HP1 controls genomic targeting of four novel heterochromatin proteins in *Drosophila*

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Heterochromatin is important for the maintenance of genome stability and regulation of gene expression; yet our knowledge of heterochromatin structure and function is incomplete. We identified four novel *Drosophila* heterochromatin proteins (HPs). Three of these proteins (HP3, HP4 and HP5) interact directly with HP1, whereas HP6 in turn binds to each of these three proteins. Immunofluorescence microscopy and genome-wide mapping of *in vivo* binding sites shows that all four proteins are components of heterochromatin. Depletion of HP1 causes redistribution of all four proteins, indicating that HP1 is essential for their heterochromatic targeting. Finally, mutants of HP4 and HP5 are dominant suppressors of position effect variegation, demonstrating their importance in heterochromatic gene silencing. These results indicate that HP1 acts as a docking platform for several mediator proteins that contribute to heterochromatin function.

The EMBO Journal (2007) **26**, 741–751. doi:10.1038/sj.emboj.7601527; Published online 25 January 2007

Subject Categories: chromatin & transcription; genomic & computational biology

Keywords: chromatin composition; DamID; position effect variegation; protein interactions

Introduction

Heterochromatin was originally defined as the densely staining regions of interphase nuclei (Heitz, 1928). In most species, these regions are largely composed of pericentromeric regions that are often aggregated in the cell nucleus to form one or more so-called chromocenters. Additionally, distinct regions on the chromosome arms and close to telomeres display molecular or morphological characteristics of heterochromatin.

Heterochromatin has diverse functions. Pericentromeric and telomeric heterochromatin are involved in the maintenance of genome stability, by ensuring proper chromosomal segregation (Ekwall *et al*, 1995; Kellum and Alberts, 1995;

Peters *et al*, 2001) and preventing telomere fusions (Fanti *et al*, 1998; Ferreira and Cooper, 2001; Sharma *et al*, 2003; Oikemus *et al*, 2004). Apart from this structural function, heterochromatin is also involved in regulating gene expression. Paradoxically, heterochromatin can mediate both repression and activation of gene expression. It is involved directly or indirectly in silencing of repetitive elements (Dorer and Henikoff, 1997; Lippman *et al*, 2004; Verdel *et al*, 2004), certain euchromatic genes (Hwang *et al*, 2001; Liu *et al*, 2005) and euchromatic genes that are artificially placed close to heterochromatic regions (reviewed in Henikoff, 1992; Weiler and Wakimoto, 1995), but it is also essential for the expression of pericentromeric genes (Wakimoto and Hearn, 1990; Lu *et al*, 2000) and several genes located on the chromosome arms (Piacentini *et al*, 2003; Cryderman *et al*, 2005; Liu *et al*, 2005). The molecular basis for this dual role of heterochromatin in gene regulation is still not understood.

In a variety of species, proteins have been identified that are important components of heterochromatin and many of them are conserved in eukaryotes. Here, we focus on heterochromatin proteins (HPs) in *Drosophila*. HP1 (sometimes referred to as HP1a) is a key player in heterochromatin formation. It binds to di- or trimethylated lysine 9 of histone H3 (H3K9me2/H3K9me3) via its chromodomain (CD) (Jacobs and Khorasanizadeh, 2002; Nielsen *et al*, 2002). The histone methyltransferase (HMT) Su(var)3-9 is to a large extent responsible for methylating H3K9 and thereby creates binding sites for HP1 (Schotta *et al*, 2002). In addition, HP1 and Su(var)3-9 interact directly (Schotta *et al*, 2004). This triangle of interactions is thought to constitute a positive feedback loop that stabilizes heterochromatin. Heterochromatin is also marked by trimethylation of lysine 20 of histone H4 (H4K20me3), which is mediated by Suv4-20, an HMT that interacts directly with HP1 (Schotta *et al*, 2004). Three other proteins, Su(var)3-7, HOAP (encoded by the gene *caravaggio* (cav)) and HP2, also interact with HP1 (Delattre *et al*, 2000; Shaffer *et al*, 2002; Badugu *et al*, 2003), and Su(var)3-7 was shown to require HP1 for its localization to heterochromatin. Furthermore, heterochromatin formation involves components of the RNA interference (RNAi) machinery (Fukagawa *et al*, 2004; Pal-Bhadra *et al*, 2004; Verdel *et al*, 2004).

Recent evidence indicates that heterochromatin—as defined by the above-mentioned proteins—is heterogeneous. Genome-wide mapping of the *in vivo* target genes of HP1 and Su(var)3-9 (Greil *et al*, 2003) has shown that many loci are bound by both proteins. However, some genes located on the chromosomal arms are bound by Su(var)3-9 alone. In addition, genes on chromosome 4 are strongly bound by HP1 in a manner that is mostly independent of Su(var)3-9 (Schotta *et al*, 2002; Greil *et al*, 2003). Thus, these HPs can form different complexes, each with distinct sets of genomic targets.

Understanding the structure and functions of heterochromatin will require complete knowledge of the composition of

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Received: 12 September 2006; accepted: 23 November 2006;
published online: 25 January 2007

heterochromatin complexes, and of the molecular interactions between the components. Here, we describe the identification and characterization of four novel *Drosophila* HPs, named HP3, HP4, HP5 and HP6. Yeast two-hybrid assay data indicate that HP3, HP4 and HP5 interact directly with HP1, whereas HP6 interacts with the other three (Giot *et al*, 2003). By large-scale mapping of their *in vivo* binding sites using the DamID technique, we show that all four proteins bind to the same genomic loci as HP1. Depletion of HP1 leads to strong redistribution of HP3, HP4, HP5 and partially of HP6, indicating that HP1 is essential for their correct targeting. Studies with available mutants of HP4, HP5 and HP6 show that HP4 and HP5 are important for silencing of a reporter gene by heterochromatin. Based on these results, we propose that

HP1 serves as a universal docking platform for proteins that are essential for heterochromatin function.

Results

Novel HP1 interacting proteins

In order to identify novel HPs, we searched the Curagen *Drosophila* Interaction Database (Giot *et al*, 2003) for proteins interacting with HP1. For this database, over 10 000 proteins were tested for direct interactions in a large-scale yeast two-hybrid screen, which identified over 4500 interactions. Besides the known HP1 partners Su(var)3-9 and HOAP, this screen identified several novel proteins that interact with HP1. Three of these, encoded by the genes *CG18468*,

Figure 1 Characteristics of putative HPs. **(A)** Part of the HP1 interactome as determined by a large-scale yeast two-hybrid screen (Giot *et al*, 2003). Four previously uncharacterized proteins, which we named HP3, HP4, HP5 and HP6, directly or indirectly interact with HP1. Solid lines mark high-confidence and dashed lines low-confidence interactions between proteins, according to Giot *et al* (2003). Black ovals highlight proteins that were included in this study. **(B)** Protein domain structure of the novel HPs. CD: chromodomain; CSD: chromoshadow domain; BESS: boundary element-associated factor 32/Su (var)3-7/Stonewall domain; NLS: nuclear localization signal. Computed gene (CG) identifiers and calculated molecular weights are listed as well. **(C)** Relative expression levels of HP transcripts during fly development, according to Stolc *et al* (2004). Black lines: expression levels in early embryos (E0), late embryos (E3), larvae (L), pupae (P) and female adult flies (A); gray lines: expression levels in male adult flies.

CG8044 and CG1745, interact not only with HP1 but also with a fourth protein encoded by CG15636 (Figure 1A). This network of interactions suggested that these four proteins might form a complex together with HP1. We therefore tentatively named these proteins HP3, HP4, HP5 and HP6, respectively.

All four proteins are rather small with a predicted size of 12–49 kDa and had previously been uncharacterized, apart from several predicted domains (Figure 1B). Interestingly, HP6 largely consists of a chromoshadow domain. The chromoshadow domain is related to the chromo (CHR)romatin Organization MOdifier domain and was so far only found in tandem with the chromodomain in HP1 and homologues of HP1. Together with part of the hinge domain, the chromoshadow domain mediates protein–protein interactions, such as dimerization of HP1 or binding of Su(var)3-9, Su(var)3-7 or HOAP to HP1 (Cowieson *et al*, 2000; Delattre *et al*, 2000; Zhao *et al*, 2000; Badugu *et al*, 2003; Yamamoto and Sonoda, 2003). HP3 contains a BESS domain, a protein–protein interaction domain that is also present in Su(var)3-7 and in several other proteins involved in regulation of transcription (summarized in the Prosite BESS domain profile at <http://www.expasy.ch>). Using the BLAST and PSI-/PHI-BLAST algorithms (Altschul *et al*, 1990; Altschul *et al*, 1997), we could identify homologues of HP3 and HP5 in *Drosophila pseudoobscura*, but not in other species.

According to a genome-wide expression database (Stolc *et al*, 2004), HP3, HP4, HP5 and HP6 are expressed throughout fly development. HP3, HP4 and HP5 show a similar expression profile as HP1: they are expressed high during embryogenesis and low during later stages of fly development (Figure 1C). In contrast, HP6 expression is inversely correlated with HP1 expression: it is expressed low during embryogenesis and high in later fly developmental stages. This divergent expression is especially prominent in male adults, where HP6 expression levels are markedly higher than those of the other HP genes, suggesting a role of HP6 during male fly development.

Epitope-tagged HP3, HP4 and HP5 are concentrated in the chromocenter

In order to test whether the novel proteins are HPs, we cloned each into the vector pDamMyc (van Steensel and Henikoff, 2000) to create fusion proteins that carry both a Myc epitope

tag and Dam methyltransferase. The Myc tag was used to study the intranuclear localization of the HP proteins, and the Dam tag was used to map genomic binding sites by the DamID technique (see below). Expression of the fusion proteins was driven by a heat-shock promoter.

We transfected the plasmids encoding the fusion proteins into *Drosophila* Kc cells, which have a clearly visible chromocenter that is marked by a DAPI-bright region and high concentration of HP1 (van Steensel and Henikoff, 2000). After heat-shock induction, the localization of the fusion proteins in the cell nucleus was visualized by staining with an anti-Myc antibody. For comparison, we stained the same cells with DAPI and with an antibody against endogenous HP1.

Tagged HP3, HP4 and HP5 clearly colocalized with HP1 in the chromocenter (Figure 2A–C). The MycDam tag alone showed a weak and diffuse staining throughout the cell, with no enrichment in the chromocenter (data not shown; van Steensel and Henikoff, 2000). HP6 however showed a uniform nuclear staining with typically three bright speckles (Figure 2D). The uniform localization of HP6 might indeed reflect the natural HP6 localization, but could also be caused by the overexpression of the Dam–HP6 fusion protein by heat-shock. In any case, these results strongly suggested that HP3, HP4 and HP5 are HPs, although this experiment provided no evidence for such role of HP6.

Genome-wide maps show co-targeting of all four novel HPs and HP1

We wanted to confirm the colocalization of HP3, HP4 and HP5 with HP1 at single-gene resolution. Additionally, we wanted to know whether HP6 still shows a uniform nuclear distribution if it is not overexpressed by heat-shock induction. Therefore, we used the DamID technique (van Steensel and Henikoff, 2000; van Steensel *et al*, 2001; Greil *et al*, 2003) to map the *in vivo* DNA-binding sites of each novel protein on a genome-wide scale. In brief, this technique involves the low-level expression of a protein of interest fused to *Escherichia coli* DNA adenine methyltransferase (Dam) from a leaky (noninduced) heat-shock promoter. Targeted adenine-methylation by this fusion protein is mapped using a microarray-based detection method, with unfused Dam serving as a control (Greil *et al*, 2006). The ratio of the Dam-fusion over the Dam-alone signal is a measure of the degree of protein

Figure 2 Immunofluorescence images of interphase *Drosophila* Kc cells transfected with DamMyc-tagged HP3 (A), HP4 (B), HP5 (C) and HP6 (D). Left panels: DAPI staining of DNA. Brightly staining regions are heterochromatic. Middle panels: endogenous HP1 staining using the anti-HP1 antibody C1A9 (red). Right panels: staining of DamMyc-tagged HP fusion proteins using an anti-Myc antibody (green, only in transfected cells).

binding to each probed locus and is referred to as the binding ratio of a protein throughout this text. For detection of the methylation patterns, we used microarrays containing about 12 000 cDNAs, which, after removal of redundant and poor-quality probes, cover ~60% of the coding part of the fly genome.

We performed these mapping experiments in Kc cells for all four candidate HPs. For comparison, we repeated the mapping of HP1 and Su(var)3-9, which we previously studied using the same approach but with much smaller (~6000 cDNA) arrays (Greil *et al*, 2003). As a negative control, we generated in parallel a binding map of the transcription factor JRA, which has not been implicated in heterochromatin and does not interact with any known HP in the Curagen yeast two-hybrid screen database (Giot *et al*, 2003). At least three independent DamID experiments were performed for each protein, and the replicates were averaged. All data are available as Supplementary data (Supplementary Table S1).

Visual inspection of the chromosomal maps (Figure 3) indicates strong similarities in the binding behavior of HP1, Su(var)3-9 and all four novel HP proteins. For each of these proteins, conspicuous binding is visible in pericentric regions of the major chromosomes and on the heterochromatic 'dot' chromosome 4. This pericentric enrichment is in agreement with the strong chromocenter staining observed for HP3, HP4 and HP5, but it is in contrast with the microscopy results for HP6 (Figure 2). Overexpression, side effects of heat-shock treatment or epitope masking (none of which can take place during DamID mapping) may have caused the failure to detect pericentric enrichment of HP6 by immunofluorescence microscopy. Importantly, the overlapping binding of all tested HPs is not restricted to pericentric regions, but is also clearly visible at individual target genes located on the chromosome arms. No similarity can be seen between the binding patterns of JRA and those of the other proteins, demonstrating that the DamID maps are specific.

Bivariate scatterplots provide more detailed insights into the relationships between the protein-binding patterns (Figure 4). As reported previously (Greil *et al*, 2003), HP1 and Su(var)3-9 share many target loci, but in addition each protein has a specific set of targets (Figure 4A). Strong correlations were observed between binding of HP1 and HP3, HP4, HP5 and HP6 (Figure 4B–E), suggesting that all five proteins interact with a common set of target loci. No positive correlation was observed between the binding of JRA and HP1 (Figure 4F). We analyzed the binding behavior of the novel HPs in more detail and confirmed that the chromosomal distribution and the enriched binding to repetitive elements are similar to that of HP1 (Supplementary Table S2). Interestingly, HP3 associates reproducibly with a small set of genes that are not or very weakly bound by HP1 or any of the four novel HP proteins (Figure 4B, dashed oval). This suggests that HP3 might associate with these genes independently of the other HPs.

HP1 is required for the heterochromatic targeting of HP3, HP4 and HP5

Because most genes bound by the novel HPs were also bound by HP1, we wondered whether HP1 might mediate the recruitment of the novel HPs. To test this, we used RNAi to reduce HP1 levels as described (Greil *et al*, 2003) and subse-

quently assessed the localization of DamMyc-tagged HP3, HP4, HP5 and HP6 by immunofluorescence (Figure 5). The successful reduction of HP1 levels was verified by immunofluorescence imaging of endogenous HP1 in Kc cells (Figure 5A and F). In order to control for nonspecific effects of dsRNA treatment, we also incubated cells with dsRNA derived from the *white* gene, which codes for an eye pigment transporter that is not expressed in Kc cells. The *white* dsRNA-treated cells showed a normal heterochromatic localization of HP1, HP3, HP4 and HP5 (Figure 5A–D). The localization of HP6 was also comparable to that of untreated cells (compare Figure 5E with 2D). The distribution of DamMyc-tagged HP3, HP4 and HP5 was strongly altered after HP1-dsRNA treatment. Instead of preferential association with the chromocenter, they now showed a diffuse staining of the entire nucleus. The already diffuse distribution of DamMyc-tagged HP6 however was not changed after depletion of HP1. These data demonstrate that HP1 is required for restricting HP3, HP4 and HP5 to pericentric heterochromatin, whereas such requirement for HP6 localization could not be deduced from this assay.

DamID mapping shows redistribution of HP3 upon loss of HP1

We wondered whether the diffuse nuclear staining of DamMyc-tagged HP3, HP4 and HP5 after HP1-RNAi treatment reflected the complete release of the HPs from chromatin, or the gain of *de novo* targets along chromosome arms. To investigate this, we used DamID to map the genome-wide changes in HP3 binding after reduction of HP1 levels. Figure 6A shows a scatterplot of the binding ratios of HP3 in HP1-dsRNA-treated cells, plotted against the HP3-binding ratios in control cells treated with *white* dsRNA. Visual inspection as well as analysis by a 'k-slopes' iterative regression algorithm (see Materials and methods) identified two major groups of genes that differ in their HP3-binding behavior after loss of HP1. First, nearly all genes with high HP3-binding ratios in control cells maintained HP3 binding after knock down of HP1 (Figure 6B, dark-gray dots and line), although on average the HP3 levels at these genes were somewhat reduced (note that most of these genes are located below the diagonal in the scatterplot). Second, a distinct group of genes with low HP3-binding ratios in control cells showed high binding ratios in HP1-depleted cells (Figure 6B, black dots and line). These are genes that acquire HP3 binding *de novo* when HP1 levels are reduced. These data show that redistribution of HP3 occurs upon loss of HP1, indicating that HP1 controls the genomic targeting of HP3.

We wanted to know whether the *de novo* HP3 target genes share common features, in order to discern a possible function of HP3 retention by HP1. We therefore investigated the expression status of the *de novo* targets using Kc cell expression data obtained on the same type of array that we used for DamID (Pickersgill *et al*, 2006; Figure 6C). We found that in non-RNAi-treated cells, their expression levels were significantly higher than the original HP3 target genes ($P = 1.9 \times 10^{-9}$), which means that HP1 likely constrains HP3 binding to genes with relatively low expression levels.

As pointed out earlier, we found in untreated Kc cells that some genes are bound by HP3 but not by HP1 and the other HPs (Figure 4B). We predicted that binding of HP3 to these genes would be independent of HP1 in contrast to those

Figure 3 Target genes of novel HPs and HP1 overlap on chromosomes 2R and 4. Chromosomal maps of the binding of HP1, Su(var)3-9, all four novel HPs and JRA on the second (right arm) and fourth chromosome. Each vertical line represents the average Dam-HP:Dam methylation ratio of a probed gene at its chromosomal position (in Mb). Genes with statistically significant elevated binding levels are shown in black and other genes in gray. Gray ovals depict centromeres. The relative position of cytologic bands on the second chromosome are shown at the bottom of the figure.

genes that are bound by HP3 and HP1. Indeed, the HP3-binding ratios of the HP1/HP3 co-target genes were decreased on average by 0.7 in HP1-depleted cells ($P = 1.8 \times 10^{-42}$, Wilcoxon signed rank test; Figure 6D, dark-gray dots). The HP1-independent binding of HP3 was as predicted not de-

creased, and was even increased on average after depletion of HP1 ($P = 4.8 \times 10^{-8}$, Wilcoxon signed rank test; Figure 6D, black dots). This strongly supports the notion that an HP1-independent mechanism exists that targets HP3 to a separate small set of genes. Furthermore, the increase in HP3 binding

Figure 4 Binding ratios of novel HPs and HP1 correlate. Scatterplot comparisons of $\log_2$ binding ratios for HP1 and (A) Su(var)3-9, (B) HP3, (C) HP4, (D) HP5, (E) HP6 and (F) JRA. The dashed oval in (B) highlights genes bound by HP3, but not by any of the other HPs or Su(var)3-9. Correlation coefficients are shown at the bottom of each plot.

Figure 5 Loss of HP1 causes subnuclear redistribution of HP3, HP4 and HP5. Immunofluorescence images of interphase *Drosophila* Kc cells transfected with DamMyc-tagged HP3, HP4, HP5 and HP6. DAPI staining of DNA is shown in gray. (A–E) *white* dsRNA-treated cells; (F–J) HP1 dsRNA-treated cells. (A, F) endogenous HP1 stained with the C1A9 anti-HP1 antibody (red). (B–E, G–J) Tagged HP fusion proteins stained with an anti-Myc antibody (green, only in transfected cells). The HP1 dsRNA-treated cells show a diffuse distribution of the DamMyc-HP fusion proteins in the nuclei of cells irrespective of the expression levels of the fusion proteins. Numbers in the lower right corners represent the percentage of nuclei with a clear chromocenter staining of the DamMyc fusion proteins (between 18 and 35 nuclei were scored).

to HP1-independent target genes after depletion of HP1 is in agreement with the redistribution of HP1-restricted HP3 to HP1-independent HP3 targets.

Subtle redistribution of HP6 after depletion of HP1

We performed similar DamID experiments to test whether HP6 localization is affected by depletion of HP1. Initial inspection of the scatterplot (Figure 6E) suggested that for most tested genes, the HP6-binding ratios in HP1-dsRNA-

treated cells remained similar to those in *white* dsRNA-treated cells. However, subtle non-random changes could not be excluded. Because HP6 and HP3 can interact directly in the yeast two-hybrid assay (Figure 1A), we tested whether HP6 was perhaps partially relocated to the same loci as HP3 after RNAi of HP1. Indeed, the *de novo* targets of HP3 showed on average a mild, but significant, increase in HP6 binding after depletion of HP1 ($P < 2.2 \times 10^{-16}$, Wilcoxon signed rank test; Figure 6F, black dots). Therefore, HP6 binding is also directly

Figure 6 Effects of HP1 depletion on the genome-wide distribution of HP3 and HP6. (A) Scatterplot of HP3 log₂ binding ratios in *white* dsRNA-versus HP1 dsRNA-treated cells. Black line depicts the diagonal (i.e., no change in binding). (B) Computational classification of two populations of genes: black dots mark *de novo* HP3 target genes and dark-gray dots mark genes that have slightly decreased HP3-binding ratios after depletion of HP1. These two groups of genes were determined by an iterative 'k-slopes' linear regression algorithm (see Materials and methods), and the corresponding fitted lines are shown. Light-gray dots mark genes that are not significantly bound by HP3. (C) Relative expression levels of the same groups of genes as in (B); according to Pickersgill *et al* (2006). (D) HP3-only targets remain unaltered in HP1-dsRNA-treated cells. Same scatterplot is shown as in (A), with HP3-only targets marked with black and HP3-HP1 co-targets marked with dark-gray. (E) Scatterplot of HP6 log₂ binding ratios in HP1 dsRNA-versus *white* dsRNA-treated cells. Black line depicts the diagonal. (F) Same as in (E), with *de novo* HP3 targets highlighted in black. A color version of this figure is available at *The EMBO Journal* Online.

or indirectly (via HP3) restricted by HP1, albeit to a much lesser extent than HP3.

Novel HPs are modifiers of position effect variegation

Position effect variegation (PEV) refers to the silencing of euchromatic genes that are placed in the vicinity of heterochromatin. The degree of silencing can vary from cell to cell within the same individual. Mutations in HPs typically alleviate silencing of a PEV reporter gene and therefore these proteins are suppressors of PEV (Eissenberg *et al*, 1990; Tschiersch *et al*, 1994; Cleard *et al*, 1997; Festenstein *et al*, 1999; Schotta *et al*, 2004). We therefore tested whether mutations in the novel HPs are also suppressors of PEV.

Although no mutants are known for HP3, we could obtain P-element insertion mutants for HP4 (*P{EPgy2}CG8044^{EY01733}*), HP5 (*P{EPgy2}CG1745^{EY10901}*) and HP6 (*P{GT1}CG15636^{BG01429}dp^{BG01429}*) from the Bloomington Stock Center. The P-elements are inserted just downstream of the start codons of the open reading frames, truncating the predicted proteins after 14 amino acids (HP4 and HP6) or 47 amino acids (HP5), most likely resulting in functional defects. The HP4, HP5 and HP6 genes are located, respectively, in cytogenetic regions 66A21, 10B14 and 25A1. The HP4 and HP5 mutants are homozygous viable and show no visible phenotype (we found no morphological changes in adult

body parts such as eyes, wings, thorax, legs, antennae, bristles, etc, nor did we observe any obvious changes in behavior or fertility), whereas the HP6 mutant is homozygous semilethal. It should be noted here that the gene coding for HP6 resides in an intron of the *dumpy* locus and it is known that truncations of *dumpy* affect the viability of *Drosophila* (Wilkin *et al*, 2000 and citations therein). It is therefore possible that the semilethality of HP6 mutants is due to a change in the expression of *dumpy*.

We used the translocation *T(2;3)Sb^v* as a PEV reporter. In this translocation, the antimorphic *Stubble* (*Sb^l*) allele of the third chromosome is placed close to pericentric heterochromatin of the second chromosome (Sinclair *et al*, 1983). This leads to variegating repression of the mutant allele, so that some bristles are short and others are as long as in *Sb⁺* flies (Sinclair *et al*, 1983). Mutations in genes that encode HPs can alleviate this repression, causing a decrease in the appearance of long bristles. This PEV model was previously used to identify Suv4-20 as an important functional component of heterochromatin (Schotta *et al*, 2004).

We scored the *Sb^v* phenotype for eight defined dorsal bristles in heterozygous mutants of HP4, HP5 and HP6. To estimate the baseline level of PEV, we scored bristle lengths in a wide range of control crosses of *T(2;3)Sb^v* with stocks of different genetic backgrounds (see Materials and methods).

Figure 7 HP4 and HP5 mutants are dominant suppressors of PEV. Scoring of the *Sb^V* phenotype in control lines (A) and heterozygous mutants of HP-encoding genes (B–F). Histograms show the frequency distributions of short bristle counts for eight defined dorsal thorax bristles. Control (A) shows pooled data from several control genotypes; *n* denotes the number of flies counted. Short bristles are the result of an active *Sb^V* allele; thus, increased frequencies of short bristles compared to the control group indicate suppression of PEV. See Materials and methods for experimental details.

As positive controls we included heterozygous mutants of HP1 (*Su(var)2-5⁰²*) and *Su(var)4-20* that are known to suppress PEV in this assay (Schotta *et al*, 2004).

Figure 7 shows that, like *Su(var)2-5* and *Su(var)4-20*, the HP4 and HP5 mutations cause significant increases in the frequencies of short bristles. Thus, both mutants are dominant suppressors of PEV. This effect is most prominent in the HP4 mutant, in which the strength of the phenotype lies between those of *Su(var)4-20* and *Su(var)2-5⁰²*, whereas the HP5 mutant has a somewhat weaker, but still highly significant effect. In contrast, we were unable to detect a significant effect in the HP6 mutant. Taken together, these data indicate that HP4 and HP5 are essential for efficient silencing of a reporter gene by heterochromatin.

Discussion

We have identified four novel *Drosophila* HPs, HP3, HP4, HP5 and HP6, by searching a yeast two-hybrid database for interactors of the HP1 and subsequent mapping of the *in vivo* binding sites of suitable candidates by DamID. This confirmed the heterochromatic localization as well as the strong colocalization with HP1 of all four candidates we tested. We additionally showed that the genomic targeting of HP3, HP4 and HP5 (and to a much lesser extent HP6) is controlled by HP1. Furthermore, we showed that mutations in HP4 and HP5 act as suppressors of PEV, arguing that the association of these proteins with heterochromatin is of functional importance. Our assay did not reveal such a function for HP6, suggesting that it is not needed for heterochromatic transgene silencing. We cannot exclude, though, that HP6 may behave as a PEV modifier in a different PEV assay.

The yeast two-hybrid data indicate that HP3, HP4 and HP5 can interact directly with HP1. Because HP3, HP4 and HP5 are partially mislocalized when HP1 is depleted, we propose

that HP1 forms a docking site for these three proteins. Interestingly, HOAP, the protein encoded by the *cav* gene, also interacts with both HP1 and HP6 (Figure 1A), and has been implicated in heterochromatin function (Badugu *et al*, 2003; Shareef *et al*, 2003). *Su(var)3-9* also interacts directly with HP1 and requires HP1 for its correct heterochromatic localization (Schotta *et al*, 2002). Thus, despite its small size (only 23 kDa), HP1 serves as a binding platform for many mediator proteins that are essential for heterochromatin function. It is unknown whether all these proteins can bind to HP1 simultaneously, or compete for available HP1 molecules.

The mechanism of HP6 targeting is less clear. The direct interactions of this protein with HP3, HP4 and HP5 suggest that each of these three proteins may help to recruit HP6 to heterochromatic loci. In agreement with this model, we find that after HP1 depletion, the mislocalized HP3 is partially accompanied by HP6, suggesting that HP3 is able to contribute to the targeting of HP6. It should be noted that although no direct interaction was reported between HP6 and HP1 (Giot *et al*, 2003), it cannot be ruled out that HP1 and HP6 are able to interact directly through their CSDs, because this domain mediates homodimerization of HP1 and therefore could also mediate heterodimerization between proteins (Cowieson *et al*, 2000; Zhao *et al*, 2000; Yamamoto and Sonoda, 2003). However, because HP6 localization was only subtly affected by HP1 depletion, it is likely that an additional (unknown) interaction plays a key role in the targeting of HP6 to heterochromatin, unless residual low amounts of HP1 are sufficient to recruit HP6.

The redistribution of the new HPs to a different set of genes upon loss of HP1 may be of functional importance during specific stages of *Drosophila* development. For example, in male adult flies, the expression level of HP1 and HP4 is reduced compared to other stages of development, whereas the level of HP6 expression is increased (Figure 1C). Such

changes in the relative abundances of the different heterochromatin components could cause a shift in the binding specificity of each protein, and possibly help to regulate specific sets of genes.

HP3 has also a small set of target genes that are not significantly bound by any of the other mapped proteins, indicating that in addition to an HP1-dependent mechanism, a second targeting mechanism exists that directs HP3 to specific genes. The nature of this mechanism and the functional relevance are at present unclear. We have not been able to identify common functions in the HP3-specific target gene set.

We demonstrated that mutations in HP4 and HP5 are dominant suppressors of PEV. We have been unable to link the genes encoding HP4 or HP5 conclusively to locations of previously identified Su(var) mutations (Reuter and Szidonya, 1983; Reuter *et al*, 1986; Szidonya and Reuter, 1988; Wustmann *et al*, 1989; Sinclair *et al*, 1992; Westphal and Reuter, 2002). Thus, these genes are most likely novel Su(var) genes.

Taken together, we have identified HP3, HP4, HP5 and HP6 as four novel HPs. DamID was an important tool in the validation of the heterochromatin targeting of these proteins and in obtaining insight into their targeting mechanism. The high resolution and genome-wide character of DamID, together with its major advantage that no antibodies are required, make this mapping technique particularly suitable for studies of novel proteins. We suggest that this approach may be generally applicable for the identification and characterization of many still unknown components of various chromatin complexes.

Materials and methods

Identification of putative HPs

The Curagen Drosophila Interaction Database (<http://portal.curagen.com/cgi-bin/interaction/flyHome.pl>) was used to search for proteins interacting with HP1.

DamID

Full-length HP3, HP4, HP5 and HP6 cDNA clones (from the BDGP, (Stapleton *et al*, 2002a)) were obtained from the Drosophila Genomics Resource Center and fused to the C terminus of Dam through a Myc epitope tag linker by cloning of the open reading frames into pNDamMyc (van Steensel and Henikoff, 2000). The Dam-HP1 and Su(var)3-9-Dam constructs were described earlier (van Steensel and Henikoff, 2000; Greil *et al*, 2003). DamID was performed as described (Greil *et al*, 2003) in embryonal *Drosophila melanogaster* Kc167 cells grown in BPYE medium (Shields and Sang M3 Insect Medium supplemented with 2.5 g/l bacto peptone, 1 g/l yeast extract and 5% heat-inactivated fetal calf serum).

Microarrays

For hybridizations, we used spotted microarrays (Genomics Facility, Fred Hutchinson Cancer Research Center, Seattle) containing 11 857 cDNA clones from Release 1 and Release 2 of the Drosophila Gene Collection (DGC1 and DGC2), as well as 126 additional cDNA and genomic fragments contributed by members of the Northwest Flychip Consortium (Rubin *et al*, 2000; Stapleton *et al*, 2002b). Annotation of the array was based on Release 3 of the *D. melanogaster* genome. For 9789 probes, reliable genomic locations genome could be determined: 255 of the probes are within 1 Mb of the centromere of chromosomes 2, 3 or X and 74 probes are located on chromosome 4. For 295 probes, multiple locations in the genome were found; of these, 70 mapped to transposable element sequences. Microarray data are available from GEO (<http://www.ncbi.nlm.nih.gov/geo/>), accession number GSE6411.

RNAi

As a template for HP1 dsRNA production, a 618 bp fragment of the open reading frame of HP1 flanked by a T7 promoter at both 5' and 3' ends was amplified using the primer pair 5'GAATTAATACCACTACTATAGGGAGAGGCAAGAAAATCGACAACCCTGAGAGC3' and 5'GAATTAATACGACTACTATAGGAGATTAATCTTCATTATCAGAGTACCAGGATAGGCGCT3'. As a template for *white* dsRNA production, a 344 bp fragment of the open reading frame of *white* flanked by a T7 promoter at both 5' and 3' ends was amplified using the primer pair 5'GAATTAATACGACTACTATAGGATCTGCTGTCGGTCTCAAG3' and 5'GAATTAATACGACTACTATAGGATCATCGGATAGGCAATCGCCG3'. RNAi treatment was performed as described by Greil *et al* (2003).

Data analysis

The R software package (<http://www.r-project.org>) was used for all data analyses. For statistical analysis, all measured ratios were log₂-transformed and normalized to the median value of the entire array. Data from three to four independent experiments were averaged, with one or two experiments performed with reversed dye orientation, respectively. To define target loci, we tested whether log-ratios were significantly greater than 0 using the CyberT algorithm (Long *et al*, 2001) followed by a correction for multiple testing (Benjamini and Hochberg, 1995), adjusting the estimated false discovery rate to 1% and applying an additional threshold of log₂(1.5).

To identify *de novo* HP3 targets after RNAi knockdown, we developed an iterative 'k-slopes' linear regression algorithm to separate *de novo* from wild-type targets. This method is analogous to k-means clustering (Hartigan and Wong, 1979). The method begins with two straight lines drawn intuitively through the two populations of probes. Then all probes are assigned to one of two groups, corresponding to the nearest line. Next, for each group, a new linear regression line is calculated. Again, all probes are reassigned to the nearest line, and for the new groups the linear regression line is calculated. This process is repeated until the algorithm has converged to an optimal separation of the two classes and stable definition of the two lines. Importantly, this iterative fitting of two lines is highly robust with respect to the initially chosen two lines. Implementation of this algorithm in the R programming language is available upon request.

PEV assay

HP4-6 mutant lines are P-element insertion lines and were obtained from the Bloomington Drosophila Stock Center. The HP4 mutant line is $y^1w^{67c23}P\{EPgy2\}CG8044^{EY01733}$ and the HP5 mutant line is $y^1w^{67c23}P\{EPgy2\}CG1745^{EY10901}$. The HP6 mutant stock is $w^{1118}; net1 P\{GT1\}CG15636^{BG01429}dp^{BG01429}/In(2LR)Gla, wg^{Gla-1}Bc^1$. The HP6-encoding gene *CG15636* consists of a single exon located in an intron of the larger *dp* gene; we cannot rule out that the expression of *dp* is affected in this mutant. Any transcript that could arise from $P\{GT1\}CG15636^{BG01429}$ is unlikely to produce a functional HP6 protein, because the open reading frame starting at the next methionine would have a truncated chromoshadow domain (the only recognizable domain in HP6). The HP1 and Su(var)4-20 mutant lines (kindly provided by Dr G Reuter, Martin-Luther-Universität, Halle-Wittenberg, Germany) have the genotypes $w^{m4h}; Su(var)2-5^{02}/SM1$ and $FM7/P\{GT1\}Sw4-20^{BG00814}w^{1118}$, respectively. The HP4 and HP5 homozygous mutant lines were first crossed to balancer stocks $w^{1118}; Sb^H/Tm6B$ and $FM7a$, respectively, to obtain heterozygous parental lines. As controls, we used the $y^1w^{67c23}P\{EPgy2\}CG5905$ $Nep1^{EY21255}$ and $y^1w^{67c23}; P\{EPgy2\}CG4109$ $Syx8^{EY21219}$ lines (kindly provided by Dr H Bellen, Baylor College of Medicine, Houston, TX, USA), which are unrelated P-element insertion mutants derived from the same parental stock as the mutant lines for HP4 and HP5. Because all HP mutants carry additional mutant alleles for *white* and/or *yellow* (w^{1118} and y^1w^{67c23}), we also used w^{1118} and y^1w^{67c23} stocks as additional controls.

For the PEV assay, we crossed 4-6 female virgins of each heterozygous mutant or homozygous control line to 3-5 males of $T(2;3)Sb^V$, $In(3R)Mo$, Sb^1 , $sr^1/TM3$, Ser (obtained from the Bloomington Drosophila Stock Center). All crosses were performed in triplicate at 25°C. In the Ser^+ progeny (i.e., flies carrying the Sb^V allele) of these crosses, we scored the length of the four dorso-central and four scutellar setae as either long (similar to wild-type flies) or short. This we did for heterozygous mutant progeny, their

balancer-carrying siblings (except for males carrying FM7, which have the *sc*⁸ bristle phenotype that precluded reliable scoring) and all progeny of crosses with the four control lines. Together, we analyzed seven different control genotypes, and these data were pooled. Male and female scores were similar in all cases (data not shown) and therefore combined, except for the HP5 mutant, which was only scored in male progeny because of the lack of a visible marker in female progeny.

The frequency distribution of long bristles (reflecting a silenced *Sb*^V allele) approximated Poisson distributions; to calculate *P*-values, we therefore used long bristle frequencies and a statistical test designed for comparing Poisson-distributed observations (Detre and White, 1970), implemented in an R-script.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

Note added in proof

Recently, it was reported that HP3 corresponds to Lhr, a *Drosophila simulans* protein that is involved in hybrid incompatibility and is located in heterochromatin (Brideau NJ, Flores HA, Wang J,

Mareshwari S, Wang X, Barbash DA (2006). Two Dobzhansky-Muller genes interact to cause hybrid lethality in *Drosophila*. *Science* **314**, 1292–1295).

Acknowledgements

We thank Gunter Reuter (Martin-Luther-Universität) and Gunnar Schotta (IMP, Vienna) for advice on PEV assays; Gunter Reuter, Karen Schulze and Hugo Bellen (Baylor College of Medicine), Paul Talbert and Steven Henikoff (Fred Hutchinson Cancer Research Center), Lee Fradkin (Leiden University) and the Bloomington *Drosophila* Stock Center for fly stocks; Jeffrey Delrow (Fred Hutchinson Cancer Research Center) for providing cDNA arrays; Lee Fradkin, Jasprien Noordermeer and Bert van Veen (Leiden University) for invaluable help with setting up our fly lab and providing fly food; Michael Hauptmann (Netherlands Cancer Institute) for advice on statistics; Kami Ahmad for help with immunofluorescence images; and members of the van Steensel lab for critically reading the article. This work was supported by the European Network of Excellence 'the Epigenome' and an European Young Investigator Award to BvS.

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