBS and divided into three parts. Each of the three parts was spun down and the cell pellet was processed for flow cytometry, total chromatin-bound histone purification or total RNA extraction as described in the following sections. Cells were also harvested at 18 hours (3T3 and MTH1c) or 21 hours (MTH1°) and processed similarly for flow cytometry as described (Brown et al., 1996).

2.2 Total RNA isolation

Total RNA was isolated from the cells using Micro-to-Midi total RNA purification system (Invitrogen Life Technologies, Carlsbad, CA). Total RNA was eluted in DEPC treated water. The purified RNA was treated with DNase I (Qiagen, Valencia, CA) and purified using the RNeasy Mini kit (Qiagen, Valencia, CA). RNA was eluted in DEPC treated water and stored at -80°C until use. The quality of the DNase I-treated total RNA was verified using Agilent Lab-on-a-chip 2100 Bioanalyzer (Agilent Technologies, Santa Clara CA).

2.3 Isolation and analysis of total histones

Total histones were purified and reverse-phase HPLC was performed as described previously (Brown et al., 1996) on a Dionex Summit ® HPLC System (Dionex Corporation, Sunnyvale, CA). The relative H1 levels are presented as a percentage of the total area under all H1 peaks. In order to resolve H1° and H1b, which co-eluted, we collected the fraction containing H1° and H1b and resolved the two variants on SDS-PAGE. The intensities of the bands corresponding to H1° and H1b were then measured using the Personal Densitometer SI (Molecular Dynamics).

2.4 Microarray design, labeling and hybridization

Double-spotted arrays containing 15,247 sequence-verified mouse expressed sequence tags from the National Institute of Aging (NIA) were obtained from University Health Network, Microarray Centre, Ontario, Canada (<http://www.microarrays.ca/products/glists.html>). Gene expression profiles of H1c overproducing cells (MTH1c) and H1°-overproducing cells (MTH1°) were compared to those of control 3T3 cells. Four biological replicates were made for each group and a pair of dye-swap experiments constituted the technical replicates of each biological replicate. For all experiments, the control 3T3 RNA used was derived from a single stock of ZnCl₂-treated cells at 3 hours and 6 hours after release from density arrest. RNA was labeled using 3DNA array detection, Array 900™ kit (Genisphere, Hatfield, PA). cDNA was synthesized from 1 μg total RNA of control cells and 1 μg total RNA of MTH1c or MTH1° cells, using provided primers. Reverse transcription was carried out using SuperScript II reverse transcriptase (Invitrogen Life Technologies, Carlsbad, CA). Each reaction was spiked with 1 ng of control *Arabidopsis* transcript prepared from linearized pARAB (<http://www.microarrays.ca/support/proto.html>) using the MEGAscript T7 kit (Ambion,

Austin, TX). Slides were hybridized with the control and experimental cDNAs and then with the capture reagents as per the manufacturer's instructions. After the hybridization and washing steps, the dried microarray was immediately dipped into dyesaver2 (Genisphere, Hatfield, PA) and then left on bench-top for 10 minutes to dry. The array was stored in a dark tube until scanned.

2.5 Scanning and quantitation

The arrays were scanned using ScanArray Express (Perkin Elmer, Wellesley, MA). Multiple spots of the *Arabidopsis* chlorophyll synthetase gene served as the scanning control. The PMT and laser power were adjusted such that the control spots had comparable intensities in both channels. The images were quantitated using QuantArray Microarray analysis software (Perkin Elmer, Wellesley, MA). The adaptive threshold method was used for quantitation (Bowtell and Sambrook, 2003).

2.6 Statistical analysis of microarray data

Bad spots and areas of the arrays were visually identified and removed from analysis. Any spot with intensity less than 1.5 times the background in either one of the channels was also not included in further analysis. Background intensities were subtracted from the foreground intensities. Print tip LOESS (locally weighted scatter plot smoothing) normalization was carried to correct for the intensity-dependent curvature seen in the ratio vs. intensity plots (Yang et al., 2002; Quackenbush, 2002). A mixed model Analysis of Variance, adjusting for dye as a fixed effect and assuming biological samples as a random factor, was performed using the statistical package SAS (<http://www.statsoftinc.com>). The intercept from the model is an estimate of the ratio of the 2 channels, adjusted for within-slide and within-biological sample variability as well as potential bias due to the dyes. We considered the distribution of standard errors and chose the 5th percentile as a "fudge factor" for a SAM (Significance analysis of microarrays) type analysis (Tusher et al., 2001). The $\log_2$ ratios were plotted against the $-\log_{10}$ p-values in a volcano plot (Cui and Churchill, 2003) to get a simple visual scale to choose differentially expressed genes (Figure 2). No attempt at adjusting the p-values or estimating false discovery rates was used in this analysis. All genes that had a p-value of less than or equal to 0.05 and $\log_2$ ratio greater than or equal to 0.6, or less than or equal to -0.6 , were selected as differentially regulated genes. The $\log_2$ ratio represents the log ratio of gene expression from experimental sample to control sample, i.e. H1/ 3T3. Ratios of gene expression were used to calculate fold changes. Fold changes were calculated from the ratios using the formula $-1/\text{ratio}$ for downregulated genes. For upregulated genes, fold changes were equal to the ratios. Total number of genes affected by H1c was calculated by combining all genes affected at both 3 and 6 hours. Common or overlapping genes were counted only once. A similar procedure was done for H1°.

2.7 Gene annotations, clustering and functional analysis

The accession IDs were annotated using Expression Analysis Systematic Explorer, EASE (Hosack et al., 2003). Clustering of genes was done using Gene Expression Statistical System for Microarrays (GESS, NCSS, Kaysville UT). Summary statistics in the form of the estimated average log expression ratios from all four groups were combined to form the database for performing a cluster analysis. In order to facilitate the analysis and eliminate difficulties with interpretation, expression ratios that were deemed insignificant in the individual groups were replaced with the null ratio value (i.e., zero). In addition, only those genes with over 2.14 fold expression differential and p-value less than or equal to 0.05 were used in the subsequent cluster analysis in order to reduce the dimensionality of the final cluster solution. 126 genes that were found to be differentially expressed by at least 2.14 fold in at least one experiment were subjected to a hierarchical clustering. We used Ward's minimum variance (Milligan, 1981)

approach to investigate cluster membership based on Euclidean distance. The cophenetic correlation for clustering the genes was $r = 0.51$. Based on the dendrogram produced, we report seven clusters of genes. The genes altered by overproduction of H1^o and H1c were classified into functional categories using the Ingenuity Pathway Analysis, IPA (www.ingenuity.com). Significance level was set at $p\text{-value} \leq 0.01$.

2.8 Real-Time PCR (RT-PCR)

Total RNA from control 3T3 and MTH1c or MTH1^o cells was reverse-transcribed to make cDNA using SuperScript II reverse transcriptase and oligo(dT)₁₂₋₁₈ primer (Invitrogen Life Technologies, Carlsbad, CA). Varying amounts of cDNA were used as templates for performing RT-PCR for each gene to assure linear range of the data, using Brilliant SYBR Green QPCR Master Mix (Stratagene, La Jolla, CA). For each gene, a non-template control was also included. All primers were designed to cross intron-exon junctions in the genomic sequences of the genes, obtained from the University of California Santa Cruz Mouse (*Mus musculus*) Genome Browser Gateway (<http://genome.ucsc.edu/cgi-bin/hgGateway>) using the Mouse February 2006 (mm8) assembly. A description of all primers used is provided in Supplemental table 12. RT-PCR was performed using the Mx3000P® QPCR System at the School of Nursing (Stratagene, La Jolla, CA). The shift in the threshold cycle between the control and the test sample was used to calculate the ratio of expression for each gene using the formula $2^{\Delta C_t}$, where ΔC_t denotes the shift in the threshold cycle. Fold changes were calculated from the ratios using the formula $-1/\text{ratio}$ for downregulated genes. For upregulated genes, fold changes were equal to the ratios. The fold changes were normalized to a control gene, *cyclophilinA* (*ppia*), whose expression was not altered as detected by microarrays and confirmed by RT-PCR in all 4 groups.

3. RESULTS AND DISCUSSION

3.1 Effects of overproduction of H1 variants on global gene expression

We made comparative measurements of the steady-state levels of approximately 15,000 gene transcripts under conditions in which either H1c or H1^o constituted a high percentage of the total H1 levels in the cells. We overproduced H1c and H1^o variants to levels comparable to those in our earlier studies (Brown et al., 1996) (Table 1). Under the conditions used, the total amount of H1 in the cells was not perturbed and the H1/core ratios remained unchanged compared to the control cells (data not shown). After release from density arrest, the cells were harvested at two early time points, three hours and six hours, in the G1 phase. This allowed us to look at gene expression changes prior to their masking by compensatory mechanisms. We confirmed that 90% or more cells were synchronized in the G1 phase of the cell cycle and that the cells were progressing through the cell cycle by FACS analysis at 18 hours (3T3 and MTH1c) or 21 hours (MTH1^o) after release from density arrest (Figure 1). Any cells remaining in G₀ do not reduce sensitivity since cells in G₀ express very little RNA and the percentage of these cells is the same as in control 3T3 cells. Genes with a fold change of 1.5 times or more (positive and negative) and a p-value of 0.05 or less were selected as genes differentially expressed relative to the control (Figure 2, Table 2). We observed that when levels of H1c are increased such that it constitutes about 70% of the total H1 content in the cells, expression of 213 genes is affected at three hours after release from density arrest (Supplemental table 1a and 1b). At six hours after release, a larger number of genes are affected (407 genes; Supplemental table 2a and 2b). Out of these 407 genes, 113 are also affected at three hours (Supplemental Table 3a and 3b). However, the expression of 100 genes whose expression was altered at three hours, returns to their normal levels by six hours. At both three hours and six hours, H1c overproduction is able to cause upregulation as well as downregulation of genes. H1^o overproduction, likewise, is able to cause upregulation as well as downregulation of genes. At three hours after release, H1^o overproduction affects the expression of 230 genes

(Supplemental table 4a and 4b). There is only a slight change in the number of genes (286 genes) affected at six hours after release (Supplemental Table 5a and 5b). Of the 286 genes affected at six hours after release, 118 genes show altered expression at three hours also (Supplemental table 6a and 6b). However, 112 genes that are affected at three hours return to their basal expression levels by six hours. These results indicate that individual variants, H1c and H1°, are specific rather than global regulators of gene expression. These data affirm the results of other studies demonstrating that H1s affect the expression of specific genes rather than global gene expression and shows their conclusions are not an artifact of compensation (Lin et al., 2004; Fan et al., 2005). Further, these other studies did not look for genes affected by knocking out specific single variants. They investigated gene expression changes caused by the deletion of two or more variants that also caused overall decreased levels of H1s in the cells and were given enough time to activate their compensatory mechanisms. Here we have studied the effects of two individual H1 variants where they constitute ~70% of the total H1 content, and where there are no changes in the total amount of H1s in the cells. We believe that the overproduced variant occupies sites that are normally bound by one of the other variants. In essence, the changes seen are a result of reduction of one type of variant and occupancy of the sites that are normally regulated by the reduced variant by the overproduced variant. Also, by looking for gene expression changes early in the cell cycle, we have excluded the effects that cellular compensatory mechanisms may have on gene expression; thus the changes seen are true effects of the individual variants.

These data further disprove our earlier suggestion (Brown et al., 1996) that H1° functions only as a repressor of transcription while H1c functions as an activator of some genes. In concordance with our older study, we do see that H1° is more likely to be a repressor than H1c. About 66 % (3 hr) – 68% (6 hr) of the affected genes are upregulated by H1c. Relative to H1c, H1° causes less upregulation: only 48% (3 hr) and 56% (6 hr) of the affected genes are upregulated by H1°. This suggests that presence of a higher percentage of H1c on chromatin may make regions of chromatin more accessible than when bound by other H1 variants. H1°, on the other hand, is unable to increase accessibility of more regions of chromatin and therefore does not cause as many genes to be upregulated as H1c does. These suggestions are supported by findings that H1c is enriched in less condensed chromatin regions (Lennox and Cohen, 1984) and H1° is enriched in more compact chromatin (van Holde, 1988). H1c is one of the major H1 subtypes present in the pre-pachytene spermatocytes (Lennox and Cohen, 1984), where it may function to maintain a less condensed chromatin for genetic recombination. H1°, on the other hand, is the major subtype present in quiescent and terminally differentiated cells, e.g. hepatocytes (van Holde, 1988). Our suggestions are also supported by a proposal made by Lennox, that subtypes H1b, H1d, H1e and H1° promote formation of compact chromatin while H1a and H1c condense chromatin less than the other somatic H1s (Lennox, 1984). Therefore, this study provides an understanding of H1 variant functions as global transcriptional regulators and is a significant advancement from the older study.

If the mechanism by which H1s affect transcription is chromatin packaging, we suggest that both variants of H1 can act to promote open or closed conformations in specific regions of the chromatin, causing either up or down regulation of genes. Our suggestion finds support in the observations that H1s are present in chromatin regions harboring both active and inactive genes and that the difference between the two types of regions lies in the mode of interaction between H1 and the template (Weintraub, 1984). In the inactive regions H1 held the nucleosomes together while in the active ones it did not. However, it would be imperative to study the changes in the structure of chromatin at individual gene loci in order to further understand the mechanism of H1 action.

One explanation for the differential effects of H1c and H1° on gene expression might be their different binding affinities for the chromatin. Although a study assessing the *in vivo* binding

of mouse variants H1c and H1^o to chromatin by fluorescence recovery after photobleaching (FRAP) analysis did not report a difference between the two variants (Misteli et al., 2000), a later study done on the six human somatic H1 variants reported differences in the binding properties of H1.0 and H1.2, human homologs of mouse variants H1^o and H1c respectively (Th'ng et al., 2005). This study reported that H1.2 (H1c) bound to chromatin with an affinity lower than that of H1.0 (H1^o). The dynamic nature of H1 binding to chromatin (Misteli et al., 2000) allows for transient removal of linker histones from chromatin, providing a window for other DNA binding proteins that are present to bind and facilitate processes like transcription. H1 variants with lower binding affinities for chromatin would be expected to be easily displaced from their positions, rendering those regions of chromatin more amenable for processes like transcription. In contrast, H1 variants with higher binding affinities for chromatin would be expected to be more difficult to displace from their positions and hence present a greater hindrance to genomic processes. This is consistent with our observations that H1c affects the expression of more genes than H1^o. H1c affects the expression of 507 genes at both three and six hours, while H1^o only affects 398 genes (Table 2, Supplemental Table 7a and 7b).

Another possible mechanism by which H1 can regulate transcription of genes is its competition with transcription factors for binding at the promoters of the genes. The similarity in structure between the core of the linker histone H5 and the winged helix transcription factors gives credence to this possibility (Ramakrishnan et al., 1993). It has been previously reported that the winged helix transcription factor HNF3 binds to the nucleosome cores at sites of H1 binding (Cirillo et al., 1998). H1 has also been shown to occlude transcription factor binding to nucleosomes (Juan et al., 1994). In the light of this evidence and to attempt to understand the mechanism of this differential control of gene expression, we looked for presence of different transcription factor binding sites in the genes affected by H1c and H1^o. We checked for the presence of binding sites for HNF3 and two basal transcription factors. We could not identify preferential transcription factors for the genes affected by H1c and H1^o (data not presented). We therefore concluded that differential gene expression by different H1 variants is not due to a competitive interaction with either of two basal transcription factor sites or HNF3 sites.

We found that 212 genes are affected by both H1c and H1^o (Supplemental table 8a and 8b); most of these genes (205 genes) are altered the same way by both the variants (Table 2). Perhaps these genes are located in chromatin milieus where both subtypes of H1 bind with similar affinities and cause either compaction or decondensation. It is possible that H1c and H1^o bind to these regions with affinities lower or higher than the other variants of H1 that are present at those sites under control conditions, and that this may lead to either looser or tighter chromatin causing up or downregulation respectively.

We also identified genes that are affected by only H1c or H1^o. There are 295 genes (58% of all H1c affected genes) that are affected by H1c only at either three hours, six hours or both time points (Supplemental Table 9a and 9b), and 185 (46% of all H1^o affected genes) genes affected by H1^o only at either or both time points (Supplemental Table 10a and 10b). These genes are unique targets of the two variants. A series of knockout studies, in which one or more of the H1 variant genes were disrupted, suggest redundant roles for H1 variants. When the expression of one or two H1 variants was disrupted, levels of other H1 subtypes increased and compensated for the deficiency (Sirotkin et al., 1995, Lin et al., 2000 and Fan et al., 2001). Our study suggests that despite having certain redundant roles in the organization of the chromatin, the H1 variants may also have unique roles in regulating gene expression.

We selected a group of genes that showed expression changes ranging from highly upregulated to highly downregulated due to overproduction of the two variants, for verification by RT-PCR (Table 3). Some of the genes tested were uniquely affected by H1c or H1^o while some were affected by both. We found that 83% of genes tested by RT-PCR were altered in the same

direction as detected by microarrays. There are several possible reasons for the discrepancy. One is that microarrays may detect less than perfect complements due to cross-hybridization events while the specificity of RT-PCR was tested by checking the amplified products using DNA gel electrophoresis. Another is that the gene region we amplified in RT-PCR may not coincide with the probe sequences on the microarray. Overall, we feel this represents a relatively low level of false positives.

3.2 H1c and H1^o affect genes that participate in common and unique functions

We determined the molecular and cellular functions associated with the genes affected by H1c and H1^o overproduction using Ingenuity Pathways Analysis, IPA (www.ingenuity.com). Out of the 33 pathways affecting molecular and cellular functions defined in the Ingenuity Pathways Knowledge Base, 24 were found to be associated with H1c and H1^o affected genes. Of these 24 functions, most of the pathways are affected by both variants and only few pathways are affected uniquely by individual variants. We found 21 functions with p-value ≤ 0.01 to be associated with the genes affected by H1c and 22 functions (p-value ≤ 0.01) to be associated with H1^o affected genes (Table 4). Of these, 19 were common to both H1c and H1^o affected genes. We found 3 functions that were only associated with H1^o affected genes and 2 that were only associated with H1c affected genes. Ingenuity pathway analysis showed a significant effect on cell cycle genes for both variants. However, we also saw an effect on many other molecular and cellular pathways, which is expected for such an abundant chromatin protein.

We selected genes that showed significant alterations in their expression levels (fold change of 2.14 or greater) in at least one group and clustered them as described in materials and methods (Figure 3). The cluster analysis allowed for a better visualization of the data and helped us demarcate groups of genes that were similarly expressed across two or more of the 4 experimental groups. We defined 7 clusters on the basis of their expression ratios across the 4 groups and found that none of the clusters affected any unique pathways. The 7 clusters are shown in Supplemental table 11.

It is clear from our findings that H1c and H1^o can cause both up and down regulation of specific genes and that these variants do not cause global gene expression changes. Our findings also clearly indicate that while some of their functions are redundant, H1c and H1^o also have some unique roles in regulation of gene expression. However, we still do not understand the molecular mechanisms underlying these differential effects. It also remains to be seen whether the other variants of H1 possess this functional heterogeneity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Abbreviations

CDS, cell dissociation solution; PBS, phosphate buffered saline; FACS, fluorescence-activated cell sorting; DEPC, diethyl pyrocarbonate; RP-HPLC, reverse phase high-performance liquid chromatography; RT-PCR, real time polymerase chain reaction.

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Fig. 1. FACS analysis confirming the synchronization of 3T3, MTH1c and MTH1° cells in the G1 phase of the cell cycle

3T3, MTH1° and MTH1c cells were density arrested and concomitantly induced with ZnCl₂. After induction for 72 hours, cells were trypsinized, diluted and replated in fresh medium. At 3, 6 and 18 hours (21 hours in case of MTH1°) after release, cells were harvested, washed with PBS, fixed with methanol and analyzed by flow cytometry.

Fig. 2. Volcano plots of microarray data for MTH1c 3 hours, MTH1c 6 hours, MTH1° 3 hours and MTH1° 6 hours

Microarray analysis was done on H1c and H1° overproducing cell lines, 3 and 6 hours after release from density arrest. The log₂ ratios (x-axis) were plotted against the negative logged p-values (y-axis) in a volcano plot for all four groups. Reference lines are displayed at the cut-offs used to define differentially expressed genes - $p\text{-value} \leq 0.05$ and $0.6 \leq \log_2 \text{ratio} \leq -0.6$.

Fig. 3. Hierarchical clustering of genes showing significant changes in gene expression

Genes showing greater than 2.14 fold change in either direction in at least one experimental group were clustered using hierarchical clustering. Each row represents a gene and each column represents an experimental group: A- H1c 3 hours, B- H1c 6 hours, C- H1° 3 hours, D- H1° 6 hours. Green represents downregulation and red represents upregulation. The 7 clusters defined on the basis of expression ratios are- Cluster 1: Genes highly downregulated by both H1c and H1° at both time points; Cluster 2: Genes downregulated at 6 hours by H1c and H1°; Cluster 3: Genes moderately downregulated by both H1c and H1° at both time points; Cluster 4: Genes downregulated by H1° only; Cluster 5: Genes upregulated by H1c only; Cluster 6: Genes upregulated by both H1c and H1°; Cluster 7: Genes upregulated by H1° only.

Table 1**Relative H1 variant levels upon induction with ZnCl₂ during density arrest in control 3T3, MTH1° and MTH1c cells**

Total chromatin-bound histones were extracted and separated by RP-HPLC as previously described (Brown et al., 1996). Data are derived from RP-HPLC profiles (not shown). H1 levels are presented as the percentage of the total area under all H1 peaks, corrected for their size as previously described (Brown et al., 1996).

Cell line	Relative H1 variant levels			
	H1°	H1b	H1d/e	H1c
3T3	11.1	27.2	43.8	17.9
MTH1°	71.3	3.2	25.5	-
	H1°/H1b		H1d/e	H1c
MTH1c	8.6		23.1	68.3

Table 2**Number of genes affected by overproduction of H1c and H1°**

Number of genes affected by either variant at one or both time points is shown. “All” refers to the total number of genes affected by each variant, calculated by combining all genes affected at both time points: 3 and 6 hours. Common or overlapping genes were counted only once. “H1c and H1° common” refers to genes affected by both H1c and H1° at either time point. (These include identified genes and expressed sequence tags, ESTs).

H1 variant	Number of genes		
H1c	Total	Upregulated	Downregulated
3h	213	140	73
6h	407	277	127
3h and 6h	113	81	31
All	507		
H1°	Total	Upregulated	Downregulated
3h	230	111	119
6h	286	160	124
3h and 6h	118	62	53
All	398		
H1c and H1° common	212	124	81

Table 3**Verification of microarray results by RT-PCR**

Fold changes of affected genes as detected by microarrays and RT-PCR are shown for all 4 groups. For RT-PCR, ratios of expression of genes in experimental to control sample were calculated using the formula $2^{\Delta C_t}$, where ΔC_t represents the shift in threshold cycle between the control and experimental sample. Fold changes were calculated from the ratios as described in materials and methods. Cyclophilin A was used as the normalizer gene. *Genes for which the microarray and RT-PCR data do not correlate.

(a)			
H1c 3 hrs			
Gene	EntrezGene ID	Arrays	RT-PCR
Pem	18617	4.56	1029.73
Gapd	14433	3.01	3.92
Eno1	13806	2.5	3.2
Scd2	20250	1.59	1.88
Anxa3	11745	1.54	2.72
Eif3s10	13669	-1.82	-1.02*
Odf1	237222	-3.23	-1.41
Dcn	13179	-3.77	-327.23
(b)			
H1c 6 hrs			
Gene	Entrez Gene ID	Arrays	RT-PCR
Pem	18617	6.21	952.62
Gapd	14433	3.04	3.52
Mif	17319	2.34	1.42
Irak1	16179	1.87	1.85
CD164	53599	1.63	1.33
Tpt1	22070	1.56	1.46
H2-D1	14964	1.52	-3.06*
Eif3s10	13669	-1.54	-1.56
Anxa3	11745	-1.6	-2
Jun	16476	-1.62	-3.12
Igf2r	16004	-1.71	-86.82
Cfl1	12631	-1.83	-2.22
Actb	11461	-2.19	-1.61
Anxa1	16952	-2.98	-5.55
Dcn	13179	-3.84	-200

(c)

H1° 3 hrs

Gene	Entrez Gene ID	Arrays	RT-PCR
Pem	18617	3.43	240.49
Hif1a	15251	1.88	4.51
Gapd	14433	1.64	1.83
Mcm2	17216	1.62	-1.96*
Eno1	13806	1.59	1.43
Ccng1	12450	1.55	2.64
Sc4mol	66234	1.54	1.22
Scd2	20250	-1.52	-1.14*
Col5a1	12831	-1.55	1.02*
Ofd	1237222	-1.65	-1.26
Irak1	16179	-1.61	-1.04*
Tgfb1i4	21807	-1.79	1.28*
Plagl1	22634	-2.27	-3.94
Dcn	13179	-4.45	-8.64

(d)

H1° 6 hrs

Gene	Entrez Gene ID	Arrays	RT-PCR
Pem	18617	2.79	218.32
Ctla2a	13024	2.56	35.98
Ofd1	237222	1.85	1*
Irak1	16179	1.73	1.35
Mif	17319	1.73	-1.28*
Gapd	14433	1.72	2.14
Fgfr1	14182	1.55	2.35
Jun	16476	-1.56	-3.44
Sc4mol	66234	-1.58	-3.46
Col5a1	12831	-1.65	-1.72
Anxa3	11745	-1.69	-2.5
Eif3s10	13669	-1.74	-1.23
Amotl2	56332	-1.96	-5.21
Actb	11461	-1.98	-1.54
Dcn	13179	-3.1	-8.33

Table 4**Functions associated with genes affected by both H1c and H1°, only H1c and only H1°**

Genes affected by H1c and H1° were classified into functional groups using Ingenuity Pathway Analysis. Functional pathways affected by both variants and those affected uniquely by either variant are shown. Significance level, that measures the likelihood of genes from the dataset participating in a function, was set at p-value ≤ 0.01 .

Common	H1c	H1°
Cellular assembly and organization	DNA replication, recombination and repair	Amino acid metabolism
Cellular compromise	Vitamin and mineral metabolism	Protein synthesis
Cancer		Protein trafficking
Cellular movement		
Cell-cell signaling and interaction		
Cell death		
Cell morphology		
Cell cycle		
Cellular development		
Cellular function and maintenance		
Cellular growth and proliferation		
Cell signaling		
Gene expression		
Molecular transport		
Small molecule biochemistry		
Drug metabolism		
Lipid metabolism		
Carbohydrate metabolism		
Nucleic acid metabolism		