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DNA microarray profiling of genes differentially regulated by three heterochromatin protein 1 (HP1) homologs in *Drosophila*

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ABSTRACT

Heterochromatin protein 1 (HP1) is an epigenetic gene silencing protein that is regulated by lysine 9 methylation of histone H3. Most eukaryotes have at least three HP1 homologs with similar domain structures but with different localization patterns and functions in heterochromatin and euchromatin. However, little is known about the genome-wide effects of the three main HP1 homologs on gene expression. Here, to gain insight into the different gene expression effects of the three HP1 homologs, we performed a comprehensive and comparative microarray analysis of *Drosophila* HP1 homologs. Bioinformatic analysis of the microarray profiling revealed significant similarity and uniqueness in the genes altered in HP1-knockdown S2 cells in *Drosophila*. Although global changes of these transcripts were surprisingly subtle (4–6%), there were ~582 common target genes for the three HP1s that showed transcript levels either reduced or induced >1.5-fold. Depletion of HP1 resulted in up-regulated and down-regulated gene profiles, indicating that HP1 mediates both repression and activation of gene expression. This study is the first to systematically analyze the bioinformatics of HP1 paralogs and provide basic clues to the molecular mechanism by which HP1 might control gene expression in a homolog-specific manner.

1. Introduction

The methylation of histones modulates chromatin structure and function. Lysine methylation of histone is a dynamic and reversible posttranslational modification (PTM) mediated by histone methyltransferases (KMTs) and histone demethylases (KDMs). The methylation of histone H3 at lysine 9 is important determinant of heterochromatin formation [1]. Di-and trimethylated H3K9 (H3K9me2 and H3K9me3) are found at pericentric heterochromatin in almost all higher eukaryotes and are a hallmark of establishment and maintenance of heterochromatin [2]. Methylated H3K9 provides a docking site for Heterochromatin protein 1 (HP1). HP1 is a conserved non-histone chromosomal protein that is encoded by *Su(var)*2–5 [3,4] and is a suppressor of position effect variegation (PEV) [5]. In *Drosophila*, HP1 possess five HP1 homologs as based on the amino acid sequence similarity and domain structure [6]. Three HP1 homologs (HP1a, HP1b, and HP1c) are ubiquitously expressed in adult flies whereas two tissue-specific homologs, HP1d/rhino and HP1e are only expressed in the ovaries and testes, respectively [7]. HP1 has two prominent structural motifs, an N-terminal chromodomain (CD) and a C-terminal chromoshadow domain (CSD), separated by a variable length hinge region (Hin), important for chromatin binding and protein–protein interaction respectively. HP1 remodels chromatin through interactions with HP1-binding proteins in a consensus sequences [8], PXVXL-dependent or independent manner [9–11]. Interestingly, these diverse interactions occur in an HP1 homolog-specific manner or in a universal manner for all three homologs, and may depend on particular post-translational modifications and involve proteins with various cellular functions [6,12]. Thus, HP1 can have multiple functions in different nuclear environments. The factors that affect these differences remain largely unknown.

HP1 plays a role in heterochromatin formation, chromosome segregation, and heterochromatic silencing in pericentric heterochromatin [3,4,13]. HP1 associates with pericentric regions through an binding of the CD of HP1 to methylated H3K9, an epigenetic marker generated by H3K9 KMT such as Su(var)3–9/KMT1 [14–16]. Molecular mapping in *Drosophila* cells has shown that HP1a and Su(var)3–9/KMT1 colocalize at most of their target loci. The localization of HP1a to heterochromatic regions and genes

Abbreviations: DEG, differentially expressed gene; H3K9me, histone H3 lysine 9 methylation; HP1, heterochromatin protein 1; KD, Knockdown; KDM, histone demethylase; KMT, histone methyltransferase.

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depends on Su(var)3–9/KMT1. Mutations of *Su(var)*2–5, HP1a gene, lead to the suppression of silencing, resulting in HP1-dependent changes in chromatin structure. Expression analysis of *Su(var)*2–5 mutant larvae demonstrated that many euchromatic genes are downregulated in these mutants [17,18]. The euchromatic functions of HP1a are concentrated within the bodies of euchromatic genes rather than in the promoter regions [19,20] and are largely independent of the localization of HP1c and Su(var)3–9/KMT1 [21]. However, the effect of HP1 on gene expression has emerged from accumulated *Drosophila* HP1a data. Little is known about the role of the homologs, HP1b and HP1c in gene regulation. To investigate these issues, we attempted to compare the gene expression profiles between the three main HP1 homologs in *Drosophila*. Our data show that HP1 regulates non-overlapping and overlapping sets of genes. Collectively, this study is the first to systematically analyze the role of HP1 paralogs in gene expression.

2. Materials and methods

2.1. dsRNA knockdown (KD) of HP1 expression in S2 Cells

dsRNA against HP1 was synthesized from PCR products by in vitro transcription with T7 promoters on both ends of the amplicons, using the MEGAscript RNA kit (Ambion). S2 cells were grown in Schneider's insect medium supplemented with 10% serum to a density of 3–6 $\times$ 10^6 cells/mL. Cells were diluted to 1 $\times$ 10^6 cells/mL in serum-free media and incubated with 10 μg dsRNA/1 $\times$ 10^6 cells for 45 min. An equal volume of Schneider media containing 20% serum was then added to the cells. dsRNA against lacZ was used as a control. Control cultures were prepared in the same manner but without addition of dsRNA. Both RNA-treated and control cells were grown for 3 days at 25 °C and then processed for total RNA extraction.

2.2. Analysis of gene expression by quantitative real time PCR

Quantitative real-time (qRT)-PCR was performed according to the methods of Kwon et al. [22]. Total RNA samples from knockdown S2 cells were isolated and first-strand cDNA was synthesized from RNA. PCR primers were designed with Primer Express software (Applied Biosystems). Reactions using the SYBR green PCR master mix (Takara) were performed in triplicate. mRNA values were normalized to those of rp49 (RpL32 – FlyBase).

2.3. DNA microarray

For control and test RNA, the synthesis of target cRNA probes for hybridization were performed using Agilent's Low RNA Input Linear Amplification kit PLUS (Agilent Technology) according to the manufacturer's instructions. Briefly, 1 μg of each total RNA and T7 promoter primer mix were incubated at 65 °C for 10 min. The cDNA master mix was prepared and added to the reaction mixer. The samples were incubated at 40 °C for 2 h, at which point the RT and dsDNA syntheses were terminated by incubating at 65 °C for 15 min. The transcription of dsDNA was performed by adding the transcription master mix to the dsDNA reaction samples and incubating the mixture at 40 °C for 2 h. Amplified and labeled cRNA was purified with a cRNA Cleanup Module (Agilent Technology) according to the manufacturer's protocol. Labeled cRNA target was quantified using a ND-1000 spectrophotometer (NanoDrop Technologies). After checking the labeling efficiency, fragmentation of cRNA was performed by adding 10 $\times$ blocking agent and 25$\times$ fragmentation buffer and incubating at 60 °C for 30 min. The fragmented cRNA was resuspended with 2$\times$ hybridization buffer and directly pipetted onto an assembled Agilent's *Drosophila* Oligo Microarray Kit (44K). The arrays were hybridized at 65 °C for 17 h using an Agilent Hybridization oven (Agilent Technology). The hybridized microarrays were washed according to the manufacturer's washing protocol (Agilent Technology).

2.4. Data acquisition and statistical analysis

The hybridized images were scanned using an Agilent Microarray Scanner (Agilent #G2565BA) and quantified using Feature Extraction Software (Agilent Technology, Palo Alto, CA). Aqll data normalization and selection of fold-changed genes were performed using GeneSpringGX 7.3 (Agilent Technology, USA). Intensity-dependent normalization (LOWESS) was performed, where the ratio was reduced to the residual of the Lowess fit of the intensity vs. ratio curve. The averages of the normalized ratios were calculated by dividing the average of the normalized signal channel intensity by the average of the normalized control channel intensity. The cDNA microarray experiments (one-channel method) were repeated twice and the average of two gene expression values for each gene was used for further analysis. For statistical analysis, ANOVA (1-way) test was used for multiple group comparison across all samples in the experiment by MeV (MultExperiment Viewer) Software. A p-value of $\leqslant$0.05 was considered statistically significant andtermed differentially expressed genes (DEGs). The resulting list of genes and associated p-values were graphically represented by Venn analysis and hierarchical clustering using MeV (MultExperiment Viewer) Software. Functional annotation of the genes was performed based on the Gene OntologyTM Consortium (<http://www.geneontology.org/index.shtml>) by Gene-SpringGX 7.3. Gene classification was based on searches of the BioCarta (<http://www.biocarta.com/>), GenMAPP (<http://www.genmapp.org/>), DAVID (<http://david.abcc.ncifcrf.gov/>) and Medline databases (<http://www.ncbi.nlm.nih.gov/>). To identify the molecular pathways, we used the Kyoto Encyclopedia of genes Genomes (KEGG) pathway database.

3. Results

3.1. Global changes in gene expression in HP1-knockdown S2 cells

To compare the effects of each HP1 homolog on gene transcription, we performed a genome-wide microarray screen of S2 cells to define a comprehensive profile of genes whose expression is altered by HP1 KD. First, we established KD cell lines for each HP1. Both the protein and the mRNA levels of each HP1 were markedly decreased by dsRNA. The same set of mRNA shown in Figure S1 was used for microarray analysis. To identify the differentially expressed genes (DEGs) among HP1 KD and control, we adopted the analysis of variance (ANOVA) model approach. Genes with a p-value of <0.05 and 1.5-fold or greater change relative to LacZ controls were considered to be DEGs. The heat maps generated from the microarray analysis in S2 cells with each HP1 homolog knockdown were subject to hierarchical clustering analysis. The hierarchical clustering heat map demonstrated that three HP1 homologs segregated independently from the control in S2 cells (Fig. 1B).

3.2. Common gene and differential gene signature of HP1 KD in S2 cells

One of the key objectives of this study was to identify a cassette of genes commonly or differentially regulated by the three HP1 homologs. Venn analysis was used to determine the overlap of genes affected by HP1 KD. The microarray results showed that the expression of a total 582 genes was changed >1.5-fold by KD of the three HP1 homologs (10 genes, up-regulated and 572 genes, down-regulated, $p < 0.05$). A combined total of 1282 genes were