Biochemical and Biophysical Research Communications 434 (2013) 820–828

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

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

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ARTICLE INFO

Article history:
Received 11 April 2013
Available online 20 April 2013

Keywords:
HP1a
HP1b
HP1c
Gene regulation
Microarray analysis

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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http://dx.doi.org/10.1016/j.bbrc.2013.04.020

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

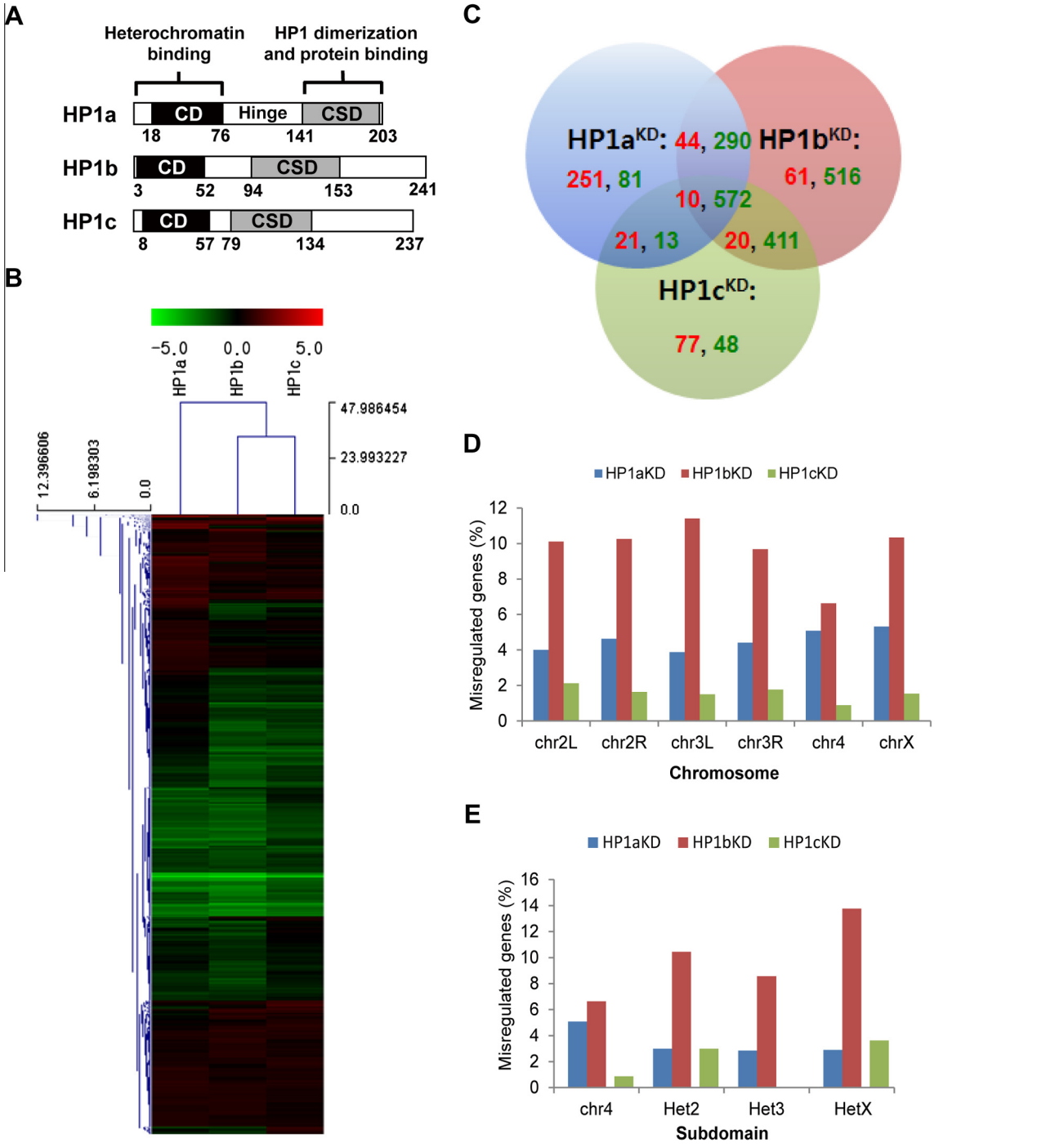

Fig. 1. Analysis of gene expression in HP1-knockdown S2 cells. (A) Schematic representation of the three HP1 proteins. Full-length HP1 (FL) contains a chromo domain (CD), a hinge domain (Hin) and a chromoshadow domain (CSD). (B) Three HP1 proteins display a distinct pattern of gene expression. Heat map microarray data is shown comparing the expression patterns from S2 cells with KD of HP1a, HP1b or HP1c. (C) Venn diagram illustrating overlap of misregulated genes from each HP1 KD. Genes that were up-regulated relative to controls are shown in red and those that were down-regulated are in green. Bar chart depicting the percentage of misregulated genes in each HP1 KD compared with controls by chromosome (D) or heterochromatin domain (E).

modulated by the KD of HP1a representing approximately 4.0% of the total gene set analyzed by the array. Within this set, 332 DEGs uniquely modulated by HP1a KD were identified, as illustrated in the Venn diagram (Fig. 1C and Table 1). Of these, 251 genes were up-regulated and 81 were down-regulated >1.5-fold. Of the 1924 genes (6.0%) modulated following KD with HP1b, 135 genes were up-regulated and 1789 were down-regulated >1.5-fold. Similarly, of the 1172 genes (3.65%) modulated by the KD of HP1c, 128 genes

Table 1
Number of genes affected by knockdown of HP1 in S2 cells.

Gene set name	Up	%	Down	%
HP1a KD	326	1.03	956	2.97
HP1b KD	135	0.43	1789	5.57
HP1c KD	128	0.40	1044	3.25

Fold change $\geqslant 1.5$, $p \leqslant 0.05$ (ANOVA).

were up-regulated and 1044 were down-regulated >1.5-fold. The top 10 genes up- and down-regulated by the three HP1 homologs were commonly modulated >1.5-fold displayed in Table 2. Also, we picked the top five genes with the obviously and uniquely up- or down-regulated expression in any of HP1a KD, HP1b KD or HP1c KD, as listed in Tables 3–5. A detailed list of the gene signatures is presented in the Table S1. These data show that the three HP1 homologs exert similar and distinct effects on gene expression.

3.3. Gene misregulation resulting from HP1 depletion by chromosome

Comparing the gene expression profiles of all chromosomes and heterochromatin domains, we found that the percentage of genes on chromosome 4 misregulated in HP1a and HP1b KDs was larger than in the HP1c KD, with 5.09% and 6.64% of the genes showing significant changes in expression (Fig. 1D and E). In knockdown of HP1a and HP1b, 1.84% and 1.14% were up-regulated, while 2.43% and 1.56% were down-regulated, respectively. This bias towards downregulation was also seen in other regions of the genome generally considered to be heterochromatic regions such as the pericentric regions of chromosome 2, 3, and X. Overall, there were relatively high numbers of genes significantly up- and down-regulated in HP1b KD. Interestingly, the misregulation profile of fourth chromosome and heterochromatic chromosome 3 in HP1c KD was different from that of the other HP1 homologs. HP1c depletion did not notably influence these chromatic regions.

3.4. Identification of biological pathways modulated by HP1

Gene ontology (GO) annotation analysis of misregulated genes revealed that with all three HP1 homologs examined, a wide variety of genes were affected. The affected gene products were associated with various cellular processes such as cell proliferation and death, signal transduction, secretion, immune and stress responses, and cell development, to name a few (Fig. 2). Specifically, proteins associated with apoptosis and cell death were overrepresented in the set of misregulated genes in HP1a KD, while pigmentation-associated proteins were underrepresented. Most noteworthy was the overrepresentation of genes associated with the GO term "cell development", "cell migration", and "neurogenesis" and the underrepresentation of genes characterized as being involved in "aging", "immune response", "response to stress", and "pigmentation" in HP1c KD. Interestingly, almost all of the genes noticeably affected by HP1b KD were underrepresented compared with similarly up- and down-regulated genes in HP1a KD and HP1b KD. Next, we investigated the expression program coregulated by the three HP1 proteins. This analysis was restricted to the 582 genes found to be differentially expressed in the same direction in all three HP1 KDs. A cluster of genes implicated in cell death, cell development, immune response, neurogenesis, response to stress and signal transduction was observed.

We further analyzed the HP1-DEGs to explore the key biological pathways modulated by HP1. We performed KEGG pathway analysis using DAVID Bioinformatics Resources 6.7 (NIAID/NIH) on the DEGs in S2 cells with KD of each HP1 homolog. Fisher's exact test was used to calculate the *p*-value to compare the genes in the dataset and the canonical pathway. Twenty-one common pathways were altered by HP1a, 16 pathways by HP1b and 16 pathways by HP1c (Fig. 3 and Table S2). Twelve overlapping and common pathways were modulated by all three HP1 proteins. Furthermore, pathways unique to each or two of the three HP1 proteins were observed. Two biological pathways modulated by both HP1a and HP1b were involved in; ECM-receptor interaction and nitrogen metabolism. Glycan degradation and methane metabolism were regulated by HP1b and HP1c while there were not found pathways modulated by both HP1a and HP1c. In the case of pathways that were uniquely regulated by each HP1 protein, we identified glycerolipid, glycerophospholipid, sphingolipid, and ether lipid metabolism, tyrosine, beta-alanine, alanine, aspartate and glutamine and pyruvate metabolism in HP1a. Interestingly, HP1b influenced the proteasome degradation pathway. In the case of HP1c, three pathways were uniquely modulated: galactose metabolism, lysosomal degradation, androgen and estrogen metabolism.

3.5. qRT-PCR validation of changes in gene expression in HP1-knockdown in S2 cells

In order to assess the robustness of the microarray analysis, qRT-PCR analysis was performed to validate a selected panel of 16 DEGs and 1 non-DEG, using the primer sets. We selected a

Table 2
Analysis of common genes with altered expression by knockdown of three HP1 proteins in S2 cells.

Accession No.	Gene symbol	Gene name	Fold change			*P*-value
			HP1a KD	HP1b KD	HP1c KD	
Induced						
NM_136851	Gal	Beta galactosidase	3.20	2.50	2.83	0.0252
NM_137413	CG14946	CG6401	7.14	4.13	1.84	0.0318
NM_137526	CG4586	CG15077	1.74	2.00	2.19	0.0382
NM_001038789	CG15343	CG34011	1.86	2.5	2.17	0.0016
NM_170542	CG4576	CG18729	6.38	2.03	1.54	0.0052
NM_136917	CG18446	Gartenzwerg	5.15	2.48	1.62	0.0262
NM_137497	CG10924	Damm, Death associated molecule related to Mch2	4.89	2.32	1.55	0.0376
NM_137518	CG30087	CG15073	3.14	2.00	1.74	0.0381
NM_001031970	CG15529	CG33767	2.53	1.67	2.75	0.0015
NM_137002	CG10621	CG4627	2.70	1.56	2.01	0.0280
Repressed						
NM_166072	CG7300	CG10205	0.06	0.06	0.09	0.0311
NM_135408	CG9466	CG9466	0.05	0.03	0.05	0.0049
NM_141936	CG32778	Protein partner of snf (Pps)	0.03	0.03	0.04	0.0073
NM_140195	CG7091	Ubiquitin protein ligase E3A (UBE3A)	0.06	0.05	0.07	0.0052
NM_166252	CG32972	Gainy head	0.09	0.07	0.17	0.0466
NM_078959	Prx2540–2	Peroxiredoxin 2540	0.11	0.06	0.12	0.0186
NM_166694	rk	Rickets, G protein-coupled receptor kinase 1	0.13	0.08	0.20	0.0143
NM_001169680	DptB	Diptericin B	0.09	0.11	0.17	0.0309
NM_137532	CG8086	CG15096	0.12	0.10	0.21	0.0383
NM_078580	CG34265	Twisted gastrulation	0.12	0.13	0.30	0.0413

Table 3
Top 5 genes whose transcript levels were uniquely up- or down-regulated in the HP1a KD samples, as identified in supplementary table S1.

Accession No.	Gene symbol	Gene name	Fold change	*P*-value
Induced				
NM_168690	CG13029	CG13029	16.473839	0.003497
NM_001201980	CG42700	CG42700	7.6794543	0.047308
NM_135442	CG4438	CG4438	7.3366213	0.006935
NM_137506	CG3884 (Atg7)	Autophagy-specific gene 7	4.7359233	0.03786087
NM_001038966	Syt7	Kibra ortholog	4.414436	0.00203841
Repressed				
NM_079857	CG11093	Tailless (tl), Nuclear receptor subfamily 2 group E (NR2E2)	0.15326016	0.04101477
NM_137217	Cpn	Ribosomal protein S14a	0.1604457	0.00484855
NM_136643	CG5693	Preli-like	0.22837697	0.00853350
NM_137222	CG13278	CG8299	0.257266	0.03232572
NM_079601	Obp18a	Seven up	0.2681758	0.04040862

Table 4
Top 5 genes whose transcript levels were up- or down-regulated in the HP1b KD samples, as identified in supplementary table S1.

Accession No.	Gene symbol	Gene name	Fold change	*P*-value
Induced				
NM_143813	Cyp303a1	CG4163	5.247604	0.007393
NM_001202031	Cng	Cyclic-nucleotide-gated ion channel protein	4.516458	0.033094
NM_001014756	Est-6	CG42687	2.3307924	0.000307
NM_001038827	CG33767	CG3405	2.5599432	0.003063
NM_057507	CG31245	Glutamate receptor I	2.0445502	0.047171
Repressed				
NM_001042846	RpL40	CG40486	0.43592167	0.022038
NM_001202390	CG10132	Tropomodulin	0.43755698	0.038497
NM_001043313	Cpr76Bc	CG34155	0.46792698	0.035046
NM_001103691	CG32391	CG34165	0.4944853	0.011015
NM_001103660	Cby	CG34181	0.5050396	0.019582

Table 5
Top 5 genes whose transcript levels were uniquely up- or down-regulated in the HP1c KD samples, as identified in supplementary table S1.

Accession No.	Gene symbol	Gene name	Fold change	*P*-value
Induced				
NM_142307	CG12783	CG12783	6.2404017	0.035588
NM_169807	gl	Glass	5.9710608	0.028921
NM_168091	NT1	Neurotrophin 1	5.3963385	0.000114
NM_140964	CG5282	CG5282	6.2404017	0.035588
NM_057740	Gycalpha99B	Guanylyl cyclase alpha-subunit at 99B	4.1556478	0.002616
Repressed				
NM_135339	CG12375	CG12375	0.1666277	0.008856
NM_137528	Ir56c	Multiple C2 domain and transmembrane region protein	0.43443662	0.038214
NM_136820	CG3699	CG13220	0.44972256	0.024604
NM_079188	CG15539	Forkhead domain 64A	0.45762095	0.049114
NM_136950	CG14069	CG8818	0.46022812	0.026940

house-keeping gene, the RpL32, rRNA gene, and used it to normalize all qPCR data. To further validate and characterize the DEGs identified by the microarray analysis, we analyzed the HP1 homolog-dependent changes in the expression of the selected DEGs compared to control. The pattern of expression showed consistent directional conformation (up- and down-regulation) between the qPCR and microarray analysis (Fig. 4). Damm (death-associated molecule related to Mch2) and Gal (beta galactosidase) were upregulated in the three HP1 KDs while protein partner of snf (Pps) and ubiquitin protein ligase E3A (UBE3A) were downregulated. Other expression changes were as follows: Atg7 (autophagy-specific gene 7) and sty7 (kibra ortholog) (up, HP1a KD); tailess (nuclear receptor subfamily 2 group E) and Cpn (ribosomal protein S14a) (down, HP1a KD); Cng (cyclic-nucleotide-gated ion channel protein) and Est-6 (esterase 6) (up, HP1b KD); Cby (chibby) and tropomodulin (down, HP1b KD); neurotrophin 1 and gl (glued) (up, HP1c KD); out (outsiders, transcription factor 23) and Ir56c (Multiple C2 domain and transmembrane region protein) (down, HP1c KD).

4. Discussion

It has been reported that HP1a impacts gene expression in both heterochromatin and euchromatin. However, most available data relates only to HP1a in *Drosophila*. A comprehensive gene expression profile of *Drosophila* HP1 homologs that would allow the elucidation of the exact overall HP1 gene expression program is not available. Here, we demonstrate the genome-wide effects of the three HP1 homologs on gene expression through microarray analysis. Based on the hypothesis that the three HP1 homologs differentially regulate gene expression, we explored the effects of HP1 depletion on gene regulation throughout the *Drosophila* genome. Indeed, HP1 KD results in the misregulation of a large of number of genes (1172–1924 genes). In concordance with the results of our study, a large number of genes affected by the depletion of HP1a in larval tissues seem to be different from that in embryonic Kc cells. The depletion of HP1a in Kc cells induced cell cycle defects and correlated with the misregulation of many cell cycle regulators such as Mcms (mini-chromosome maintenances), ORC4, CAF1,

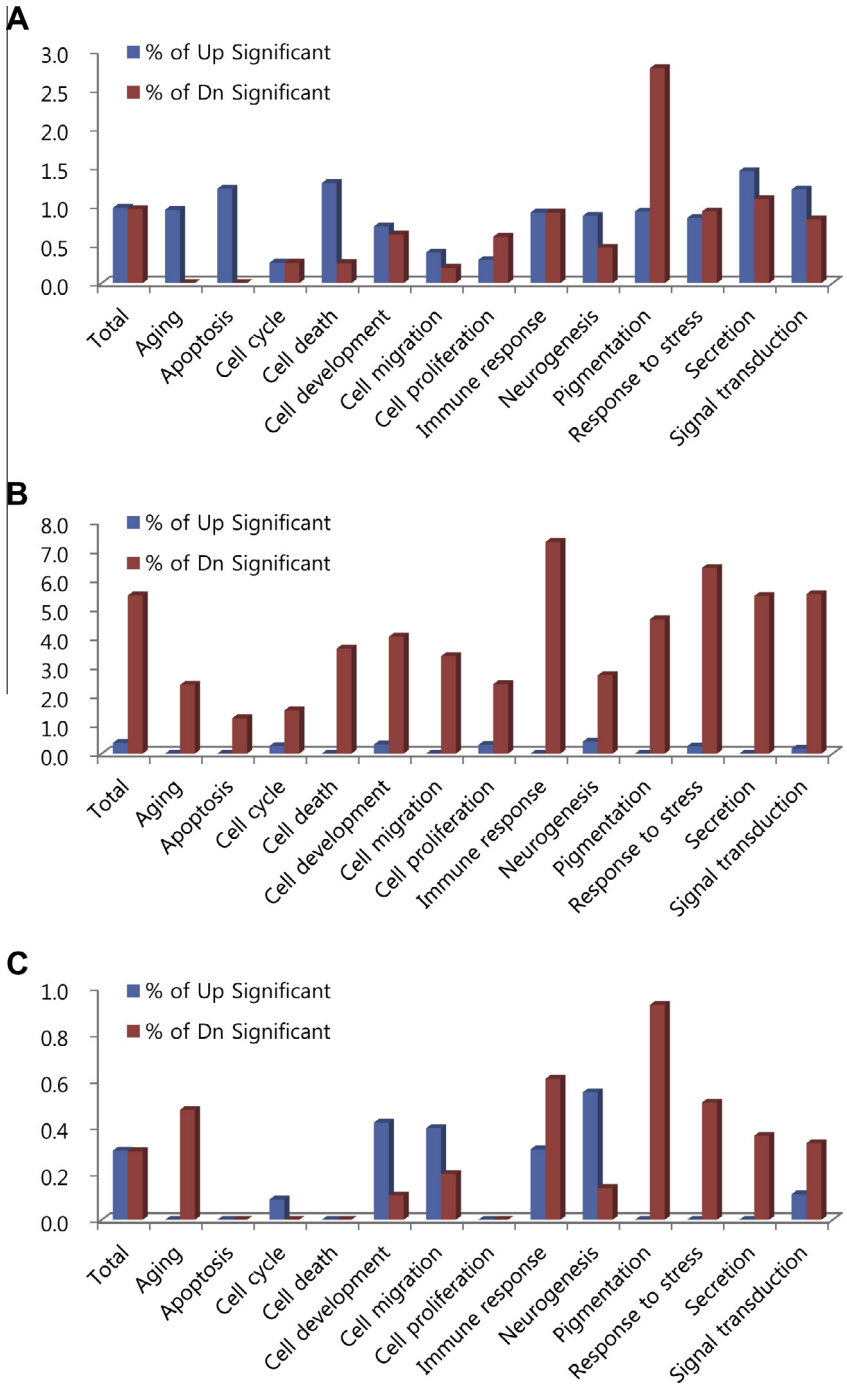

Fig. 2. Gene ontology categories for the *Drosophila* oligomicroarray (32,162 genes). GO clustering of HP1a, HP1b and HP1c KDs. GO term enriched in the set of HP1a (A), HP1b (B) and HP1c (C) KDs. The percentage of changed genes in each GO category is shown on the *X*-axis.

Cdc45 and Aurora B [18]. However, this change in the transcription of these cell cycle regulators in Kc cells among the HP1a-affected genes at the larval stage has not been previously reported [17]. In our study, significant changes of cell cycle regulators were not detected in the HP1-homolog-dsRNA-treated S2 cells. This may be due to the specific role that each HP1 homolog plays in the different stages of development. It is also possible that the effect of HP1 in the transcription of cell cycle regulators in proliferating cells is underestimated when performing analyses on mixed populations of both proliferating and differentiating/differentiated cells.

Several studies have suggested a role for HP1 in euchromatic gene regulation as well as in heterochromatic gene regulation. Thus, we analyzed the expression profiles of the other *Drosophila* HP1 homologs, HP1b and HP1c, in addition to HP1a. Consistent with other reports, our gene expression analysis showed up-regulation and down-regulation of genes in all three HP1 KDs. Contrary to expectations regarding its gene silencing role, there were more down-regulated genes (572 genes) than up-regulated genes (10 genes) in all three HP1 KDs. This data strongly suggest that the three HP1 proteins work together to enhance the expression of most of their common target genes, although they could also act to repress the expression of a subset of genes in *Drosophila*. Furthermore, the microarray profiling showed that HP1 regulates different target genes as well as common target genes. The

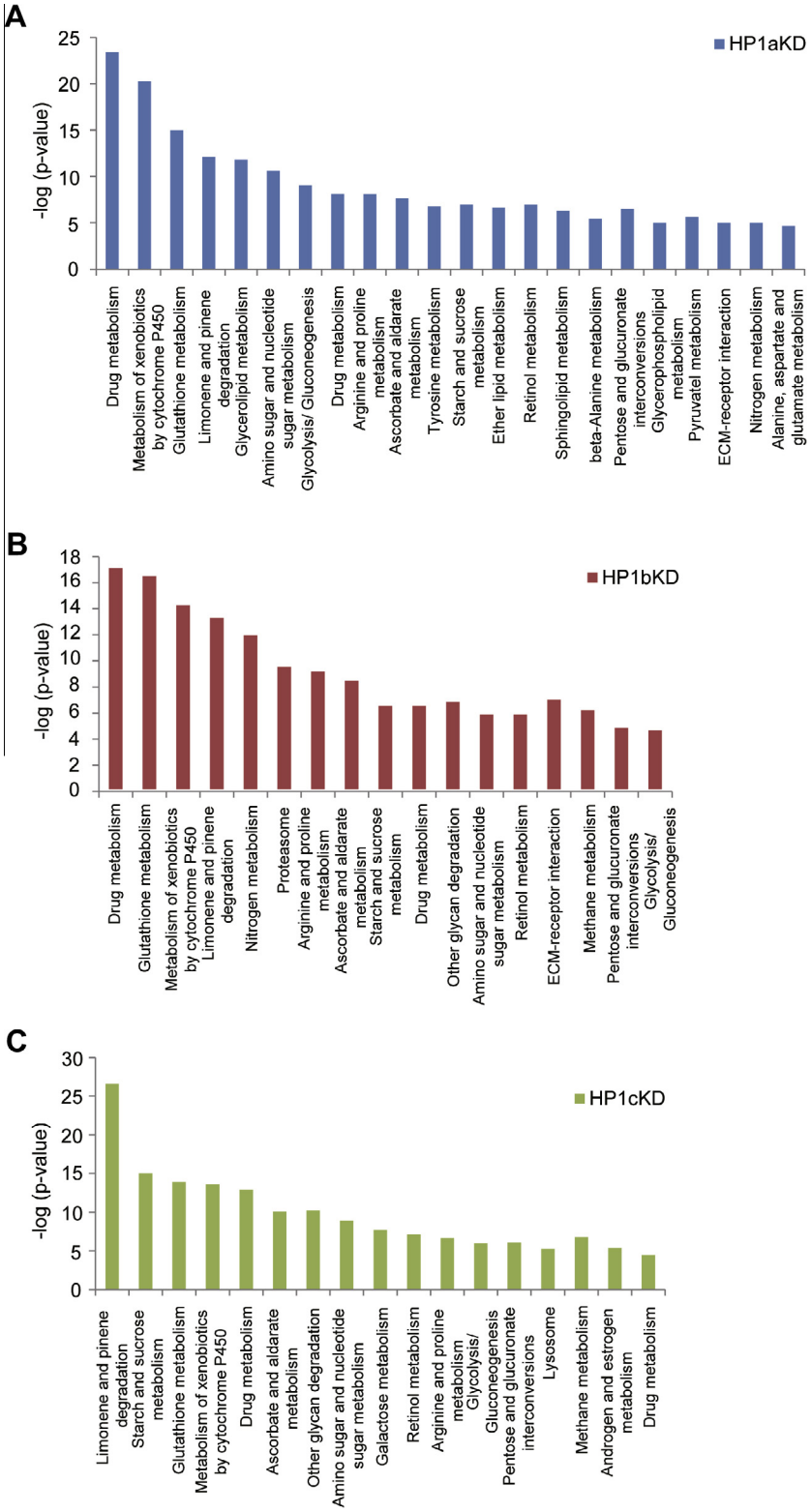

Fig. 3. Canonical pathways that were significantly modulated by HP1 as identified by the KEGG pathway. HP1a (A), HP1b (B), or HP1c (C) knockdown S2 cells. Fisher's exact test was used to calculate a *p*-value determining the probability that the association between the genes in the dataset and the canonical pathway is explained by chance alone.

microarray analysis identified that all three HP1s induce potent up-regulation of Damm. qPCR confirmed that this gene is significantly up-regulated in all HP1 KDs (Fig. 4A). In *Drosophila* melanogaster seven caspases, namely Damm, DCP-1, DREDD/DCP-2, DRICE, DRONC, DECAY, and STRICA have been cloned [23]. Damm is a *Drosophila* effector caspase and controls apoptosis. This result suggests that HP1 plays an important role in cell survival, by supporting cell proliferation as previously reported. Within this gene expression overlap, we identified another interesting target gene, UBE3. The depletion of all three HP1 homologs caused

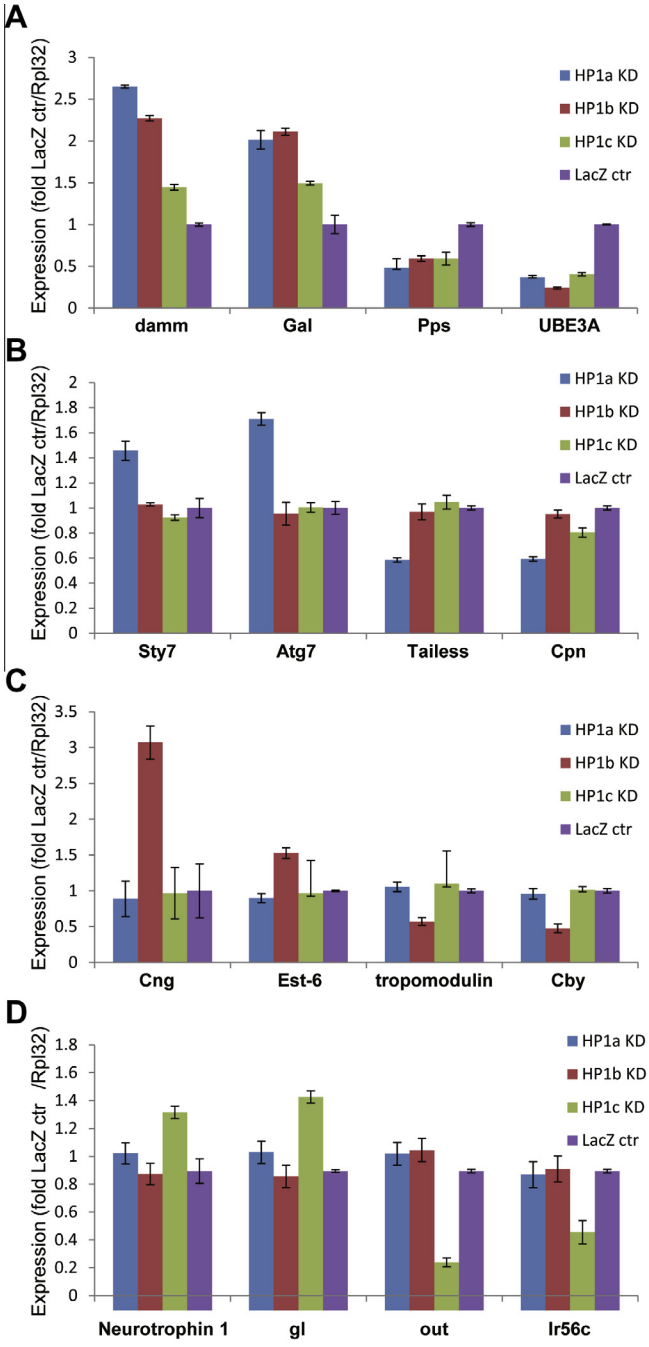

Fig. 4. Validation of microarray data by qRT-PCR. (A–D) Knockdown was performed for each HP1 homolog in S2 cells and total RNA was extracted and reverse transcribed prior to qPCR analysis. The change in qRT-PCR is presented as the average of three independent experiments. All genes were normalized to Rpl32. Error bars denote ± s.d. of mean.

down-regulation of UBE3A. UBE3A, which is the *Drosophila* homologue of the Angelman syndrome (AS) ubiquitin ligase, regulates the formation of terminal dendritic branches [24]. Since the mental retardation disorder AS is primarily caused by the loss of the UBE3A protein product [25], it is of great importance to understand the normal function of UBE3A in neuronal development and synaptic plasticity. *Drosophila* is one of the excellent model systems to understand the genes and molecular pathways that are misregulated in human disease conditions. The down-regulation of UBE3A suggests that it may control neuronal development through HP1. A study of the relationship between HP1 and UBE3A would provide further insights into the molecular pathogenesis of AS.

In addition to the overlapping target genes of the three HP1 proteins, target genes unique to each HP1 protein were observed. For example, gl and neurotrophin 1 were the most heavily up-regulated genes in HP1c KD (>2.0-fold). Neurotrophins (NTs) are the main secreted signaling molecules that link nervous system structure and function in vertebrate. NTs regulate neuronal survival, development, targeting, synaptic plasticity, memory and cognition [26,27]. Consistent with our results, Font-Burgada et al. reported that HP1c, WOC and ROW coregulate genes related to the development and functionality of the nervous system in RNAi fly lines [28]. The up-regulation of NT1 suggests that it may regulate the nervous system in part via HP1c.

Collectively, the results of this study demonstrate that HP1 plays an essential role in gene expression. This information will assist in the identification of novel pathways and targets modulated by each HP1 homolog. Further study may provide much-needed information on mechanism of action of HP1 in both heterochromatin and euchromatin.

Acknowledgments

We wish to thank Dr. Lori Wallrath for contributing the anti-HP1a monoclonal antibody (C1A9). This research was supported by the Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (2012013998).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.04.020.

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